The study of nuclear targets of Phosphatidylinositol-3 Kinase in lymphocytes

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SUMMARY

Pathways regulated by Phosphotidylinositide-3-kinase (PI3K) have emerged as important mediators of cell proliferation and survival. When altered, several components of this pathway have been identified to contribute towards a wide range of human malignancies. PI3K has been implicated in the development of several EBV-associated malignancies of both lymphoid and epithelial origin. These include Burkitt's lymphoma, Hodgkin's disease and nasopharyngeal carcinoma. Although progress has been made in dissecting the pathways regulated by PI3K, the key components contributing to lymphocyte transformation have not been fully characterised. This study sought to investigate downstream targets of PI3K in lymphocytes in order to further our understanding of the contribution of PI3K signalling to lymphocyte proliferation and survival, particularly within the context of EBV-associated B-cell lymphomas.

Initial work in this study revealed that a component of the mammalian ribosome, S6-ribosomal protein, is a major target for PI3K activation in transformed lymphocytes. In order to study PI3K and EBV regulated proteins on a larger scale, the technology of two-dimensional electrophoresis (2DE) was employed. The use of 2DE in combination with a PI3K inhibitor did not allow the identification of PI3K regulated proteins. However, three EBV regulated proteins were detected in the B-lymphocyte nucleus using this technology. The technology was further developed to study the post-translational modifications of DNA bound transcription factors. This detected multiple isoforms of the cAMP-response element binding protein (CREB), signal transducers and activators of transcription 1 (STAT1) and forkhead box O (FOXO) transcription factors in the nuclei of EBV immortalized lymphocytes.

More detailed analysis of the PI3K regulated pro-apoptotic transcription factor, FOXO1, revealed that this protein is downregulated in EBV positive cells at both the transcriptional and translational levels. This downregulation was shown to directly correlate with the protein expression of a known target gene activated by FOXO1, Bcl-6, and to inversely correlate with protein levels of Cyclin D2, a target transcriptionally repressed by FOXO1. Further investigations into the mechanisms by which EBV downregulates FOXO1 implicated a role for two EBV encoded proteins, Latent membrane protein-1 (LMP1) and LMP2A in the downregulation of both FOXO1, and its target gene, Bcl-6.

In conclusion, this work has explored the use of antibody detection and proteomic techniques for the identification and analysis of nuclear proteins and transcription factors regulated by PI3K and EBV. Together, these investigations have deepened our understanding of the molecular changes that occur in lymphocytes in response to EBV infection, and how EBV may influence malignancy.

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ABBREVIATIONS

2DE	Two-dimensional electrophoresis
4E-BP	4E-binding protein
AGC	cAMP-dependent protein kinase A/protein kinase G/protein kinase C
AIDS	Acquired immune deficiency syndrome
AML	Acute myeloblastic leukaemia
AP	Activating protein
APA	Alkaline phosphatase assay
APS	Ammonium perusulphate
ARMS	Alveolar rhabdomyosarcomas
ATF	Activator-transcription factor
BART	BamHI-A rightward transcript
Bcl-6	B-cell lymphoma-6
BCR	B-cell receptor
BL	Burkitt's lymphoma
BSA	Bovine serum albumin
CAM-KII	Ca^{2+}/calmodulin-dependent protein kinase II
CDK	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
CHD	Classic Hodgkin's disease
CRE	Cyclic AMP-responsive element
CREB	Cyclic AMP responsive element binding protein
CSK-1	Casein kinase-1
CTAR	C-terminal activating regions
CTL	Cytotoxic T-lymphocyte

DLBCL	Diffuse large B-cell lymphoma
DMSO	Dimethyl sulfoxide
DNA-AP	DNA-affinity precipitation
DTT	Dithiothreitol
DYRK-1	Dual tyrosine phosphorylated regulated kinase-1
EAP	Epstein-Barr virus encoded RNA associated protein
EBER	Epstein-Barr virus encoded RNA
EBNA	Epstein-Barr virus nuclear antigen
EBNA-LP	Epstein-Barr virus nuclear antigen- leader protein
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetra acetic acid
ERK	Extracellular signal-regulated protein kinase
Fas-L	Fas-ligand
FCS	Foetal calf serum
FOXO	Forkhead box-O
GAB	Growth factor receptor-bound protein-2 associated binding protein
GC	Germinal centre
gp	Glycoprotein
GRB2	Growth factor receptor-bound protein-2
GSB	Gel sample buffer
GSK-3	Glycogen-synthase kinase 3
HD	Hodgkin's disease
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
HRS	Hodgkin and Reed-Sternberg
HTLV-1	Human T-cell leukaemia virus 1

IARC	International agency for research on cancer
IκB	IkappaB
IKK	IkappaB kinase
ICAM	Intercellular adhesion molecule
ICAT	Isotope Coded Affinity Tags
IMAC	Immobilized metal-affinity chromatography
IEF	Isolelectric focusing
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILK	Integrin-linked kinase
IM	Infectious mononucleosis
IPG	Immobilized pH gradient
IR	Internal direct repeats
ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
JNK	Jun N-terminal kinase
Kb	Kilobasepairs
KSHV	Kaposi's sarcoma-associated herpesvirus
LAR	Luciferase assay reagent
LB	Luria-Bertani
LCL	Lymphoblatoid cell line
LCV	Lymphocryptoviridae
LFA	Lymphocyte-function-associated antigen
LMP	Latent membrane protein
LUR	Long unique region
MAPK	Mitogen-activated protein kinase

MAPKAP	Mitogen-activated protein kinase activated protein kinase
MDM2	Murine double minute-2
MESNA	2-mercaptoethansulphonate
MHC	Major histocompatibility complex
MLL	Mixed lineage leukaemia
Mr	Molecular weight
MS	Mass spectrometry
mTOR	Mammalian target of rapamycin
NES	Nuclear export signal
NFκB	Nuclear factor-kappa B
NHL	Non-Hodgkin's lymphoma
NLPHD	Nodular lymphocyte predominant hodgkin's disease
NLS	Nuclear localization signal
NPC	Nasopharyngeal carcinoma
OHL	Oral hairy leukoplakia
OriP	Origin of replication
p70S6K	p70 ribosomal protein-S6 kinase
PARP	Poly-ADP-ribose polymerase
PAX	Paired box
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKR	Protein kinase R
PH	Pleckstrin-homolgy
PHA	Phytohaemagglutanin
pI	Isoelectric point

PI3K	Phosphotidylinositol-3-kinase
PIP	Phosphatidylinositol-phosphate
PMSF	Phenylmethylsulfonyl fluoride
PTEN	Phosphatase and tensin homologue deleted on chromosome ten
PTK	Protein tyrosine kinase
PTLD	Post-transplant lymphoproliferative disorder
PTM	Post-translational modification
PVDF	Polyvinylidene diflouride
RB	Retinoblastoma
RBP-Jκ	J-kappa recombinantion-binding protein
RDV	Rhadinoviridae
RTK	Receptor tyrosine kinase
S6	S6-ribosomal protein
SAP	Signalling lymphocytic activation molecule – associated protein
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SELDI-ToF	Surface enhanced laser desorption/ionization time of flight
SGK	Serum and glucocorticoid-stimulated kinase
SH2	Src homology 2
SHIP	SH2-containing inositol-5'-phosphatase
SHM	Somatic hypermutation
SILAC	Stable Isotope Labelling by Amino Acids in Cell Culture
STAT	Signal transducer and activator of transcription
TCA	Trichloroacetic acid
TE	Tris-EDTA
TEF	Transformation effector site
TEMED	NNN'N-tetramethyl-ethylenediamine
TGF	Transforming growth factor
TNF	Tumour necrosis factor

TNF-R	Tumour necrosis factor-receptor
TR	Tandem direct repeats
TRAIL	Tumour necrosis factor-related apoptosis-induced ligand
TRADD	Tumour necrosis factor-receptor associated death domain
TRAF	Tumour necrosis factor-receptor associated factor
TSC	Tuberous sclerosis complex
tTA	Tetracycline-regulated transactivator
WHO	World health organization
WT	Wild type
XLP	X-Linked lymphoproliferative syndrome

TABLE OF CONTENTS

CONTENTS **PAGE NO.**

1.2 Phosphotidylinositol-3-Kinase

1.3 FOXO transcription factors

CHAPTER 2 Materials and methods

Appendix I

List of suppliers

Appendix II

List of publications

Appendix III

Publication: Shore *et al.*, 2006, Journal of Virology, *Manuscript in press*.

LIST OF FIGURES

CHAPTER 4

CHAPTER 5

LIST OF TABLES

CHAPTER 1

Review of the literature

1.1 Epstein-Barr Virus (EBV)

1.1.1 Discovery of EBV

Denis Burkitt, a British missionary surgeon working in equatorial Africa first described an aggressive B cell lymphoma prevalent in children in this region in the late 1950s and early 1960s. He suspected an environmental or infectious etiology of the lymphoma due to its climatic and geographical distribution, but was unsuccessful in identifying a candidate agent (Burkitt, 1962, Burkitt & Wright, 1966). In 1964, Anthony Epstein and his colleagues Yvonne Barr and Bert Achong successfully cultured cells from Burkitt's lymphomas patient biopsies and identified herpesvirus particles in electron micrographs of the cultured lymphoblasts. This virus was shown to be distinct from previously identified herpesviruses as it did not react with antibodies towards known human herpesviruses and was unable to replicate in other cultured cells (Epstein *et al*, 1964). The transforming ability of EBV was subsequently demonstrated by two studies that reported blast transformation and continual proliferation of infected B lymphocytes (Pope *et al*, 1968, Pattengale *et al*, 1973). Thus, EBV was the first virus to be implicated with the development of human cancers.

1.1.2 EBV classification and structure

EBV is a member of the lymphocryptovirus (LCV) genus of the gamma subfamily of herpersviruses. LCV are found exclusively in primates, with EBV being the only LCV known to infect humans. EBV is also known as human herpesvirus-4 (HHV4) due to taxonomist classification.

Gammaherpesviruses are characterized by their tropism for lymphoid cells and ability to efficiently immortalize B lymphocytes of their hosts. Other features that characterize gammaherpesviruses relate to their genomic composition, encoding several early regulators of viral gene expression and a homologue of bcl-2, an anti-apoptotic protein (Kieff & Rickinson, 2001). In addition, integral membrane proteins capable of triggering intracellular signaling pathways are encoded by the long unique region (LUR) of the viral genome (Brinkmann & Schultz, 2006).

EBV has a toroid shaped protein core that is wrapped with the linear double stranded DNA genome of approximately 185 kb. This is surrounded by an icosahedral nucleocaspid of approximately 100-110nm in diameter that is composed of 162 caspomeres. A protein tegument lies between the nucleocaspid and the envelope, and is encapsulated by an outer envelope that contains external viral glycoprotein spikes on the surface. A schematic representation of the structure of EBV is shown in Figure 1.1. The most abundant EBV envelope and tegument proteins are glycoproteins of 350 (gp350), 220 (gp220) and 152 (gp152) kDa. Additional viral envelope proteins expressed at lower levels include gp85, gp25, gp110, gp84/113, gp43/38 and gp15. The structure of the EBV genome is characterized by short and long unique sequence domains (U_S and U_L) that contain most of the genome coding capacity. Other features include tandem direct repeats (TR) of 0.5 kDa at both termini and internal direct repeats (IR-1) consisting of 6 to 12 tandem repeats of 3-kb (Kieff & Rickinson, 2001).

1.1.3 Infection, persistence and transmission of EBV

EBV is a ubiquitous virus that persistently infects greater than 95% of the population worldwide. The virus is largely lymphotropic but can also infect other cell types such as epithelial cells (Kieff & Rickinson, 2001). EBV gains entry into the B lymphocyte via an interaction between the viral envelope glycoprotein BLLF1 (gp350/220) and human complement receptor type 2 (CD21) molecules expressed at the B cell surface (Fingeroth *et al*, 1984, Nemerow *et al*, 1985, Tanner *et al*, 1987) . The normal function for CD21 is

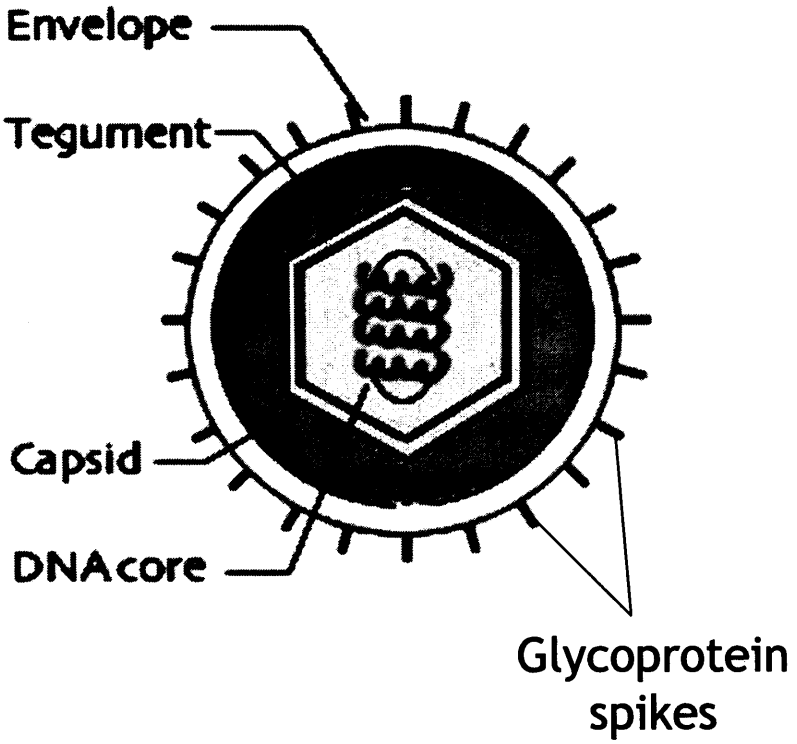


Figure 1.1 Schematic representation of the structure of Epstein-Barr virus.

Adapted from http://www.stden.lanl.stdgen/bacteria/prv/herpes.html.

the receptor for the complement component Cd3 (Rickert, 2005). A complex formed between the viral glycoproteins gp25, gp42/38 and gp85 also contribute towards the adsorption of EBV by forming an interaction with major histocompatibility complex (MHC) class II molecules expressed on the surface of B cells (Knox & Young, 1995). This interaction functions as a co-receptor for the entry of EBV into B cells. Aggregation of CD21 molecules within the plasma membrane follows, which induces the internalization of EBV within cytoplasmic vesicles (Nemerow *et al*, 1984, Carel *et al*, 1990). Alternative mechanisms by which EBV infection occurs are also likely, as one study has demonstrated that EBV lacking gp350/220 was able to infect B cells and epithelial cells (Janz *et al*, 2000). Upon entry of EBV into a cell, the linear viral genome circularizes due to the joining of terminal repeats, and extrachromosomal copies of the viral episome persist within the host nucleus. Viral DNA is replicated by host cellular machinery and is transmitted to daughter cells by the process of cell division (Kutok & Wang, 2006).

1.1.3.1. EBV latent infection *in vitro*

A unique characteristic of EBV is its ability to infect and transform primary B lymphocytes *in vitro* into permanently proliferating lymphoblastoid cell lines (LCLs). LCLs are a good model for cells in the polyclonal phase of post-transplant lymphoproliferative disorder (PTLD); an EBV associated malignancy observed in immunocompromised individuals. LCLs are therefore a powerful *in vitro* model of EBV latent infection, and have proved valuable for the study of the transforming effects of the virus. EBV readily infects primary B lymphocytes derived from peripheral blood, tonsils, or foetal cord blood *in vitro*. (Kieff and Rickinson, 2001).

Successful *in vitro* infection of primary B lymphocytes by EBV results in the generation of latently infected cells. Approximately 100 genes have been identified in the EBV genome, however, only a small proportion are actively transcribed during EBV latent infection of B cells, and encode proteins that induce cell cycle entry, continual proliferation and the maintenance of viral latency. These include six EBV nuclear antigens; EBNA1, EBNA2, EBNA3A,

EBNA3B, EBNA3C and EBNA-leader protein (EBNA-LP), three latent membrane proteins; LMP1, LMP2A and LMP2B, two small nonpolyadenylated RNA molecules (EBERs) and transcripts spliced from the *Bam*HIA region of the viral genome (BARTs). This pattern of EBV gene expression is characteristic of LCLs and is termed latency III (Rowe *et al*, 1992). The latency programmes expressed by EBV are summarized in Table 1.1. LCLs are similar in phenotype to lymphocytes proliferating in response to antigenic or mitogenic stimulation in that they express high levels of the B cell activation markers CD23, CD30, CD39, CD70 and also adhesion molecules CD11a/18 (LFA-1, lymphocyte-function-associated antigen-1), CD58 (LFA-2) and CD54 (ICAM-1- intercellular adhesion molecule 1) that induce inter-cellular adhesion (Rowe *et al*, 1987).

The *in vitro* infection of EBV negative Burkitt's lymphoma (BL) cell lines with EBV can also be achieved but at a reduced efficiency compared to peripheral blood lymphocytes (Kieff & Rickinson, 2001). The continual proliferation of BL cell lines is driven by the constitutive expression of *c-myc*, a transcription factor that promotes cell proliferation by regulating the expression of numerous target genes involved in cell cycle progression (Dang, 1999). This is a result of the characteristic reciprocal chromosomal translocation detected in BL cells that places *c-myc* at chromosome 8q24 adjacent to one of three immunoglobulin heavy or light chain genes on chromosome 2, 14 or 22 (Hecht & Aster, 2000). EBV infection of BL *in vitro* initially leads to the establishment of a latency III pattern of EBV gene expression as in LCL's, which can be maintained in culture. However, continual culture of EBV infected BL cells can also lead to the loss of the EBV genome from a proportion of the cells. Another proportion of the cells may also maintain the EBV episome, but revert to a different pattern of EBV gene expression referred to as latency I. During latency I, only EBNA-1, EBERs and BARTs are expressed. Cell lines generated from the *in vitro* infection of BL cells with EBV are therefore often heterogeneous in their EBV status (Kieff & Rickinson, 2001).

Table 1.1 Patterns EBV gene expression during latency

Latency programme	Gene expression	Examples of latency pattern
I	EBNA1, EBERs	Burkitt's lymphoma
II	EBNA1, LMP1, LMP2, EBERs	Hodgkin's lymphoma Nasopharyngeal carcinoma T cell lymphomas
III	EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, LMP1, LMP2, EBERs	Lymphablastoid cell lines Post-transplant lymphoroliferative disease X-linked lymphoproliferative disease AIDS-associated B cell lymphomas

1.1.3.1.1 EBNA1

EBNA1 is a DNA binding nuclear phosphoprotein expressed in all virus infected cells. The central role of EBNA1 is the maintenance and replication of the viral episome during the cell division cycle. This is achieved by the sequence specific binding of EBNA1 dimers to the plasmid origin of replication (oriP) of the EBV episome, resulting in the tethering of the viral episome to chromosomes (Marechal *et al*, 1999, Lee *et al*, 1999). EBNA1 can also act as a transcriptional regulator of other EBNAs, LMP1, LMP2B as well as EBNA1 itself by binding to certain viral promoters (Kieff & Rickinson, 2001). The EBNA1 mature protein is stabilized by glycine-glycine-alanine repeat sequences that separate the N- and C-terminal domains of the protein. This domain protects EBNA1 from proteasomal-mediated degradation necessary for MHC-class I restricted epitope presentation to cytotoxic T lymphocytes (CTL) (Levitskaya *et al*, 1995). EBNA1 has therefore evolved to be poorly recognized by the immune system.

One study has addressed the role of EBNA1 in the immortalization of primary B lymphocytes by generating a mutant strain of EBV lacking the entire EBNA1 open reading frame. The infection of primary human B cells with this mutant strain did result in the outgrowth of LCLs, although at a much reduced efficiency compared to wild type virus (Humme *et al*, 2003). These results suggested that EBNA1 is not absolutely required for the *in vitro* growth

transformation of primary B cells with EBV. However, the inhibition of EBNA1 function has been demonstrated to impair the growth of EBV positive BL cells both *in vitro* and *in vivo* (Kennedy *et al*, 2003, Nasimuzzaman *et al*, 2005). In addition, B-cell specific expression of EBNA1 in transgenic mice induced the development of B-cell lymphomas, further indicating a role for EBNA1 in EBV mediated cell transformation (Wilson *et al*, 1996a).

1.1.3.1.2 EBNA2 and EBNA-LP

EBNA2 and EBNA-LP (EBNA-leader protein) are the first viral proteins to be expressed after the infection of resting B cells (Allday *et al*, 1989). EBNA2 is crucial for the immortalization process by EBV. This was first demonstrated by the inability of the P3HR-1 laboratory strain of EBV, which lacks the gene encoding EBNA2 as well as the last two exons encoding EBNA-LP, to transform B cells *in vitro* (Miller *et al*, 1974). Homologous recombination experiments restoring EBNA2 gene expression by P3HR-1 resulted in the transformation of primary B cells into LCLs, confirming the importance of EBNA2 in this process (Cohen *et al*, 1989). EBNA-LP is not essential for B cell transformation *in vitro*, but is required for the efficient outgrowth of LCLs (Mannick *et al*, 1991). EBNA2 and EBNA-LP are nuclear proteins that co-operate to act as transcriptional activators of both viral and cellular genes involved in the initiation and maintenance of cell proliferation. The expression of viral latent membrane proteins, LMP1 and LMP2 are upregulated by transactivation by EBNA2, as well as the other EBNA proteins by binding of EBNA2 to the viral Cp promoter. Cellular gene targets of EBNA2 include *c-myc*, *c-fgr, EBI1/BLR2* and the B cell activation markers, *CD21* and *CD23* (Kutok & Wang, 2006). EBNA2A and EBNA-LP have also been shown to co-operate to induce the transition of infected cells from G_0 to G_1 by inducing the expression the cyclin D2 cell cycle protein (Sinclair *et al*, 1994).

EBNA2 does not bind to DNA directly, but interacts with a ubiquitous cellular transcription factor, Jκ-recombinantion-binding protein (RBP-Jκ). RBP-Jk is a downstream component of activation through the Notch signaling pathway, which plays a role in many developmental processes including early

embryogenesis and lineage commitment of lymphocytes. In the absence of Notch activation or EBNA2, RBP-Jκ acts as a transcriptional repressor by recruiting histone deacetylase complexes to promoter sites. An interaction between RBP-Jκ and activated Notch or EBNA2 relieves this repressive effect, and leads to the modulation of cellular and viral promoters containing Jκ cognate sequences. For these reasons, EBNA2 has been implicated as the viral functional homologue of a constitutively active Notch receptor (Zimer-Strobl & Strobl, 2001).

1.1.3.1.3 EBNA3A, EBNA3B and EBNA3C.

Genes encoding EBNA3A, 3B and 3C are tandemly placed in the EBV genome and encode hydrophilic nuclear proteins. Studies with EBV recombinants have demonstrated the requirement for EBNA3A and EBNA3C for B cell transformation *in vitro*, whereas EBNA3B is not necessary for this effect (Tomkinson *et al*, 1992, Tomkinson *et al*, 1993). EBNA2A, 3B and 3C all form a stable association with RBP-Jκ, thereby limiting its binding to EBNA2 and cognate Jκ sequences. This consequently has the potential to represses EBNA2 mediated transcriptional activation of viral and cellular target genes (Robertson *et al*, 1996). All EBNA3 proteins have been demonstrated to reduce the activation of the EBNA Cp promoter by EBNA2 (Murray & Young, 2001). One study investigating the effects of EBNA3A overexpression in an LCL reported that the disruption of EBNA2-RBP-Jκ association was accompanied by a downregulation in c-myc, CD21 and CD23 along with the initiation of G_0/G_1 growth arrest (Cooper *et al*, 2003). EBNA 3C can also co-operate with EBNA2 in upregulating LMP1 (Lin *et al*, 2002). Therefore, the EBNA3 proteins co-operate with EBNA2 in regulating the balance of viral and cellular promoter activation by RBP-Jκ.

1.1.3.1.4 LMP1

LMP1 is a 63kDa integral membrane phophoprotein that is essential for EBV mediated transformation of B cells. This was demonstrated with the use of a recombinant virus lacking functional LMP1. This mutant form of EBV was

unable to induce the growth transformation of primary B cells *in vitro* (Kaye *et al*, 1993). Studies using an inducible LMP1 gene have demonstrated that this protein is also required for the continued proliferation of B cells *in vitro* (Kilger *et al*, 1998).

The LMP1 protein consists of a three segments. The short amino terminal consists of 24 amino acids and functions to orientate and stabilize LMP1 within the plasma membrane. Six hydrophobic transmembrane-spanning domains (residues 24-186) induce the oligomerization of LMP1 molecules, which is required for LMP1 function. The third segment consists of a long carboxy-terminal cytoplasmic tail (residues 187-386) that can be further subdivided into three C-terminal activating regions (CTAR 1,2 and 3) (Li & Chang, 2003) CTAR1 and CTAR2 have been shown to be crucial for the transforming effects of LMP1, and serve as docking sites for the recruitment of specific cellular proteins that constitutively transduce survival and proliferative signals to infected cells (Kaye *et al*, 1995, Izumi & Kieff, 1997). For this reason they are also referred to as transformation effector sites (TEFs).

LMP1 activation is functionally analogous to activated CD40, a member of the tumour necrosis factor receptor (TNF-R) family, as the CTAR regions of LMP1 recruit components of the TNF-R signaling pathway to activate downstream signalling pathways. CTAR1 is located proximal to the membrane and activates signaling pathways via an interaction with molecules of the TNF-R associated factor (TRAF) family. CTAR2 is located at the extreme C-terminus of LMP1 and activates signaling pathways by binding to TNF-R-associated death domain (TRADD) proteins (Li & Chang, 2003).

The Nuclear Factor-κB (NFκB) transcription factor pathway is one of the major signaling pathways activated by the CTAR1 and CTAR2 domains of LMP1 (Huen *et al*, 1995, Busch & Bishop, 2001). This effect contributes towards many of the phenotypic consequences of LMP1 expression, including the induction of various anti-apoptotic genes such as *A20*, *bcl-2*, *blf-1* and *Mcl-1*, as well the production of the pro-inflammatory cytokines IL-6 and IL-8 (Li & Chang, 2003). Both CTAR1 and CTAR2 also engage the p38/mitogen-

activated protein kinase (MAPK) cascade leading to the activation of activator-transcription factor-2 (ATF-2) (Eliopoulos *et al*, 1999a). Janus kinase (JAK) activation of the signal transducers and activators of transcription (STAT), and c-Jun N-terminal kinase (JNK) mediated activation of the activating protein-1 (AP-1) transcription factor are also pathways activated by CTAR1 and CTAR2, that contribute towards the transforming effects of LMP1 (Kieser *et al*, 1997, Gires *et al*, 1999, Busch & Bishop, 2001). One study employed a mutant form of LMP1, containing inactivating mutations of both CTAR1 and CTAR2, to investigate the signalling properties of these domains. This mutant form of LMP1 inhibited LMP1-stimulated transcription of NFκB, STAT and Jun, demonstrating the importance of these domains for effective signalling through LMP1 (Brennan *et al*, 2001, Zhang *et al*, 2004). The CTAR1 has also been associated with the activation of the Phosphotidylinositol-3-Kinase (PI3K) pathway, which mediates several signalling pathways leading to cell survival and proliferation (Dawson *et al*, 2003, Maniou *et al*, 2005). The CTAR3 region of LMP1 lies between the CTAR1 and CTAR2 regions within the carboxy-terminal cytoplasmic tail of LMP1. This region remains relatively poorly characterized with respect to its signaling properties, but has been suggested to bind JAK3 and mediate DNA binding of the STAT1 transcription factor (Gires *et al*, 1999).

1.1.3.1.5 LMP2A and LMP2B

The gene encoding LMP2 yields two distinct transmembrane proteins, LMP2A and LMP2B. LMP2A and LMP2B are structurally similar, containing twelve transmembrane spanning domains and a 27 amino acid cytoplasmic C-terminus. Exon 1 of *LMP2A* encodes a 199 amino acid N-terminal hydrophilic cytoplasmic domain, whereas exon 1 of *LMP2B* is non-coding and the protein therefore lacks this domain. LMP2B is thought to function as a negative regulator of LMP2A (Longnecker & Miller, 1996). The N-terminal domain of LMP2A contains eight tyrosine residues. Two of these tyrosines at positions 74 and 85 are constitutively phosphorylated and form an immunoreceptor tyrosine-based activation motif (ITAM). During normal B cell signalling, phosphorylated ITAM molecules present in the B cell receptor (BCR) mediate

the recruitment and activation of the Syk protein tyrosine kinase (PTK) as well as the Src family PTKs, which play an important role in the mediation of lymphocyte proliferation and differentiation in response to BCR ligation. LMP2A stably associates with the Src family PTKs, Lyn and Fyn, and also Syk via its phosphorylated ITAM motif, blocking normal signalling through the BCR (Burkhardt *et al*, 1992, Miller *et al*, 1995). This was demonstrated by a study showing that normal BCR cross-linking is blocked in LCLs. BCR signalling was, however, found to be normal in a recombinant form of EBV lacking expression of LMP2A as measured by calcium mobilization, cellular kinase activation and induction of tyrosine phosphorylation (Miller *et al*, 1993, 1995, Fruehling *et al*, 1996). The Lyn and Syk binding regions of the ITAM motif of LMP2A were subsequently found to be required for this effect (Fruehling & Longnecker, 1997, Fruehling *et al*, 1998). *In vivo*, LMP2A provides developmental and survival signals to BCR-deficient B cells, allowing their survival in the periphery (Caldwell *et al*, 1998). LMP2A therefore has a role in driving the proliferation and survival of B cells in the absence of signaling through the BCR. By blocking signalling through the normal BCR, LMP2A also has a role in maintaining the virus in its latent phase by preventing the activation of resting lymphocytes that induces the reactivation of the lytic cycle of EBV. Indeed, inhibition of BCR-signalling by LMP2A correlates with lower levels of EBV lytic replication (Miller *et al*, 1994).

In addition to blocking normal signalling through the BCR, LMP2A has also been shown to induce constitutive signalling through the PI3K/PKB (protein kinase B) pathway in B cells (Swart *et al*, 2000) and epithelial cells (Scholle *et al*, 2000, Morrison *et al*, 2003). Activation through this pathway normally provides a survival signal in response to BCR signaling. In B cells, the constitutive phosphorylation of PKB via PI3K required the recruitment of Syk and Lyn to the ITAM motif (Swart *et al*, 2000). A subsequent study demonstrated that LMP2A inhibits Transforming Growth Factor – β1 (TGF-β1) mediated apoptosis through activation of the PI3K/AKT pathway in a BL cell line (Fukuda & Longnecker, 2004). The role of LMP2A mediated activation of the PI3K/PKB pathway in promoting B cell survival has also

been demonstrated *in vivo*, as B cells from LMP2A transgenic mice are sensitive to apoptosis in the presence of specific inhibitors of PI3K and PKB (Portis & Longnecker, 2004a). Neither of the LMP2 proteins are absolutely essential for EBV induced B cell-transformation *in vitro* (Longnecker *et al*, 1993). However, these proteins do greatly enhance the efficiency of B cell immortalization (Brielmeier, 1996).

1.1.3.1.6 EBERs

The two small nonpolyadenylated RNA molecules, EBER1 and EBER2, are the most abundant EBV RNAs expressed in latently infected cells and are expressed in all forms of latency. The EBERs co-localize in the nucleus and assemble into stable ribonuclear complexes with the autoantigen La, ribosomal protein L22 and EBER associated protein (EAP). EBERs also bind the double-stranded RNA activated protein kinase R (PKR) to inhibit its function. PKR is an interferon-α (IFN-α)-inducible serine/threonine kinase, and is a key mediator of the antiviral activities of IFN-α. EBER inhibition of PKR may therefore play a role in EBV persistence (Nanbo *et al*, 2002). EBERs are not essential for the transforming effect of EBV on primary B cells *in vitro* (Kieff & Rickinson, 2001). However, the expression of EBERs in BL cell lines has been shown to increase the survival potential of these cells, as well as induce the expression of the anti-inflammatory cytokine, interlukin-10 (IL-10) (Nanbo & Takada, 2002). EBERs are therefore postulated to contribute towards the persistence and survival of EBV infected cells.

1.1.3.2 EBV latent infection *in vivo*

Primary EBV infection is believed to occur predominantly in the oropharynx and is transmitted through saliva. Shedding of the virus is detected in the saliva of patients with acute infectious mononucleosis (IM) as well as healthy long term carriers of the virus (Niederman *et al*, 1976, Yao *et al*, 1989). It remains to be defined whether EBV directly infects its target cells, which are B cells of the lymphoid tissue underlying the submucosal epithelium of the throat, or whether this occurs via an initial infection of oropharyngeal

epithelium cells. Since B cells residing beneath this region are predominantly naïve B cells, EBV target cells are thought to be mainly in a resting state (Thorley-Lawson & Babcock, 1999). Subsequent events leading to the persistence of EBV in healthy individuals have been studied by the group of Thorley-Lawson. This group analysed the presence and frequency of EBV-infected cells from distinct human B-cell subsets as well as the expression pattern of EBV-encoded genes in these cells. On the basis of these studies, a model of latent EBV infection *in vivo* was proposed (Thorley-Lawson, 2001, Thorley-Lawson, 2005), and is summarized below.

The infection of naïve B cells *in vivo* with EBV leads to a latency III pattern of EBV gene expression, as described in section 1.3.1 (Joseph *et al*, 2000). This is thought to activate the proliferation and clonal expansion of infected cells without the requirement for external signalling. Proliferating B cell blasts latently infected with EBV are continuously produced in the tonsils, and are susceptible to elimination via cytotoxic T-lymphocyte (CTL) mediated attack (Rickinson & Moss, 1997, Khanna *et al*, 1999). However, a proportion of the proliferating cells migrate into the follicles, and undergo germinal centre (GC) differentiation to increase the pool of EBV-infected cells. In peripheral blood, EBV is found exclusively in these cells (Babcock *et al*, 1998). In GC B cells, the viral transcription programme switches to latency II, where the only viral proteins expressed are EBNA1, LMP1 and LMP2A (Babcock *et al*, 2000). EBNA1 is required for the replication of viral DNA (Marechal *et al*, 1999, Lee *et al*, 1999). LMP1 and LMP2 are thought to co-operate to drive the latently infected B cell through the GC reaction by driving immunoglobulin gene mutation (Casola *et al*, 2004), isotype switching (He *et al*, 2003) and downregulating the expression of bcl-6, which signals the exit of memory B cells from the GC (Panagopoulos *et al*, 2004). GC B cells subsequently differentiate into memory B cells, forming a long-term reservoir of the virus. The precise mechanism by which this occurs is controversial, however, this transition does require that the transcription of EBNA2 is switched off, as EBNA2 blocks differentiation (Polack *et al*, 1996). Memory B cells are typically in a resting state and are long lived in the periphery. This provides a setting whereby EBV can persist in the memory B cell pool throughout the

lifetime of the host. Viral gene expression in the EBV-positive memory B cell may be restricted to EBNA1 (Hochberg *et al*, 2004), or may be completely absent (Babcock *et al*, 1999).

The shutting down of viral gene expression plays an important role in the persistence of EBV in long-lived EBV positive memory B cells, by evading the immune recognition by EBV-specific CTLs. In addition, as the growth promoting latent genes are not expressed, infected cells are non-pathogenic and so can persist in the memory B cell pool in a benign state (Thorley-Lawson, 2001). To facilitate virus transmission, a proportion of the infected memory B cells enter the viral lytic cycle for the production of progeny virus particles. This may be triggered in response to the terminal differentiation of memory B cells into antibody plasma cells (Laichalk & Tholey-Lawson, 2005). This provides a mechanism whereby the virus can be amplified for the infection of new naïve B cells in the oropharynx, to replenish the pool of infected cells.

A schematic illustrating a proposed model of EBV infection, persistence and transmission in the human host is shown in Figure 1.2.

1.1.3.3 EBV lytic infection.

Infectious viruses are produced during the lytic phase of the EBV viral life cycle. During lytic viral replication, approximately 80 viral genes are expressed and encode structural proteins, including the viral capsid antigens, as well as proteins involved in transcriptional activation and DNA replication (Kutok & Wang, 2006). They key mediators of the switch from viral latency to lytic cycle are the BZLF1 and BRLF1 proteins encoded by early-immediate genes of the viral genome. Both proteins are transcriptional activators that potently induce the transcription of the full array of EBV lytic genes that result in the generation of new viral infectious particles (Amon & Farrel, 2005). The viral genome is then packaged within a capsid to generate transmissible virions that can infect new hosts.

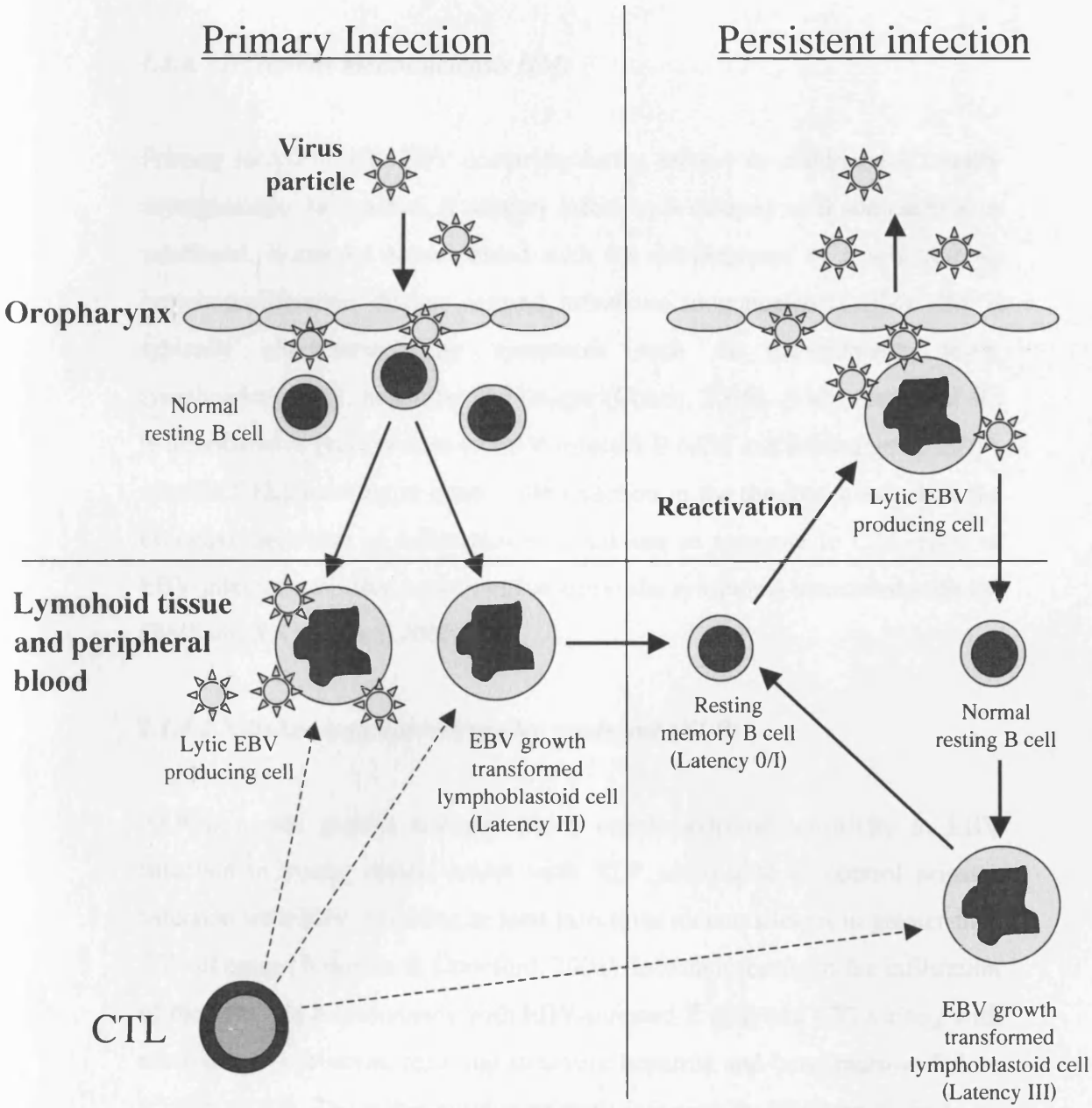


Figure 1.2 Schematic illustrating a proposed model of EBV infection, persistence and transmission in the human host. Adapted from Rickinson & Kieff, 2001

1.1.4 Clinical features of EBV infection

1.1.4.1 Infectious mononucleosis (IM)

Primary infection with EBV occurring during infancy or childhood is usually asymptomatic. In contrast, if primary infection is delayed until adolescence or adulthood, it can be accompanied with the development of a self-limiting lymphoproliferative disease termed infectious mononucleosis (IM). IM is typically characterized by symptoms such as sore throat, fever, lymphoadenopathy, headache and fatigue (Cohen, 2005). A key feature of IM is the extensive proliferation of EBV-infected B cells, and subsequently EBV-specific CTLs working to control the infection in the tonsillar tissue. It is the excessive secretion of inflammatory cytokines in response to CTL lysis of EBV-infected cells that is believed to cause the symptoms associated with IM (Williams & Crawford, 2006).

1.1.4.2 X-linked lymphoproliferative syndrome (XLP)

XLP is a rare genetic disorder which causes extreme sensitivity to EBV infection in young males. Males with XLP are unable to control primary infection with EBV, resulting in fatal infectious mononucleosis in greater than 50% of cases (Williams & Crawford, 2006). Infection results in the infiltration of the liver and bone marrow with EBV-infected B cells and CTLs along with macrophage activation, resulting in severe hepatitis and bone marrow failure (Cohen, 2005). Those that survive primary infection by EBV are likely to go on to develop hypogammaglobulinemia and/or malignant lymphoma. The mutated protein in XLP is the signalling lymphocytic activation molecule – associated protein (SAP), which is expressed and activated in T cells and natural killer cells. SAP regulates cell signaling in B cells, T cells and dendritic cells during immune system responses. Mutations in this protein therefore result in the impaired regulation of B and T cell responses during acute infection (Williams & Crawford, 2006).

1.1.4.3 Oral hairy leukoplakia (OHL)

OHL is a nonmalignant disorder that presents in severely immunocompromised individuals, and was first identified in AIDS patients (Greenspan *et al*, 1985). OHL is characterized by white corrugated hairy lesions of the squamous epithelium of the tongue. EBV may enter the tongue epithelium from EBV infected B cells circulating in the blood (Walling *et al*, 2004). The disorder is unique in being the only EBV-associated disorder in which the virus is actively replicating in the lytic cycle (Kutok & Wang, 2006).

1.1.5 EBV-associated malignancies

EBV was the first virus to be implicated as the causative agent of human cancer due to its association with the development of Burkitt's lymphoma (BL) (Epstein *et al*, 1964). In addition to BL, EBV-associated B cell lymphomas include Hodgkin's disease (HD), post-transplant lymphoproliferative disease (PTLD), and AIDS-associated lymphomas. EBV has also been associated with the development of malignancies of epithelial and T cell origins. These include Nasopharyngeal carcinoma (NPC) and T-lymphocyte Non-Hodgkins lymphoma (NHL) respectively. The high prevalence of EBV in these tumours and the ability of EBV to efficiently transform primary human B cells *in vitro* supports the hypothesis that the virus contributes towards the development of these malignancies. However, a role for additional co-factors is likely as of the 95% of people infected with the virus, only a minority develop virus-associated malignancies. Therefore, whether EBV is the causative agent of lymphomagenesis, or whether it is secondary to genetic alterations remains controversial.

1.1.5.1 Burkitt's lymphoma (BL)

According to the World Health Organization (WHO) classification, BL is a highly aggressive mature B cell neoplasm of which there are three clinical

variants. These are endemic, sporadic and immunodeficiency-associated, that differ with respect to their geographical and clinical presentation, and association with EBV (Kutok & Wang, 2006). The endemic form of BL is the type originally described by Denis Burkitt that has high incidence rates in children throughout equatorial Africa and New Guinea. The incidence of BL in these regions is 50-100 cases per million individuals per year. The most compelling evidence for the involvement of EBV in BL pathogenesis is the clonal presence of the virus in almost 100% of BL tumours within endemic areas (Kutok and Wang, 2006). BL also occurs as a rare, sporadic lymphoma outside endemic areas and is mainly seen in young adults and children. In contrast to the endemic form of BL, the sporadic form has no specific geographical distribution. Incidence rates of the sporadic BL in the United States are 2-3 cases per million individuals per year and their association with EBV is also much lower, at 15-30% of cases in the United States and Europe. BL is also observed as a consequence of Human Immuodeficiency Virus (HIV) infection, and frequently occurs prior to the development of AIDS. However, only 30-40% of these immunodeficiency-associated BL cases are associated with EBV infection (Rickinson & Kieff, 2001).

The defining feature of BL, irrespective of geographical location or immunodeficiency-association, are chromosomal translocations involving the long arm of chromosome 8 (8q24) in the region of the *c-myc* proto-oncogene. These chromosomal translocations place *c-myc* under the control of the immunoglobulin (Ig) heavy chain (14q32) or less frequently under the control of one of the Ig light chain loci (2p11 or 22q11) (Bhatia *et al*, 1992). This results in the inappropriate expression and constitutive activation of c-myc, which promotes uncontrolled cell proliferation by driving cells through the cell cycle and activating anti-apoptotic pathways. In addition, mutations in the gene encoding the tumour suppressor p53 (Bhatia *et al*, 1992) and the putative tumour suppressor retinoblastoma-like 2 (RB2) (Cinti *et al*, 2000) are also observed in cases of BL. In the case of p53, this occurs in a third of cases and appears to be independent of geographical location (Bhatia *et al*, 1992). In contrast, mutations in the *RB2* gene was found in most cases of endemic BL

and only in a small subset of sporadic cases (Cinti *et al*, 2000). These genetic events may therefore facilitate tumour progression in some cases of BL.

Phenotypic studies have provided evidence that BL cells originate from germinal centre B-cells (Gregory *et al*, 1987, Ling *et al*, 1989, Onizuka *et al*, 1995). BL cells express the germinal centre centroblast specific markers CD10, CD77, and bcl-6 and lack the activation markers associated with proliferating, non-germinal centre B cells such as CD21 and CD23. This theory is further supported by evidence of somatic hypermutation (SHM) of immunoglobulin genes in BL cells, a characteristic of germinal centre B cells (Chapman *et al*, 1995. 1996, Harris *et al*, 2001, Sale and Neuberger, 1998). Studies analysing the chromosomal breakpoints of c-myc-immunoglobulin translocations have indicated that these translocations occur either as a mistake of SHM or class-switch recombination (Goossens *et al*, 1998, Kuppers & Dalla-Favera, 2001), providing yet further evidence that BL cells stem from germinal centre B cells.

Southern-blot hybridization of DNA from EBV positive BL biopsies have indicated that tumour cells are monoclonal with respect to EBV infection (Raab-Traub & Flynn, 1986), suggesting that EBV infection occurs prior to tumour development and may therefore play a role in the transformation process. However, EBV-positive tumours display a highly restricted latency I pattern of EBV gene expression, where only EBNA1 and EBERs are expressed (Rowe *et al*, 1987, Niedobitek, 1995). The role of EBV in the pathogenesis of BL is therefore controversial. It has been proposed that EBV may have an initiating role in establishing a pool of growth-transformed infected cells that are prone to subsequent *c-myc* translocations (Polack *et al*, 1996). Alternatively, evidence exists that suggests that EBNA1 and EBERs may have a more direct role in promoting tumourigenesis by providing a survival advantage. EBNA1 has been shown to be essential for the continued survival of EBV-infected BL cells (Kennedy *et al*, 2003) and moreover, tissue specific expression of EBNA1 in transgenic mice leads to the development of B cell lymphomas (Wilson *et al*, 1996a). In addition, EBERs can induce IL-10 expression and mediate resistance to IFN-α induced apoptosis in BL cells

(Kitagawa *et al*, 2000, Nanbo *et al*, 2002) which may contribute towards tumour growth and the survival of malignant cells. A crucial role for PI3K in the survival of BL cells has also been implicated, as the inhibition of PI3K leads to the rapid apoptosis of both EBV negative and EBV positive BL cell lines (Brennan *et al*, 2002). In EBV positive BL cells, this effect may be mediated via LMP2A, as this protein has been demonstrated to inhibit Transforming Growth Factor – β1 (TGF-β1) mediated apoptosis in a PI3K dependent manner in an EBV positive BL cell line (Fukuda & Longnecker, 2004). These observations point to a mechanism whereby PI3K activity may contribute towards the survival of BL cells.

1.1.5.2 Hodgkin's disease (HD)

Hodgkin's disease is characterized by atypical, large, tumour cells referred to as Hodgkin and Reed-Sternberg (HRS) cells. HRS cells typically represent less that 1% of the cells in the tumour tissue, with the remaining tumour mass being composed of non-malignant reactive inflammatory cells including T-lymphocytes, B-lymphocytes, eosinophils, granulocytes and plasma cells (Harris *et al*, 1994, Drexler *et al*, 1992). Based on differences in the histology and the reactive background, HD is distinguished into two major categories. These are nodular lymphocyte predominant HD (NLPHD), accounting for 5% of cases and classic HD (CHD), which account for 95% of cases (Harris *et al*, 1999). In general NLPHD follows an indolent course, in contrast to CHD, which is fatal without therapy.

The incidence of HD occurs at 2-3 cases per 100,000 individuals annually and has a worldwide distribution. CHD is more common in males and demonstrates a bimodial age distribution (<10 and >50 years of age). The association of HD with EBV varies according to pathological subtype and geographical location. Approximately 40% of cases of CHD are associated with EBV, in contrast to the NLPHD subtype where less than 10% of cases are EBV-associated. In western countries up to 50% of HD cases carry the virus, compared with near 100% of cases in other populations in underdeveloped countries (IARC, 1997).

In EBV-associated HD, the viral genomes are found in monoclonal form, suggesting that EBV infection of the tumour cell has occurred prior to clonal expansion, and thus EBV may have a role in the carcinogenic process (Anagnostopoulos *et al*, 1989). The viral genome is present in every HRS cell and generally expresses a latency II pattern of viral genes (EBERs, EBNA1, LMP1 and LMP2A) in EBV positive tumours (Herbst *et al*, 1991, Deacon *et al*, 1993, Grasser *et al*, 1994, Niedobitek *et al*, 1997). LMP1 is expressed at a high level in HRS cells (Pallesen *et al*, 1991, Murray *et al*, 1992) and may therefore contribute towards the pathogenesis of EBV-positive tumours. HRS cells show many characteristics of LMP1-induced phenotypic changes including the strong activation of NFκB (Bargou *et al*, 1996, 1997). The PI3K/PKB pathway has also been demonstrated to be constitutively activated in HRS cell lines and in most primary HRS cells, and contributes towards their survival (Morrison *et al*, 2004, Dutton *et al*, 2005, Nagel *et al*, 2005, Georgakis *et al*, 2006). Nagel *et al* demonstrated that constitutive PI3K activation in HRS cell lines may enhance the expression of the pro-inflammatory cytokine, IL-6 (Nagel *et al*, 2005). IL-6 plays and important role in the growth of EBV infected cells (Tosato *et al*, 1990, Scala *et al*, 1990) and is therefore believed to have a pathological role in HD as well as other EBV associated tumours. Recently, a role for LMP2A in the constitutive activation of PI3K in B cells has been demonstrated (Swart *et al*, 2000), highlighting another potential mechanism whereby EBV may contribute towards the survival of infected B cells.

Because of the co-expression of several cell lineages, the cellular origin of HRS cells has long been unclear. However, the detection of Ig rearrangements and somatically mutated Ig genes in isolated HRS cells has provided strong evidence that these cells are B cells derived from the germinal centre (Kuppers *et al*, 1994, Kanzler *et al*, 1996, Braeuninger *et al*, 1997, Foss *et al*, 1999, Maratofi *et al*, 2000). It is now widely thought that HRS cells are derived from pre-apoptotic, germinal centre B cells that have acquired 'crippling' mutations in their Ig genes (Kanzler *et al*, 1996, Kuppers, 2002). Such B cells are usually efficiently eliminated by apoptosis. However, HRS cells are rescued from

apoptosis by a transforming event. A study by Bechel *et al* demonstrated that EBV can rescue BCR-deficient germinal centre B cells from apoptosis, further implicating a role for EBV in the pathogenesis of HD. Furthermore, LMP2A, which can mimic the BCR, can induce the survival of BCR-deficient immature B cells in transgenic mice (Cladwell *et al*, 1998, Casola *et al*, 2004). The constitutive activation of NFκB in HRS cells has also been shown to prevent HD tumour cells from undergoing apoptosis (Bargou *et al*, 1997). Thus, in EBV positive HRS cells, LMP1 and LMP2A expression may therefore substitute B cell survival signals, allowing the survival of mutated germinal centre B-cells and tumour progression.

1.1.5.3 Post-transplant lymphoproliferative disease (PTLD)

The T-cell immunosuppressive therapy given to patients after transplantation is associated with a greatly increased risk of developing lymphoproliferative disease. These post-transplantation lymphoproliferative diseases (PTLDs) are of B cell origin and can be of clonal or polyclonal morphology (Loren & Tsai, 2005). The detection of somatic hypermutation activity (Brauninger *et al*, 2003, Timms *et al*, 2003, Capello *et al*, 2003) and the expression of the centroblast marker CD77 (Randhawa *et al*, 1994, Arbus *et al*, 2000) in PTLD B cells provides strong evidence that these cells are derived from the germinal centre.

Nearly all PTLDs are associated with EBV (Knowles, 1998). PTLD is thought to arise as the immunosuppressive regime impairs the function of EBV specific CTLs, which usually work to control the proliferation of EBV infected B cells (Rickinson & Moss, 1997, Khanna *et al*, 1999). Thus, in the immunocompromised host, the normal control over excessive proliferation of infected cells is lost and leads to the onset of PTLD (Haque *et al*, 2002). This model is supported by the observation that in many cases of PTLD, the relaxation of immunosupression can lead to the elimination of EBV positive cells and tumour regression (Starzl *et al* 1984, Tsai *et al*, 2001).

The majority of EBV positive B cells in PTLD express a latency III pattern of viral gene expression, where the full complement of latent genes are expressed (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA-LP, LMP1, LMP2, EBERs and BamHI A rightward transcripts. This situation is modeled *in vitro* by LCLs, which can be established by infecting primary human B cells with EBV (Rowe *et al*, 1998). PI3K has been shown to be essential for the proliferation of LCLs (Brennan *et al*, 2002). Inhibition of PI3K in these cells induces growth arrest due to a decrease in the expression of two G_1 cyclins required for cell cycle progression, cyclin D2 and cyclin D3, and an increase in the expression of the cell cycle inhibitor, $p27^{kip1}$. This implicates a mechanism whereby PI3K may influence the proliferation of EBV infected B cells in PTLD.

1.1.5.4 Nasopharyngeal carcinoma (NPC)

Nasopharyngeal carcinoma (NPC) is an epithelial tumour of the nasopharynx. According to the WHO classification of NPC, there are three histopathological subtypes; keritinising squamous cell carcinoma, differentiated non-keritinising carcinoma and undifferentiated carcinoma. NPC is a rare malignancy in most parts of the world, with incidence rates under 1 per 100,000 individuals per year. However, NPC is frequently observed in China, particularly within southern regions, with incidence rates at 20-30 cases per 100,000 individuals per year. Other populations with elevated rates include the natives of the Arctic, Southeast Asia and North African regions (Yu & Yuan, 2002). The increased risk of developing NPC in these regions is associated with cultural, dietary and genetic pre-dispositions (Niedobitek, 2000). The undifferentiated carcinoma form of NPC shows consistent association with EBV, regardless of geographical location, and the virus is present in all tumour cells (Niedobitek *et al*, 1996, Niedobitek, 2000). EBV association with the more differentiated forms of NPC (keritinising squamous cell carcinoma and differentiated non-keritinising carcinoma) have been shown, especially in geographical regions with high incidence of undifferentiated NPC (Pathmanathan *et al*, 1995b).

Southern-blot hybridization of DNA from NPC tissues has demonstrated that these tumours are monoclonal with respect to resident EBV genomes (Raab-Traub & Flynn, 1986, Pathmanathan *et al*, 1995a). This suggests that EBV infection occurs prior to the clonal expansion of malignant cells. EBV latent-gene expression in NPC is predominantly restricted to the latency II pattern of viral gene expression (EBNA1, LMP1, LMP2A and EBERs), similar to that observed in HD (Niedobiteck, 2000). EBNA1 and EBERs are expressed in all EBV positive cases of NPC, whilst LMP1 and LMP2A protein expression can only be detected in around 35% and 50% of cases respectively (Heussinger *et al*, 2004, Niedobitek *et al*, 1992, Young *et al*, 1988, Gulley *et al*, 1995). However, LMP1 can be detected in all pre-invasive lesions, suggesting that LMP1 may have an initiating role in the carcinogenic process, but is not be essential in established cancers (Pathmanathan *et al*, 1995a). Downstream components of the PI3K/PKB pathway have been shown to be constitutively activated in specimens from NPC tumours (Morrison *et al*, 2004), indicating a potential role for this pathway in the development and pathogenesis of this malignancy. Furthermore, LMP1 and LMP2A have been demonstrated to induce constitutive PI3K activation in epithelial cells (Scholle *et al*, 2000, Morrison *et al*, 2003, Dawson *et al*, 2005). LMP2A has also been demonstrated to activate mTOR, a downstream component of the PI3K pathway in NPC cell lines (Moody *et al*, 2005). Thus, these studies suggest a mechanism whereby EBV may induce constitutive PI3K activity in the subset of EBV positive NPC tumours expressing these viral proteins.

In summary, the consistent detection of EBV in several human malignancies points to a role for this virus in the pathogenesis of these tumours. As a result the WHO have classified EBV as a class I carcinogen. Constitutive activation of the PI3K pathway seems to be a feature in HD and NPC, and is a major contributor towards the survival of BL cells, and the proliferation of LCLs. These observations therefore suggest an important role for PI3K in the pathogenesis and development of several EBV-associated tumours.

1.2 Phosphotidylinositol-3-Kinase (PI3K)

1.2.1 Catalytic activity of PI3K

Phosphatidylinositol-3-kinases (PI3Ks) are an evolutionary conserved family of intracellular lipid kinases that catalyze the phosphorylation of inositol lipids at the D3 position of the inositol ring to generate the 3-phosphoinosities (Whitman *et al*, 1988). PI3K family members have been identified in species ranging from yeast to humans and have been found to be present in every eukaryotic organism examined to date (Engelman *et al*, 2006). The inositol ring can be phosphorylated on any of the free hydroxyl carbons resulting in the generation of phosphatidylinositol-3-phosphate (PI(3)P), phosphatidylinsoitol-3,4-phosphate ($PI(3,4)P_2$) and phosphatidylinositol-3,4,5-phosphate ($PI(3,4,5)P_3$) molecules located in the inner leaflet of the plasma membrane. $PI(3,4)P_2$ and $PI(3,4,5)P_3$ are often referred to as second messenger molecules, acting as molecular scaffolds for the recruitment of specialised protein-lipid binding domains. The structure and the metabolism of phosphoinositides by PI3K are illustrated in Figure 1.3.

The most studied protein-lipid binding domains that mediate signalling downstream of $PI(3,4,5)P_3$ formation are pleckstrin-homology (PH) domains. PH domains are composed of a sequence of 100-120 amino acids that form a structurally conserved module that bind inositol lipid head groups phosphorylated at the D3 position (Lemmon & Ferguson, 2000). This domain is found in more than 500 proteins of diverse cellular functions including protein kinases, guanine nucleotide exchange factors, GTPase activating proteins, lipid transport proteins and phospholipases (Balla, 2005). However a smaller subset of PH domains are specific for the D3 lipids and preferentially bind to $PI(3,4)P_2$ and $P(3,4,5)P_3$. Among these are the most important mediators of signalling downstream of PI3K in lymphocytes, the protein kinase B (PKB)/AKT family.

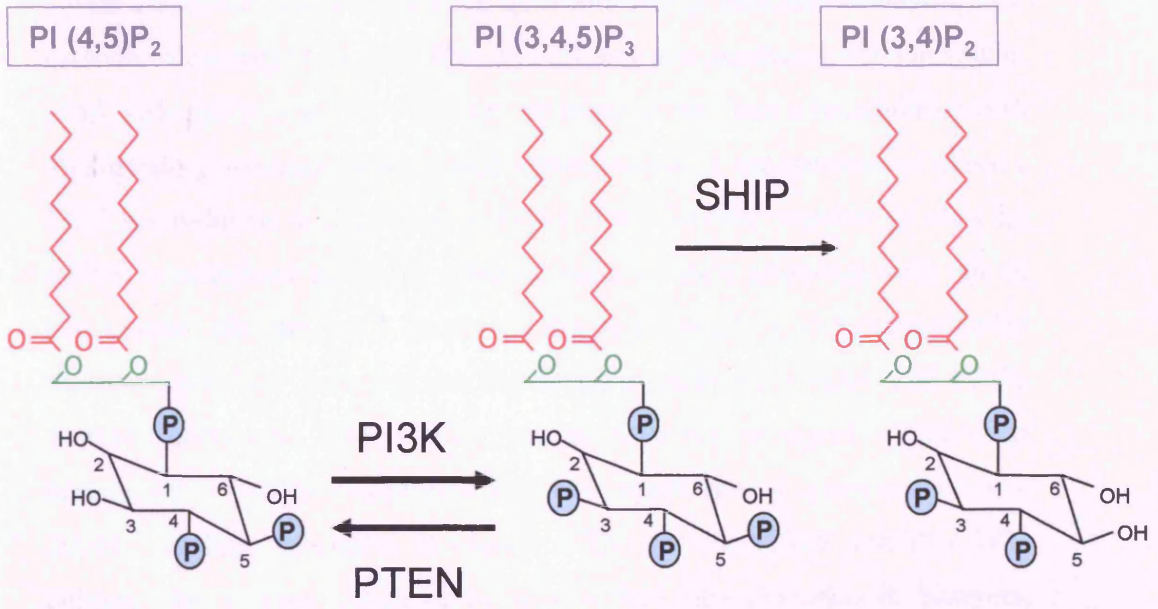


Figure 1.3 Regulation of the phosphorylation of phosphoinositide lipids by PI3K, PTEN and SHIP.

Adapted from Vivanco & Sawyers, 2002.

Phosphoinositol lipids are composed of a membrane associated fatty acid groups (red) and a glycerol moiety (green) that this linked to a cytosolic phosphorylated inositol head group (black). PI3K catalyses the phosphorylation of $PI(4,5)P_2$ at the D3 position of the insoitol ring to form $PI(3,4,5)P_3$. $P(3,4,5)P_3$ molecules that can subsequently act as second messengers via the recruitment of pleckstin homology (PH) domain containing proteins. Dephosphorylation of $PI(3,4,5)P_3$ to regenerate $PI(4,5)P_2$ is accomplished by the 3-phosphatase, PTEN. $PI(3,4,5)P_3$ can also be dephosphorylated at the D5 position by the 5-phosphatase, SHIP, to generate $PI(3,4)P_2$.

1.2.2 Classes of PI3K

The PI3Ks are divided into four classes, class I_A, I_B, II and III and are classified according to their structure, mechanism of activation and substrate specificities (Koyasu, 2003). The Class I_A PI3Ks are activated by tyrosine-kinase-associated receptors and are heterodimeric in structure containing one catalytic subunit (p110α, p110β or p110δ) and one regulatory subunit (p85α, p85β, p55α, p55γ or p50α). All catalytic subunits can form a heterodimer with all regulatory subunits. A schematic representation of the structure of class1_A PI3K heterodimers is illustrated in Figure 1.4. The only member of class I_B, PI3Kγ, is composed of a 110γ catalytic subunit which associates with one of two adaptor subunits, p101 or p84. PI3Kγ is activated by G-protein coupled receptors. All class I enzymes can also be activated by Ras (Bader *et al*, 2005), a small monomeric GTP binding protein involved in signal transduction pathways that mediate cell proliferation and suppression of apoptosis (Bianco, 2006). The lipid substrates of class I PI3Ks are PI, PI(4)P and $PI(4,5)P_2$, although the primary substrate *in vivo* is $PI(4,5)P_2$ (Vivanco & Sawyers, 2002). Three isoforms of class II PI3K have been identified in mammals, PI3K-C2α and β are ubiquitously expressed, whilst PI3K-C2γ is liver specific. Class II enzymes are currently believed to be monomeric as a regulatory subunit has not been identified. Consequently little is known about the molecular mechanisms underlying their activation. However, the substrates of class II PI3Ks have been identified to be PI and PI(4)P. Class III PI3Ks are heterodimeric comprising analogues of the yeast Vps34p PI3K subunit and a p150 adaptor subunit. Class III catalyse the formation of PI(3)P only (Hennessy *et al*, 2005).

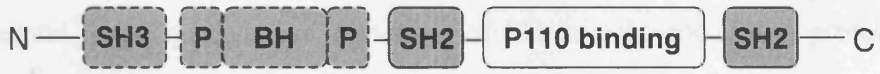
1.2.3 Activation of Class I_A PI3K

Although multiple forms of PI3K exist, the class I_A PI3Ks are primarily responsible for the formation of D3 phosphoinosities at the plasma membrane in response to tyrosine kinase activation (Cantley, 2002). This activation occurs in response to a wide range of extra-cellular signals including growth factors, chemokines, cytokines, the ligation of B cell and T cell receptors and

co-stimulatory molecules (Koyasu, 2003, Ruggero & Sonenberg, 2005). Ligand binding to a receptor tyrosine kinase (RTK) at the cell surface induces the activation and dimerisation of the receptor. This causes the autophosphorylayion of the RTK at tyrosine residues which in turn serve as high affinity docking sites for Src homology 2 (SH2) domain containing molecules (Cully *et al*, 2006). The current and widely accepted view is that in resting cells, heterodimeric PI3K molecules reside in the cytoplasm where they lack catalytic activity. However, upon stimulation, the docking sites created by RTK phosphotyrosine molecules recruit PI3K molecules to the plasma membrane via a physical interaction that occurs between the phosphotyrosine molecules and two SH2 domains and an inter-SH2 domain located within the p85 regulatory subunit of PI3K (Vivanco & Sawyers, 2002). The specificity of interaction occurs in the context of a YXXM consensus sequence (X denotes any amino acid) surrounding the phosphotyrosine residue (Songyang *et al*, 1993). Two mechanisms have been proposed that lead to the increase in PI3K activity following binding of p85 to phosphorylated RTK molecules. Firstly, PI3K molecules are now located within close proximity to their lipid substrates at the plasma membrane. Secondly, this interaction is believed to induce a conformational change in the PI3K heterodimer that relieves the *trans*-inhibitory effect of p85 on p110, thereby releasing p110 catalytic activity (Schlessinger, 2000).

Two other pathways of PI3K activation occur that are dependent on an interaction between growth factor receptor-bound protein 2 (GRB2) adaptor proteins and RTK phosphotyrosine molecules. This interaction occurs in the context of a YXN motif surrounding the phosphotyrosine residue (Pawson, 2004). GRB2 molecules interact with GRB2 associated binding protein (GAB), a scaffolding protein that can bind p85 molecules leading to the activation of p110 catalytic subunits (Ong *et al*, 2001). The interaction between GAB and p85 can occur either directly, or indirectly via an interaction with Ras. Activation of p110 through Ras is believed to occur independently of p85 (Cully *et al*, 2006).

a) Regulatory (p85α/p55α/p50α, p85β, p55γ)



b) Catalytic (p110α, p110β, p110δ)

Figure 1.4 Schematic representation of the domain structure of class I_A PI3K heterodimers.

Adapted from Engelman *et al*, 2006.

a) All class I_A regulatory subunits have a common core structure consisting of two tandem src homology 2 (SH2) domains that allow binding to phosphotyrosine containing sequences and recruitment to receptor signalling complexes. Between the SH2 domains is an inter-SH2 sequence that is responsible for binding of the p110 catalytic subunit. The p85 isoforms (α and β) also contain an extended N-terminal region (outined by a dashed line) that contains a src homology 3 (SH3) domain that binds poly-proline rich motifs, and two such polyproline-rich motifs (P) that flank a region that has homology with the breakpoint cluster region of BCR-Abl (BH).

b) The N-terminus of the p110 catalytic subunit contains a motif that binds to p85 which lies adjacent to a sequence that binds the small GTPase, Ras. Within p110 also lies a phospholipid binding C2 domain, a phosphotidylinositol kinase homology (PIK) domain and the catalytic (kinase) domain at the C-terminus.

1.2.4 Negative regulation of $PI(3,4,5)P_3$ formation

PI3K catalytic activity is tightly regulated in normal cells by various mechanisms. Subcellular localisation has an important role in the regulation of PI3K activity. Only PI3K molecules situated at the plasma membrane within close proximity to their lipid substrates are actively involved in the generation of $PI(3,4,5)P_3$. $PI(3,4,5)P_3$ generated at the plasma membrane acts as potent second messenger for the activation of PH domain containing proteins that mediate downstream signalling through PI3K. The $PI(3,4,5)P_3$ molecules are therefore major targets for attenuation of signalling through PI3K. This is achieved through the activities of two lipid phosphatases; phosphatase and tensin homologue deleted on chromosome ten (PTEN) and the *SH2*-containing inositol-5'-phosphatase (SHIP) protein. The regulation of $PI(3,4,5)P_3$ phosphorylation by PTEN and SHIP is illustrated in Figure 1.3.

PTEN is a 54 kDa protein that contains an N-terminal phosphatase domain with specificity towards $PI(3,4,5)P_3$ and also phosphotyrosine molecules to a lesser extent. PTEN molecules are ubiquitously expressed and are located in the cytosol and the nucleus. Localisation at the membrane occurs due to an interaction between C2 domains located at the C-terminus of the protein and phospholipid components of the membrane (Sly *et al*, 2003, Stiles *et al*, 2004). The main lipid substrate of PTEN is $PI(3,4,5)P_3$ and catalyzes its conversion into $PI(4,5)P_2$ through dephosphorylation at the D3 inositol position (Maehama & Dixon, 1998). PTEN is stabilized at the membrane by elevated $PI(3,4,5)P_3$ due to phosphorylation events at its C-terminal domain mediated by protein kinase C (PKC) or protein kinase CK2 (Torres & Pulido, 2001, Torres *et al* 2003).

PI3K activity is also negatively regulated by SHIP. SHIP is a 145kDa protein that is restricted in expression to heamatoipoietic cells. SHIP possesses a central phosphoinositol phosphatase domain that selectively catalyses the hydrolysis of the D5 phosphate of $PI(3,4,5)P_3$ resulting in the formation of $PI(3,4)P_2$ (Sly *et al*, 2003). SHIP molecules have been shown to translocate to the membrane in response to cellular stimulation (Phee *et al*, 2001) and is

likely to occur at sites of $PI(3,4,5)P_3$ production. PTEN and SHIP removal of a phosphate from $PI(3,4,5)P_3$ inhibits the PI3K pathway by preventing the localisation of proteins containing pleckstrin homology domains at the cell membrane.

1.2.5 Signalling networks downstream of PI3K – PKB/Akt

Several families of PH domain containing proteins mediate events downstream of PI3K activation. The most important and widely studied of which is the PKB/AKT family. This family has an important role in the coordination of cellular events downstream of PI3K including cell survival, cell proliferation, protein synthesis and cell cycle entry with the overall effect of increasing the survival potential of the cell.

PKB/Akt belongs to a subfamily of protein kinases termed the AGC (cAMP-dependent protein kinase A/ protein kinase G/protein kinase C) protein kinases that also includes protein kinase A (PKA) and protein kinase C (PKC). In fact, PKB/Akt was first identified as a novel protein kinase with high homology to PKA and PKC and was therefore referred to as PKB (Jones *et al*, 1991). Other studies also identified PKB/Akt to be the cellular homologue (*c-Akt*) of the retroviral oncoprotein (*v-Akt*), and was therefore also named Akt (Staal *et al*, 1977, Bellacosa *et al*, 1991). There are three mammalian isoforms of PKB/Akt encoded by separate genes, *Akt1*, *Akt2* and *Akt3*. The gene products (PKBα PKBβ and PKBγ) are conserved proteins of 57kDa that have a broad tissue distribution (Cooray, 2004). All isoforms are composed of three domains, an N-terminal PH domain, a central catalytic domain and a C-terminal hydrophobic regulatory domain. The domain structures of the three human PKB isoforms are illustrated in Figure 1.5.

1.2.5.1 Activation of PKB/Akt

Activation of PKB/Akt occurs rapidly after cell stimulation. Stimulation of RTKs results in the recruitment of PKB to the plasma membrane within minutes after ligand binding via a PH domain as described in section 1.2.1.

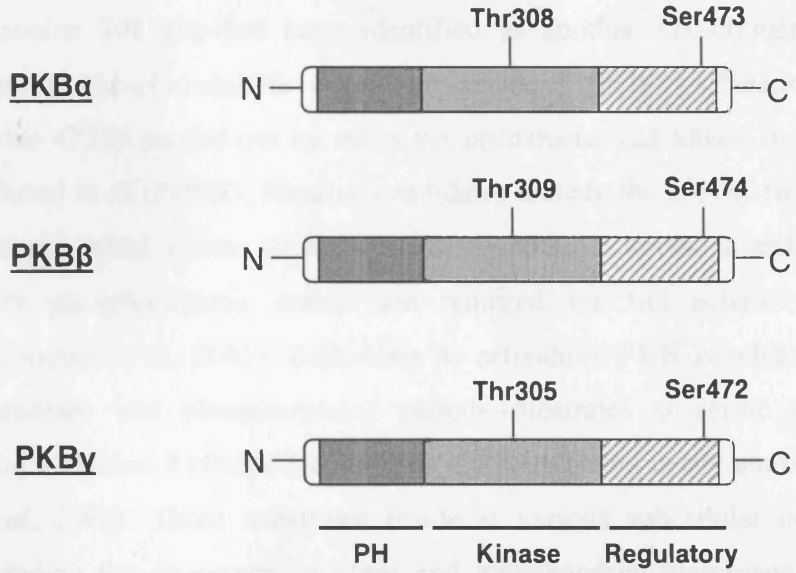


Figure 1.5 The domain structure of the human PKB isofroms.

Adapted from Osaki *et al*, 2004.

Each PKB isoform (α,β and γ) consist of three functional domains: The N-terminal pleckstrin homology (PH) domain that mediates binding to inositol phospholipids phosphorylated at the D3 position (see figure 1.3), a central kinase domain, and a C-terminal regulatory domain. Activation of all PKB isoforms require phosphorylation at two critical resides. The equivalent sites of phosphorylation on threonine (Thr) and serine (Ser) are indicated.

Mutations within the PH domain of PKB block the activation of PKB catalytic activity (Franke *et al*, 1995, Klippel *et al*, 1997). Once at the plasma membrane, PKB undergoes a conformational change that exposes two amino acids that become phosphorylated. In PKBα, these residues are threonine 308 located within the catalytic domain and serine 473 located within the hydrophobic region. The kinase responsible for phosphorylation at the threonine 308 site has been identified as another PH domain containing protein, phosphoinositide dependent kinase 1 (PDK1). Phosphorylation at serine 473 is carried out by an as yet uncharacterized kinase, which is often referred to as (PDK2). Putative candidates include the mTOR rictor complex, integrin-linked kinase (ILK), PKCβII, MAPKAP kinase 2 and PBK itself. Both phosphorylation events are required for full activation of PKB. (Hennessy *et al*, 2005). Following its activation, PKB is released from the membrane and phosphorylates various substrates at serine or threonine residues within RxRxxS/T consensus motifs, where x is any amino acid (Kane *et al*, 2002). These substrates reside at various subcellular compartments including the cytoplasm, nucleus and mitochondrial membrane and usually become inhibited by the phosphorylation event. PKB target proteins have diverse cellular functions that are involved in the regulation of cell survival, cell proliferation, cell growth, protein synthesis and progression through the cell cycle. A schematic of signalling downstream of PI3K/PKB activation is illustrated in Figure 1.6.

1.2.5.2 Regulation of cell survival by PKB.

The role of PKB in the mediation of cell survival pathways downstream of PI3K was established by several studies demonstrating the protective effect of PKB against apoptosis in epithelial, neuronal and myeloid progenitor cells (Khwaja *et al* 1997, Kauffman-Zeh *et al*, 1997, Dudek *et al*, 1997, Songyang *et al*, 1997). Subsequently studies into the molecular mechanisms by which PKB carries out this effect have led to the identification of several PKB targets involved in apoptosis.

One of the first substrates of PKB to be identified was the bcl-2 family member Bad. Bad promotes apoptosis by forming a heterodimer with the survival factor Bcl-X_L on the mitochondrial membrane inducing the release of cytochrome *c*. The phosphorylation of Bad by PKB prevents this interaction, allowing suppression of apoptosis by Bcl-X_L (Datta *et al*, 1997). The catalytic activity of another component of the apoptosis machinery, the pro-death caspase-9 has been shown to be inhibited by phosphorylation by PKB (Cardone *et al*, 1998). In addition, PKB has been shown to phosphorylate and activate MDM2 (murine double minute-2), a protein which regulates the activity of the pro-apoptotic tumour suppressor protein p53 (Mayo & Donner, 2001). MDM2 negatively regulates p53 by its targeting for degradation via the ubiquitin ligase proteasome pathway (Zhou *et al*, 2001a). Thus PKB promotes the destabilization of P53, thereby increasing the survival potential of the cell.

PKB also controls cell survival indirectly through the regulation of transcription. Phosphorylation of the forkhead box O (FOXO) family of transcription factors (FOXO1, FOXO3 and FOXO4) by PKB prevents their nuclear localization, where they direct the transcription of genes encoding several pro-apoptotic proteins including the ligand for the death receptor Fas - Fas-ligand (Fas-L), and the pro-apoptotic BH3 only protein, bim (Downward, 2004). The activities and the regulation of FOXO transcription factors by PKB are discussed in more detail in section 1.3. The IkappaB kinase α (IKKα) protein has also been identified to be a substrate for PKB (Ozes *et al*, 1999, Romashkova *et al*, 1999). IKK is activated in response to phosphorylation by PKB, resulting in the phosphorylation and subsequent degradation of its substrate IkappaB (IκB) through the proteasomal pathway (Kane et al, *1999*). IκB in its unphosphorylated form negatively regulates the activity of the nuclear factor-kappa-B (NFκB) transcription factor by anchoring the p50 subunit of NFκB to the cytoplasm. The degradation of IκB in response to PKB activation therefore leads to the release of the p50 subunit of NFκB. Once released from IκB, p50 NFκB can travel into the nucleus and form a functional transcriptional unit with its dimerization partner, p65 (also known as REL-A) (Li & Verma, 2002). This leads to the activation of NFκB, which directs the transcription of several antipoptotic genes including the bcl-2 family member,

Bcl-X_L, and the caspase inhibitors of apoptosis, *c-IAP1* and *c-IAP2* (Barkett *et al*, 1999, Caamano & Hunter, 2002).

The activity of the cAMP responsive element binding protein (CREB) transcription factor is also partly mediated by PKB phosphorylation. CREB mediates cyclic AMP (cAMP), growth factor and calcium dependent gene expression through binding to consensus cAMP responsive elements (CREs). CREB transcriptional activity is activated through phosphorylation of the critical ser 133 residue. This has been shown to occur in response to several protein kinases including protein kinase A (PKA), mitogen activated protein kinases (MAPKs), Ca^{2+}/calmodulin dependent protein kinases (CaMKs), and also PKB (Johannessen *et al*, 2004). This phosphorylation event has been shown to regulate the expression of several immediate early growth response genes such as *c-fos* and *junb* (Dey *et al*, 1991, Amato *et al*, 1996). Ser 133 phosphorylated CREB also binds to the promoters of anti-apoptotic genes such as *Bcl-2* and *Mcl-1* to regulate their expression (Wilson *et al*, 1996b, Wang *et al*, 1999, Joo *et al*, 2004)

1.2.5.3 Regulation of cell growth and proliferation by PKB.

Activated PKB modulates the function of numerous substrates related to the regulation of cell growth and proliferation. One mechanism by which PKB stimulates cell growth is by enhancing the activity of the mammalian target of rapamycin (mTOR) protein. mTOR is a serine/threonine kinase that acts as a molecular sensor and regulates protein synthesis according to nutrient availability (Bjornsti & Houghton, 2004). The kinase activity of mTOR is negatively regulated by a heterodimer consisting of hamartin and tuberin, encoded by the *tuberous sclerosis complex-1 (TSC1)* and *TSC2* tumour suppressor genes. PKB activation has been shown to induce the phosphorylation of tuberin (TSC2), which induces its dissociation from its binding partner, hamartin (TSC1), and promotes their degradation (Dan *et al*, 2002, Manning *et al*, 2002). This subsequently releases the kinase activity of mTOR, leading to the phosphorylation and subsequent activation of its substrates. The major targets of mTOR are the ribosomal protein S6 kinase

(p70S6K) and 4E-binding protein (4E-BP) which regulate components of the translational apparatus (Richardson *et al*, 2004). Thus stimulation via the PI3K/PKB pathway in the presence of sufficient nutrients stimulates the translation of proteins which are required for cell cycle progression from G_1 to S phase.

The first physiological substrate of PKB to be identified was glycogen-synthase kinase 3 (GSK-3) (Cross *et al*, 1995). PKB activation results in the phosphorylation and subsequent inhibition of GSK-3 activity. GSK-3 in its active form phosphorylates several proteins regulating cell cycle progression and proliferation to maintain them in an inactive state or to target them for degradation. Examples include the cytosolic signalling protein β-catenin and c-myc (Cantley, 2002). Prevention of β-catenin degradation by GSK-3 inactivation has been shown to induce its nuclear translocation, where it induces the expression of cyclin D1, mediating progression through the cell cycle (Diehl *et al*, 1998). Another mechanism whereby PKB can direct progression through the cycle is by phosphorylating and hence inactivating two cell cycle inhibitors, $p21^{cip1}$and $p27^{kip1}$. PKB mediated phosphorylation of both $p21^{cip1}$ and $p27^{kip1}$ induces their nuclear exclusion and subsequent cytosolic sequestration or proteasomal degradation (Zhou *et al*, 2001b, Liang *et al*, 2002, Shin *et al* 2002). This results in progression through the cell cycle and increased cellular proliferation due to the increased availability of active cyclin molecules.

1.2.6 PI3K in lymphocytes

In cells of the immune system, PI3K is activated by the binding of a ligand to antigen receptors, antibody receptors, cytokine receptors, and co-stimulatory molecules (Fruman & Cantley, 2002). In B-cells and T-cells, PI3K is activated within seconds of receptor ligation (Ward *et al*, 1993, Gold *et al*, 1994, Astoul *et al*, 1999, Costello *et al*, 2002). Activation through the BCR or TCR and co-stimulatory receptors such as CD19 on B cells and CD28 on T cells mediates the activation of class I_A PI3K (Okkenhaug *et al*, 2001, Wang *et al*, 2002).

Gene knockout studies in mice have provided information on the importance of specific isoforms of PI3K in the maintenance of a normal immune response. Investigations into the loss of the PI3K class I_A regulatory subunit demonstrated that mice lacking p85α, but retaining the expression of p50α and p55α were viable but had defects in B cell development and proliferative responses and were susceptible to infection (Fruman *et al*, 1999, Suzuki *et al*, 1999). T cell development and proliferation were unaffected in these mice (Fruman *et al*, 1999). A subsequent study has demonstrated enhanced T cell proliferation in mice lacking p85α (Deane *et al*, 2004), implying that PI3Ks have both positive and negative roles in leuokocytes. Mice lacking all three regulatory subunits died shortly after birth, suggesting that p50α and p55α expression are critical *in vivo*. Bi *et al* investigated the loss of isoforms of the p110 catalytic subunit of class I_A PI3K, and demonstrated that the loss of the p110α and p110β isoforms in mice is embronically lethal (Bi *et al*, 1999, Bi *et al*, 2002). The expression of the p110δ isoform is restricted to leukocytes and has therefore been proposed to play an important role in PI3K mediated signaling in the immune system (Chantry *et al*, 1997). Two independent investigations into the function of the p110δ by the generation of p110δ knockout mice demonstrated that this isoform is crucial in regulating B cell function (Clayton *et al*, 2002, Jou *et al*, 2002). In both studies mice lacking p110δ were viable but displayed reduced B cell numbers, reduced antibody responses, impaired B cell proliferation and defective PKB phosphorylation. Furthermore, mice expressing a catalytically inactive form of p110δ due to a point mutation ($p110^{D910A}$) display impaired B-cell and T-cell function and developed a mild form of inflammatory bowel disease (Okkenhaug *et al*, 2002). B cells and T cells purified from $p110^{D910A}$ mutant displayed reduced proliferation in response to IgM and anti-CD3 stimulation respectively (Okkenhaug *et al*, 2002).

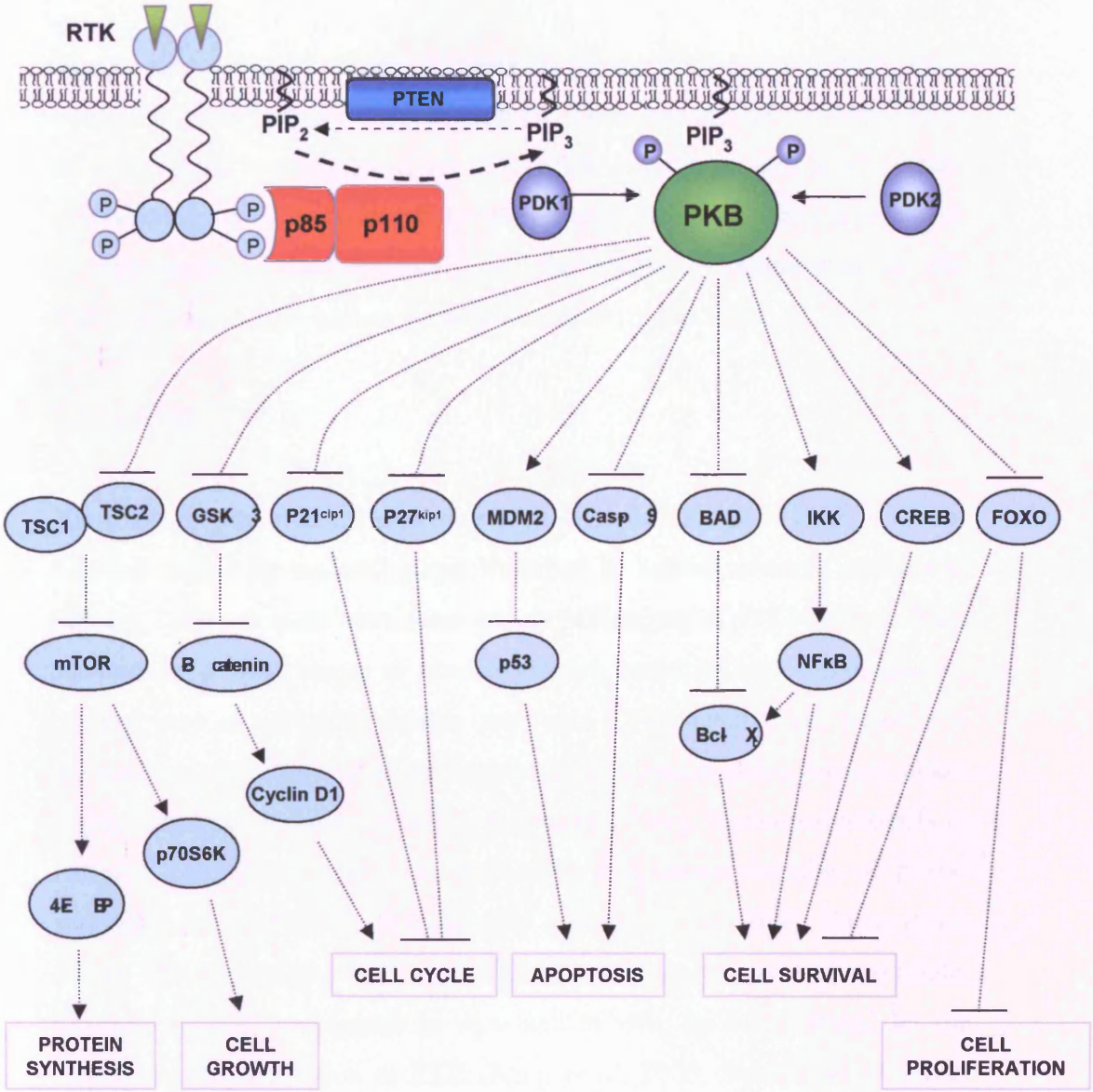


Figure 1.6 Signalling downstream of PI3K/PKB activation

Activation of class I_A phosphotidylinositol-3-kinases (PI3Ks) (p85-p110) occurs through stimulation of receptor tyrosine kinases (RTKs) and the concurrent assembly of receptor-PI3K complexes at the plasma membrane. When localized at the membrane, the p110 catalytic subunit of PI3K catalyses the conversion of $PI(4,5)P_2$ (PIP_2) to $PI(3,4,5)P_3$ (PIP_3). PIP_3 molecules serve as second messengers by recruiting and activating pleckstrin homology (PH) domain containing proteins (PKB, PDK1). PKB becomes fully activated once phosphorylated at two sites (Thr308 and Ser473 for PKBα) by PDK1 and PDK2. Activated PKB mediates the activation and inhibition of several target proteins involved in pathways that lead to protein synthesis, apoptosis and cellular growth, survival and proliferation. The formation of PIP_3 molecules at the plasma membrane is tightly regulated by two phosphatases (PTEN) that catalyses the conversion of PIP_3 to PIP_2, negatively regulating the activation of PI3K/PKB and downstream pathways.

1.2.7 PI3K and cancer

Genetic aberrations in several components of the PI3K pathway have been detected in a wide range of human cancers. These include, p85, p110α, PTEN and PKB. Viral modulation of the PI3K/PKB pathway is also an important mechanism by which viral transformation is achieved, and is often a precursor to tumorigenesis. The frequency of alterations in components of the PI3K/PKB pathway in human cancers is summarized in Table 1.2.

1.2.7.1 PI3K

The *PIK3CA* gene that encodes the p110α catalytic subunit of PI3K is one of the most frequently mutated genes identified in human cancers (Samuels & Ericson, 2006). Somatic mutations and amplifications of *PIK3CA* have been detected in a wide range of human cancers including colorectal, gastric, cervical, ovarian and breast cancers (see Table 1.2 for details). The majority of the mutations detected to date are heterozygous missense alterations and affect highly conserved residues (Samuels & Ericson, 2006). Mutational hotspots in *PIK3CA* have been mapped to the helical and kinase domains of p110α suggesting an increase in PI3K activity (Samuels *et al*, 2005, Bader *et al*, 2005). This is in accordance with studies reporting increased of enzymatic function of *PIK3CA* mutants *in vitro* and *in vivo*, as demonstrated by the increased phosphorylation of PKB (Kang *et al*, 2005, Samuels *et al*, 2005, Ikenoue *et al*, 2005). Furthermore, *PIK3CA* mutants have been shown to be oncogenic both *in vitro* and *in vivo* (Kang *et al*, 2005, Bader *et al*, 2006).

The p85 regulatory subunit of PI3K is also targeted for mutation in human cancer. A truncated form of p85 – p65 PI3K was originally isolated from transformed thymic cell lines and was shown to induce constitutive activation of PI3K contributing towards transformation (Jimenez *et al*, 1998). Mutations in the p85α domain are relatively rare but have been associated with the some cases of ovarian and colon cancer (Philp *et al*, 2001). A truncated form of p85 resulting from a frame-shift mutation has also been detected in a human lymphoma cell line derived from a patient with Hodgkins lymphoma (Jucker

et al, 2002). These alterations are believed to release the regulatory activity of p85, thereby promoting transformation by allowing increased survival signalling through p110 PI3K (Vivanco & Sawyers, 2002).

1.2.7.2 PTEN

Inactivation of the PTEN lipid phosphatase is the most common mechanism of activation of the PI3K pathway in human cancers. In fact, PTEN is the most commonly mutated tumour suppressor in humans after p53, demonstrating the importance of regulation of the PI3K/PKB pathway. Loss of PTEN hetreozygosity is detected in a significant proportion of several human cancers including glioblastoma, melanoma, prostate cancers, and some cases of B-cell chronic lymphocytic leukaemia (B-CLL) (see Table 1.2 for details). In addition, germline PTEN mutations have been detected in over 80% of individuals with Cowdens disease (Altomare & Testa, 2005), a familial disease which is associated with a high risk to develop breast, thyroid and endometrial cancers (Eng, 2003). Biallelic inactivation of PTEN is rarely detected in human tumours, indicating that PTEN haploinsufficiency is sufficient to promote tumour formation (Engelman *et al*, 2006). This model is supported by a study showing that a progressive reduction in PTEN gene dosage correlates with an increase in the aggressiveness of mouse prostate neoplasia (Trotman *et al*, 2003). In normal cells, PTEN activity works to tightly regulate the levels of $PI(3,4,5)P_3$ at the cell surface. Inactivating mutations or loss of heterozygosity of PTEN therefore leads to an increase in the basal levels of $P(3,4,5)P_3$ and these levels are maintained for a longer period of time following stimulation compared to wild type cells (Parsons, 2004). This in turn prolongs the activities of PH domain containing proteins that transduce survival and proliferative signals to the cell (Di Cristofano & Pandolfi, 2000).

1.2.7.3 PKB

No mutations of the gene encoding PKB - *Akt* have been reported in human cancers. However, *Akt* gene amplifications have been frequently detected in various human cancers. The first study to implicate PKB as a potential oncogene was the isolation of *AKT* as a retroviral oncogene (v-*Akt*) encoded by the genome of a murine lymphoma virus. The v-*Akt* was later found to be the viral homologue of cellular PKB (c-*Akt*) (Staal *et al*, 1977, Bellacosa *et al*, 1991). The same group detected *Akt1* amplification in primary human gastric carcinoma (Staal *et al*, 1987). Subsequently, *Akt2* gene amplifications have been detected in ovarian, breast, pancreatic and gastric cancers (Bellacosa *et al*, 1995, Cheng *et al*, 1996, Ruggeri *et al*, 1998) suggesting that *Akt2* amplification is a frequent event in human cancers. Up-regulation of *Akt3* mRNA has been reported in cases of breast and prostate cancers (Nakatani *et al*, 1999).

In addition to *Akt* amplifications, PKB overactivation occurs due to aberrant upstream molecules of PKB. *PIK3CA* and *PTEN* mutations, the overexpression or mutations in RTKs and the overexpression of growth factors can all result in the overactivation of PKB (Altomare & Testa, 2005). A large body of data demonstrates that many solid tumours and haematological malignancies display an increase in the protein expression and catalytic activity of PKB, including breast, colon, ovarian, pancreatic and prostate cancers and acute myeloid leukaemia (Bellacosa *et al*, 2005). Increased PKB activity has been reported to be prevalent in high grade, late stage and metastatic tumours (Altomare & Testa, 2005). However, increased levels of phosphorylated PKB have also been detected in pre-neoplastic lesions (Tsao *et al*, 2003, Balsara *et al*, 2004), suggesting that deregulation of PKB can also be an early event during tumour formation. Furthermore, ectopic expression of constitutively active and wild type PKB are sufficient to induce oncogenic transformation *in vitro* and tumour formation *in vivo* (Cheng *et al*, 1997, Hutchinson *et al*, 2001, Malstrom *et al*, 2001, Mende *et al*, 2001, Sun *et al*, 2001, Majumder *et al*, 2003).

Table 1.2 Frequency of mutations in the PI3K/PKB pathway in cancers

Adapted from Engelman *et al*, 2006.

Genetic Mutations	Cancer	Percentage Frequency	References
PIK3CA (p110α)			
Mutations	Breast	26%	Samuels *et al*, 2004 Wu *et al*, 2005a Levine et al, 2005 Lee *et al*, 2005 Bachman *et al*, 2004 Campbell *et al*, 2004 Saal *et al*, 2005
	Colon	26%	Samuels *et al*, 2004 Velho *et al*, 2005
	Glioma	8%	Samuels *et al*, 2004 Hartmann *et al*, 2005 Knobbe *et al*, 2005
	Hepatocellular	36%	Lee *et al*, 2005
	Ovarian	10%	Campbell *et al*, 2004 Levine *et al*, 2005
	Gastric	7%	Samuels *et al*, 2004 Lee *et al*, 2005 Velho *et al*, 2005 Li *et al* 2005
Amplifications	Head and Neck	42%	Pedrero *et al*, 2005 Woenckhaus *et al*, 2002
	Thyroid	9%	Wu *et al*, 2005a
	Squamous cell	66%	Pedrero *et al*, 2005 Woenckhaus *et al*, 2002
	Breast	9%	Wu *et al*, 2005b
	Gastric	36%	Byun *et al*, 2003
	Cervical	69%	Ma *et al*, 2000
PTEN			
Loss of heterozygosity	Glioblastoma	54%	Wang *et al*, 1997 Chariello *et al*, 1998 Smith *et al*, 2001
	Prostate	35%	Cairns *et al*, 1997 Feilotter *et al*, 1998 Pesche *et al*, 1998 Gray *et al*, 1998
	Breast	23%	Feilotter *et al*, 1999 Freihoff *et al*, 1999
	Melanoma	37%	Birck *et al*, 2000 Celebi *et al*, 2000 Reifenberger *et al*, 2000 Pollock *et al*, 2002
	Gastric	47%	Byun *et al*, 2003
	B-CLL	28%	Leupin *et al*, 2003
PKB			
Amplifications	Ovarian	12%	Bellacosa *et al*, 1995 Cheng *et al*, 1992
	Pancreatic	20%	Ruggeri *et al*, 1998
	Breast	3%	Bellacosa *et al*, 1995
	Gastric	20%	Staal *et al*, 1987
	Head and Neck	30%	Pedrero *et al*, 2005
PIK3R1 (p85α)			
Mutations	Ovarian	4%	Philp *et al*, 2001
	Colon	2%	Philp *et al*, 2001

1.2.7.4 Viral activation of PI3K

Viruses have long been implicated in the pathogenesis of several human cancers. Viral transformation is often achieved through modulation of host cell signalling pathways, particularly those that mediate cell survival and proliferation. The PI3K/PKB pathway is utilized to this effect by several viruses and is an important mechanism by which viruses achieve inhibition of apoptosis during acute infection, long-term virus survival and transformation (Cooray, 2004). Epstein-Barr Virus (EBV), human T-cell leukaemia virus 1 (HTLV-1) and human papilloma virus (HPV) all have the capacity to modulate PI3K. In the case of EBV, several gene products have been shown to activate PI3K activity to increase the survival potential of infected B cells during latent phase, and to ensure reactivation of the lytic phase for viral propagation (Cooray, 2004). The activation of PI3K by EBV is discussed in more detail in section 1.2.8 below. The high risk HPV type 16 (HPV-16), which is frequently detected in cervical and urogenital cancers, encodes a putative integral membrane protein, E5, that enhances signalling through PI3K/PKB (Zhang *et al*, 2002a). The ability of the HTLV-1 encoded protein Tat to induce cellular transformation has also been attributed to its capacity to enhance the activity of PI3K and contributes towards the development of adult T-cell leukaemia (Liu *et al*, 2001).

1.2.8 PI3K and Epstein-Barr Virus (EBV)

Several studies have implicated a role for PI3K in the survival and proliferation of EBV transformed cell lines and EBV associated tumours. Constitutive activation of the PI3K/PKB pathway has been detected in the EBV associated B cell lymphoma, Hodgkin's Disease (Dutton *et al*, 2005, Nagel *et al*, 2005, Georgakis *et al*, 2006) and nasopharyngeal carcinoma, an EBV associated malignancy of epithelial origin (Morrison *et al*, 2004). Activation of the PI3K/PKB pathway may contribute significantly towards cell survival and the morphological changes observed during B cell transformation by EBV, as inhibition of PI3K has been shown to reverse the transformed phenotype (Dawson *et al*, 2003). Furthermore, inhibition of PI3K in EBV

immortalized B cell lines induces growth arrest due to a decrease in the expression of two cyclins required for cell cycle progression, cyclin D2 and cyclin D3, and an increase in the expression of the cell cycle inhibitor, $p27^{kip1}$ (Brennan *et al*, 2002).

A number of studies have implicated a role for two EBV encoded latent transmembrane proteins in the constitutive activation of PI3K. Latent membrane protein-1 (LMP1) has been demonstrated to induce constitutive activation of PI3K in epithelial (Dawson *et al*, 2003) and fibroblast cells (Maniou *et al*, 2005). This increase in PI3K activity correlated with increased cell survival in epithelial cells and contributed towards the transformation of both human and rodent fibroblasts by EBV. The cytosolic C-terminal activating region-1 (CTAR1) has been suggested to be essential for these effects (Dawson *et al*, 2003, Maniou *et al*, 2005). This may be due to an interaction between CTAR1 of LMP1 and the p85 regulatory subunit of PI3K (Dawson *et al*, 2003). Another EBV encoded latent membrane protein, LMP2A, has also been demonstrated to induce constitutive signalling through the PI3K/PKB pathway in B cells (Swart *et al*, 2000) and epithelial cells (Scholle *et al*, 2000, Morrison *et al*, 2003). A subsequent study demonstrated that LMP2A inhibits Transforming Growth Factor – β1 (TGF-β1) mediated apoptosis through activation of the PI3K/PKB pathway in a Burkitt's lymphoma cell line (Fukuda & Longnecker, 2004). Another component of the PI3K pathway, mTOR has also been shown to be activated by LMP2A in epithelial carcinoma lines (Moody *et al*, 2005). In addition, B cells from LMP2A transgenic mice are sensitive to apoptosis in the presence of PI3K and PKB inhibitors (Portis & Longnecker, 2004a). Collectively, these studies indicate a role for EBV induced activation of the PI3K/PKB pathway in regulating the survival of EBV transformed cells.

1.2.9 Pharmacological inhibitors of PI3K

Pharmacological inhibitors of PI3K have contributed towards our understanding of the biological role of PI3Ks and their substrate proteins. Two inhibitors have been used extensively in this context. Wortmannin is a

hydrophobic fungal metabolite isolated from *Taloromyces wortmannii*, that irreversibly inhibits PI3K activity (Ui *et al*, 1995,). Wortmannin has proved useful in investigating PI3K regulated signal transduction pathways. However, this inhibitor has been shown to have several targets (Nakanishi *et al*, 1995, Ferby *et al*, 1996). Other limitations of wortmannin are its short half life and instability in aqueous solution. LY294002 is structurally distinct PI3K inhibitor developed as a synthetic analogue of quercetin, a naturally occurring bioflavinoid. It is an ATP-competitive inhibitor and is therefore reversible, but is more stable in solution and has a longer half life compared to wortmannin (Vlahos *et al*, 1994). Characterisation of the specificity of LY294002 indicated that this compound is specific for PI3Ks (Vlahos et al, 1994). Neither LY294002 nor wortmannin are isoform specific, and can therefore inhibit other members of the PI3K superfamily other than class I_A PI3K (Finan & Thomas, 2004). Despite their limitations, both of these compounds have proved to be useful tools for the study of PI3K signalling in mammalian cells, and have been used to implicate PI3K in cell survival and proliferation in a wide variety of systems.

1.3 FOXO transcription factors.

The FOXO transcription factors belong to the large family of functionally diverse Forkhead proteins that act as transcriptional regulators. Forkhead proteins are characterized by the presence of a highly conserved monomeric 110 amino acid DNA binding domain that is also known as the 'forkhead box' (FOX) (Kauffmann & Knochel, 1996). Over a 100 members of the forkhead transcription factor family have been identified in species ranging from yeast to humans, and 39 distinct members have been identified in humans to date. Forkhead proteins have been divided into 19 subgroups according to phylogenetic analysis, FOX A-S, and are therefore classified according to structure rather than function (Greer & Brunet, 2005). Among the forkhead family, only the FOXO subclass has been demonstrated to be regulated by the PI3K/PKB pathway (Burgering & Medema, 2003). FOXO proteins are highly conserved and have been identified in a range of organisms including *Caenorhabditis elegans*, zebrafish, *Drosophila*, mouse, rat and humans. The FOXO subgroup contains four members, FOXO1, FOXO3, FOXO4 and FOXO6, which contain a unique five amino acid insertion (GDSNS) immediately prior to the DNA binding helix (H3) within the forkhead domain. FOXO transcription factors are expressed in an overlapping range of human tissues (Anderson *et al*, 1998, Biggs *et al*, 2001) and have important roles in several biological processes including cell cycle arrest and apoptosis (Greer & Brunet, 2005).

1.3.1 Regulation of FOXO transcriptional activity by PI3K/PKB.

Genetic studies in *C.elegans* initially established a role for the PI3K/PKB pathway in the regulation of daf-16, a member of the FOXO subfamily, in development and longevity (Lin *et al* 1997, Ogg *et al*, 1997). Subsequent studies in mammalian cells have shown that PKB directly phosphorylates FOXO transcription factors in response to activation of the PI3K pathway by growth factors and hormones (Biggs *et al*, 1999, Brunet *et al*, 1999, Kops & Burgering 1999, Rena *et al*, 1999, Tang *et al*, 1999, Takaishi *et al*, 1999).

1.3.1.1 FOXO1, FOXO3 and FOXO4.

Phosphorylation of FOXO1, FOXO3 and FOXO4 by PKB occurs at three key conserved regulatory sites (Thr24, Ser253 and Ser 316 in the FOXO1 sequence) located within the consensus sequence for PKB phosphorylation (RxRxx(S/T)) (Alessi *et al*, 1996, Arden, 2006). The first PKB phosphorylation motif is located in the region immediately downstream of the start codon, the second is located in the forkhead domain, and the third is in the region downstream of the forkhead domain (Jacobs *et al*, 2003). These phosphorylation events inhibit the ability of FOXO transcription factors to transactivate target genes (Brunet *et al*, 1999, Kops *et al*, 1999, Biggs *et al*, 1999, Rena *et al*, 1999).

The mechanism whereby FOXO1, FOXO3 and FOXO4 activity is suppressed by PKB has been elucidated (Biggs *et al*, 1999, Brunet *et al*, 1999). In the absence of cellular stimulation and PKB activity, these FOXO factors are predominantly localized within the nucleus where they are capable of inducing the expression of target genes and are therefore presumed to be active. However, upon activation of PKB by growth or survival factors, FOXO phosphorylation triggers the rapid relocalization of FOXO proteins from the nucleus to the cytoplasm. Jacobs *et al*, observed that epithelial cells transfected with GFP-tagged FOXO1 and FOXO3 constructs displayed predominantly cytosolic localization of FOXO1 and FOXO3 molecules in 80% of cells in response to growth factor stimulation for 24 hours. Subsequent serum starvation resulted in the predomninant nuclear localization of these proteins (Jacobs *et al*, 2003). The requirement for PI3K for FOXO nuclear exclusion was demonstrated by a studies showing that the inhibition of PI3K with LY294002 dramatically impairs FOXO nuclear export (Brownawell *et al*, 2001) and leads to increased active nuclear FOXO (Chandramohan *et al*, 2004). The change in the subcellular localization of FOXO transcription factors to the cytoplasm prevents their ability of to bind and transcriptionally activate target gene sequences. Phosphorylated FOXOs have been demonstrated to specifically interact with 14-3-3 proteins in the nucleus, and function as chaperone molecules to promote FOXO nuclear exclusion (Brunet

et al, 1999, 2002). A schematic of the regulation of FOXO transcriptional activity by PKB is illustrated in Figure1.7.

The precise mechanism whereby 14-3-3 proteins mediate FOXO1, FOXO3 and FOXO4 nuclear exclusion is currently unclear. The binding of 14-3-3 proteins to FOXO proteins has been proposed to decrease the ability of FOXO factors to bind DNA (Cahill *et al*, 2001). It has also been suggested that a conformational change in FOXO, induced by the binding of 14-3-3, may expose the nuclear export signal (NES), allowing interaction with the export machinery protein, Exportin/Crm1 (Brunet *et al*, 2002). The phosphorylation of FOXO factors has been shown to induce an interaction with Exportin/Crm1 and the small GTPase, Ran, a regulator of Exportin/Crm1 activity , thereby accelerating the relocalization of FOXO factors to the cytoplasm (Rena *et al*, 2002). Once, in the cytoplasm, 14-3-3 binding may play a role in the cytoplasmic sequestration of FOXO factors by masking the nuclear localization signal (NLS) (Brownawell *et al*, 2001, Rena *et al*, 2002), maintaining FOXO factors in an inactive state. Protein phophatases involved in the dephosphorylation of FOXO transcription factors to counteract the effect of PKB have not been identified. Proteasomal degradation is an additional mechanism whereby FOXO1 and FOXO3 transcription factors are irreversibly inactivated. This is mediated by the ubiquitin-dependent proteasome pathway and is dependent on phosphorylation by PKB (Matsuzaki *et al*, 2003, Plas & Thompson, 2003, Aoki *et al*, 2004, Hu *et al*, 2004, Huang *et al*, 2005). Whether ubiquitination plays a role in the degradation of FOXO4 remains to be examined.

1.3.1.2 FOXO6

The regulation of the latest member of the FOXO family to be identified, FOXO6, by PKB appears to be distinct from that observed for the other FOXO members (Jacobs *et al*, 2003). This protein contains the first and second regions containing PKB phosphorylation motifs, but lacks the third, located in the region downstream of the forkhead domain in the other FOXO members. FOXO6 remains predominantly nuclear in the presence and absence of growth

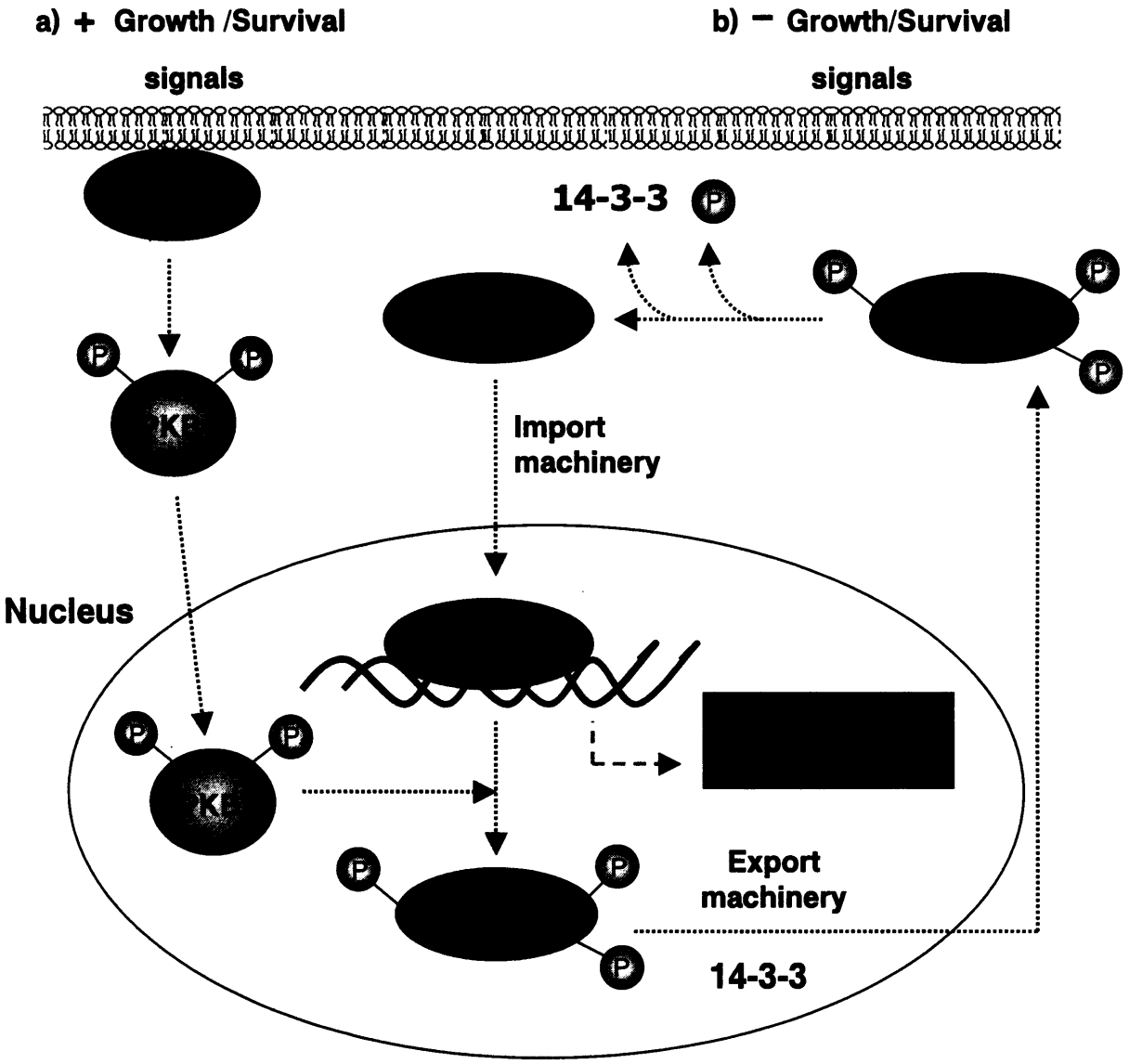


Figure 1.7 Schematic of regulation of FOXO transcriptional activity by PKB. Adapted from Birkenkamp and Coeffer, 2003.

a) In the presence of growth factors and survival signals PI3K is active and results in the activation of PKB. Active PKB proteins phosphorylate FOXO proteins at three conserved sites, which induces the release of FOXO proteins from DNA target sequences. 14-3-3 proteins and export machinery mediate the translocation of phosphorylated FOXO proteins to the cytoplasm, where they are unable to bind target sequences and are therefore inactive.

b) In the absence of growth and survival signals, FOXO proteins are unphosphorylated and 14-3-3 proteins are released. Under these conditions, import machinery mediate the translocation of FOXO transcription factors into the nucleus where they are transcriptionally active and induce the transcription of genes regulating cell cycle arrest and apoptosis.

factor stimulation, and is therefore considered to be constitutively nuclear (Jacobs *et al*, 2003). Investigations into the transcriptional activities of FOXO6 have revealed that this protein does function as a transcriptional activator of FOXO target genes, but is not constitutively active (Jacobs *et al*, 2003, Van der Heide *et al*, 2005). Growth factor stimulation inhibits FOXO6 activity in a Thr26 and Ser184 dependent manner, but is independent of nuclo-cytoplasmic shuttling (Van der Heide *et al*, 2005).

1.3.2 PKB independent phosphorylation of FOXO

Although the main regulator of FOXO function is the PI3K/PKB pathway, additional pathways are also involved in regulating FOXO activity. The structurally related protein kinase, serum and glucocorticoid-stimulated kinase (SGK), also phosphorylates FOXO transcription factors at identical sites to PKB, but preferentially phosphorylates the C-terminal PKB motif (Brunet *et*, 2001). SGK activation is also dependent on PI3K (Kobayashi & Cohen, 1999, Park *et al*, 1999), and is primarily active within the nucleus (Buse *et al*, 1999). It has therefore been proposed that PKB and SGK may phosphorylate FOXO proteins at different locations within the cell (Burgering & Medema, 2003). FOXO1 has also been shown to be phosphorylated on two additional residues, Ser 322 and Ser 325, by casein kinase 1 (CSK1) (Rena *et al*, 2002). In addition, the Ser239 residue in the FOXO1 sequence can be phosphorylated by the dual tyrosine phosphorylated regulated kinase 1 (DYRK1), a member of the MAP kinase family (Woods *et al*, 2001). This phosphorylation event appears to prime phosphorylation by CSK1 (Rena *et al*, 2002). A more recent study has demonstrated that FOXO3 can be phosphorylated by IκB kinase-β (IKK-β), resulting in FOXO3 nuclear exclusion and proteasomal mediated degradation (Hu *et al*, 2004). These additional phosphorylation events can therefore also participate in the regulation of FOXO subcellular localization (Van der Heide *et al*, 2004). It has been proposed that the phosphorylation of distinct sites by various protein kinases may allow the differential activation FOXO proteins in distinct cellular contexts (Nakae *et al*, 2000). The structure and the phosphorylation sites of FOXO proteins are illustrated in Figure 1.8.

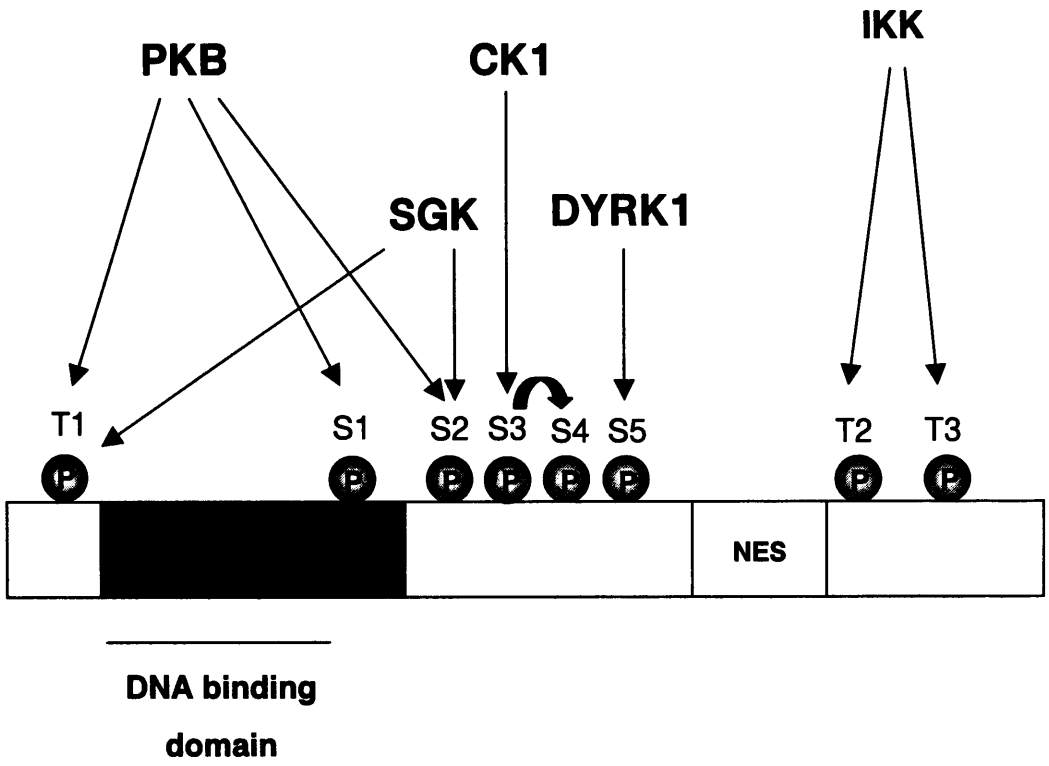


Figure 1.8 The structure and phosphorylation sites of the FOXO transcription factors.

Adapted from Arden, 2006.

FOXO transcription factors contain an N-terminal forkhead domain that mediates DNA binding to target sequences. This domain lies adjacent to a nuclear localization sequence (NLS). FOXO proteins also contain a nuclear export sequence (NES) which is located towards the C-terminal end of the protein. FOXO subcellular localization and transcriptional activity is regulated by phosphorylation in response to growth factor stimulation of various signalling pathways. The kinases responsible for FOXO phosphorylation and their threonine (T) and serine (S) target sites are indicated. PKB: Protein kinase B, SGK: Serum and glucocorticoid-stimulated kinase, CK1: Caesin kinase 1, DYRK1: Dual tyrosine phosphorylated regulated kinase 1, IKK: IκB Kinase.

1.3.3 Transcriptional targets of FOXO

When present in the nucleus, FOXO transcription factors act as transcriptional activators of a diverse set of genes involved in various cellular processes including cell cycle arrest and cell death (Greer & Brunet, 2005). Gene array analyses have indicated that FOXO proteins can also act as transcriptional repressors (Ramaswamy *et al*, 2002). The FOXO target genes mediating these effects are described below. A summary of identified FOXO target genes involved in cell cycle control and apoptosis is given in Table 1.3.

1.3.3.1 FOXO mediated regulation of cell proliferation

Studies in mammalian cells have shown that the overexpression of FOXO1, FOXO3 and FOXO4 causes a strong inhibition of cell proliferation (Collado *et al*, 2000, Medema *et al*, 2000, Nakamura *et al*, 2000, Kops *et al*, 2002). In the immune system, optimal proliferation of primary B cells requires the inactivation of FOXO transcription factors in a PI3K dependent manner (Yusuf *et al*, 2004). FOXO overexpression promotes cell cycle arrest at the G_1/S boundary in a variety of cell lines, including B cells (Yusuf *et al*, 2004), T cells (Fabre *et al*, 2005), Ras transformed and PTEN deficient cell lines (Medema *et al*, 2000, Nakamura *et al*, 2000). In several cases, this effect was demonstrated to be dependent on FOXO mediated transcriptional activation of the cyclin dependent kinase (cdk)-inhibitor, $p27^{kip1}$ (Medema *et al*, 2000, Nakamura *et al*, 2000 Yusuf *et al*, 2004). $p27^{kip1}$ regulates G_1/S transition through its cdk-inhibitory activity, which blocks the cell in G_1 phase by preventing cdk-dependent phosphorylation of the retinoblastoma protein (Rb), and subsequent progression through the cell cycle (Toyoshima & Hunter, 1994, Nakayama & Nakayama, 1998). $p27^{kip1}$ independent mechanisms for FOXO mediated cell cycle arrest also exist. Conditional activation of FOXO3a and FOXO4 can upregulate mRNA and protein expression of the Rb family member p130 (Kops *et al*, 2002). This effect was accompanied by cell cycle arrest and a sustained inhibition of cell proliferation. In its hypophosphorylated form, p130 binds to E2F transcription factor molecules, resulting in the repression of genes required for cell cycle progression (Smith

et al, 1996). The critical roles for $p27^{kip1}$ and p130 in FOXO mediated cell cycle arrest was further demonstrated as FOXO induced G_1 arrest is diminished in $p27^{kip1}$ and p130 deficient fibroblasts (Kops *et al*, 2002). FOXO factors are also capable of binding and transactivating the promoter of another cdk-inhibitor, $p21^{cip1}$, resulting in cell cycle arrest at the G_1 phase (Seoane *et al*, 2004).

Another mechanism whereby FOXO proteins mediate inhibitory effects on cell cycle progression is by transcriptional repression of D-type cyclins. D-type cyclins are critical mediators of cell cycle progression in response to mitogens, and are induced as quiescent cells are stimulated to enter the cell cycle. When present at sufficient amounts, D-type cyclins activate cdk activity, critical for G_1-S phase progression (Sherr, 1995). Constitutively active forms of FOXO1 and FOXO3, in which all three PKB phosphorylation sites are mutated, are strong inhibitors of cyclin D1 and cyclin D2 mRNA and protein expression (Ramaswamy *et al*, 2002, Schmidt *et al*, 2002). The overexpression of FOXO4 in mouse embryonic fibroblasts was shown to induce similar effects on cyclin D1 and cyclin D2 expression levels (Schmidt *et al*, 2002). A subsequent study by Fernandez de Mattos *et al* indicated that repression of cyclin D2 by FOXO3 may occur indirectly, via bcl-6 binding to the *Cyclin D2* promoter (Fernandez de Mattos *et al*, 2004). Bcl-6 is a transcriptional repressor which has itself been shown to be transcriptionally activated by FOXO4 (Tang *et al*, 2002). Therefore, the FOXO transcription factors play a major role in the inhibition of cell cycle progression by both upregulating cell cycle inhibitors ($p27^{kip1}$ and $p21^{cip1}$) and by transcriptionally repressing proteins required for cell cycle progression (Cyclin D1 and Cyclin D2).

1.3.3.2 FOXO mediated regulation of cell survival

In a number of cell types, particularly those of haematopoietic origin, FOXO transcription factors have been reported to induce apoptosis rather than cell cycle arrest. The regulation of programmed cell death is an important feature of the immune system in maintaining self-tolerance and the size of

haematopoietic subpopulations during immune responses. The expression of constitutively active forms of FOXO proteins have been reported to trigger cell death in some lymphocyte cell lines by the transcriptional activation of pro-apoptotic proteins (Brunet *et al*, 1999, Dijkers *et al*, 2002, Stahl *et al*, 2002). Bim, a pro-apoptotic bcl-2 family member is transcriptionally activated at the mRNA and protein levels by FOXO3 in cytokine dependent T and pro-B cell lines (Djikers *et al*, 2000, Stahl *et al*, 2002) as well as in neuronal cells (Gilley *et al*, 2003). This correlated with the induction of apoptosis. Furthermore, apoptosis induced by inhibition of the PI3K/PKB pathway is reduced in lymphocytes from bim-deficient mice, highlighting the role for bim as an important target of FOXO mediated induction of apoptosis (Bouillet *et al*, 1999, Greer & Brunet, 2005). The induction of bcl-6 expression by FOXO4 (Tang *et al*, 2002) demonstrates a further role for FOXO proteins in the regulation of bcl-2 family members. This induction of bcl-6 by FOXO4 was shown to negatively regulate the expression of the anti-apoptotic protein, Bcl-X_L. Therefore, one way in which FOXO factors regulate cell survival is by modulating the balance between pro and anti-apoptotic members of the bcl-2 family.

FOXO induced apoptosis in some cell types is also dependent on the induction of death cytokines. The Fas-Ligand (Fas-L) promoter, a member of the death inducing TNF family, contains putative FOXO binding sites and is responsive to FOXO3 activation in fibroblast cell lines (Brunet *et al*, 1999). FOXO1 and FOXO3 have also been demonstrated to upregulate TRAIL (Tumour-necrosis factor-related apoptosis-induced ligand) in prostate cancer cell lines (Modur *et al*, 2002). A subsequent study demonstrated that cytokine mediated inhibition of TRAIL is mediated by the phosphorylation of FOXO3 in various cytokine dependent haematopoietic cell lines (Ghaffari *et al*, 2003). Fas-L and TRAIL binding to death receptors result in activation of the caspase cascade leading to subsequent cell death (Wallach *et al*, 1999). These studies therefore indicate additional ways by which FOXO factors can influence cell death pathways.

Table 1.3 FOXO target genes regulating cell cycle control and cell death-
Adapted from Birkenkamp& Coffer, 2003

Target gene	Cellular function	References
Fas-Ligand (Fas-L) (↑)	Induction of death-receptor-mediated apoptosis	Brunet *et al*, 1999
Tumour-necrosis factor-related apoptosis-induced ligand (TRAIL) (↑)	Induction of apoptosis	Modur *et al*, 2002 Ghaffari *et al*, 2003
Bim (↑)	Induction of intrinsic apoptosis pathways	Dijkers *et al*, 2000 Stahl *et al*, 2002 Gilley *et al*, 2003
$p27^{KIP1}$ (↑)	Cell cycle inhibition – G_1 arrest	Dijkeres *et al*, 2000b Medema *et al*, 2000 Stahl *et al*, 2002
$p21^{CIP1}$ (↑)	Cell cycle inhibition – G_1 arrest	Seoane *et al*, 2004
p130 (↑)	Cell cycle inhibition – G_1 arrest	Kops *et al*, 2002
Cyclin B (↑)	Completion of cell cycle progression	Alvarez *et al*, 2001
Cyclin D1 (↓)	Cell cycle progression	Schmidt *et al*, 2002 Ramaswamy *et al*, 2002
Cyclin D2 (↓)	Cell cycle progression	Schmidt *et al*, 2002 Ramaswamy *et al*, 2002 Fernandez de Mattos *et al*, 2004
Cyclin G2 (↑)	Cell cycle inhibition – G_2 arrest	Ramaswamy *et al*, 2002 Martinez-Gac *et al*, 2004
Polo-like kinase (↑)	Completion of cell cycle progression	Alvarez *et al*, 2001
Bcl-6 (↑)	Transcriptional repression	Tang *et al*, 2002

↑ - Upregulated by FOXO ↓ - Downregulated by FOXO

1.3.4 FOXO transcription factors and cancer

Initial identification of the FOXO transcription factor family in humans occurred as three members were identified at chromosomal translocations in human tumours, namely FOXO1 in alveolar rhabdomyosarcomas (ARMS), FOXO3a in acute myeloblastic leukaemia (AML) and FOXO4 in acute lymphocytic leukaemias (Galili *et al*, 1993, Davis *et al*, 1994, Parry *et al*, 1994, Hillion *et al*, 1997, Borkhardt *et al*, 1997). These discoveries were the first indications that FOXO transcription factors have a role in tumour development.

These chromosomal translocations result in the formation of chimeric fusion proteins in which the C-terminal domains of FOXO transcription factors are fused to the N-terminal domain of other transcriptional regulators. These are paired box (PAX) 3 or PAX7 with FOXO1, and Mixed Lineage Leukaemia gene (MLL) with FOXO3 and FOXO4 (Galili *et al*, 1993, Davis *et al*, 1994, Parry *et al*, 1994, Hillion *et al*, 1997, Borkhardt *et al*, 1997, Anderson *et al*, 1998). The resulting fusion proteins retain the DNA binding domains of PAX3, PAX7 and MLL, whereas the FOXO transactivation domains are lost (Barr *et al*, 2001, Khan *et al*, 1999, Keller *et al*, 2004). Of the three PKB phosphorylation sites present in the intact FOXO proteins, only two are retained in the PAX3-FOXO1 and PAX7-FOXO1 fusion proteins. These proteins are therefore no longer regulated by PKB and are constitutively located in the nucleus (del Peso *et al*, 1999). The PAX3-FOXO1 fusion protein has been shown to be stronger transcriptional activators of PAX3 genes than the individual protein alone (Khan *et al*, 1999). These genes include myogenic transcriptional mediators such as *MyoD* and Myogenin, and the growth factor gene *Igf2*. It is therefore possible that these effects are responsible for the cancer phenotypes observed.

It has also been proposed that the loss of a functional FOXO allele may potentiate the tumourigenicity of the fusion proteins due to loss of its normal function in restraining cell cycle progression and promoting cell death pathways. This theory has not been supported by mouse model experiments as human PAX3-FOXO1 expression in transgenic mice is not sufficient to promote tumourigenesis (Anderson *et al*, 2001, Lagutina *et al*, 2002, Keller *et al*, 2004). Furthermore, tumour incidence was not increased in mice in which one FOXO1 allele has been knocked out and also expressed the PAX3-FOXO1 fusion protein (Keller *et al*, 2004). These experiments indicate that the loss of one FOXO1 allele is not sufficient to increase tumour incidence, and therefore suggests that additional events are required for tumuorigenesis, such as the loss of the remaining FOXO1 allele.

In addition to chromosomal translocations several other lines of evidence have indicated a role for FOXO transcription factors in tumour progression. A role

for the tumour suppressor, PTEN, in the negative regulation of FOXO transcription factors has been established and has therefore implicated a role for FOXO factors in tumour progression in PTEN deficient cells. This is supported by the observation that FOXO1 is cytosolic and therefore inactive in PTEN-negative renal and prostate carcinoma cells (Nakamura *et al*, 2000). The overexpression of FOXO proteins in PTEN-negative tumour cells mimics the effect of the restoration of a functional PTEN in these cells, inducing cell cycle arrest in the G_1 phase due to an increased expression of $p27^{kip1}$ (Medema *et al*, 2000, Nakamura *et al*, 2000). The overexpression of FOXO proteins in PTEN-deficient prostate cancer cells can also induce apoptosis through the direct activation of TRAIL (Modur *et al*, 2002). Furthermore, the expression of a constitutively active form of FOXO1 can reduce tumours in PTEN-deficient nude mice (Ramaswamy *et al*, 2002). The inactivation of FOXO transcription factors has therefore been proposed to be an important mechanism by which PTEN-deficient cells are transformed (Burgering & Kops, 2002). These studies also demonstrate that the nuclear exclusion of FOXO factors not only regulates cell proliferation and survival in normal cells but also contributes towards the pathogenesis of cancer (Nicholson & Anderson, 2002).

More recent studies have demonstrated that loss of FOXO activities due to protein degradation contributes towards cellular transformation of primary breast cancer tumours (Hu *et al*, 2004) and mouse primary lymphomas (Huang *et al*, 2005). Immunohistochemical staining of primary breast cancer specimens revealed that cytoplasmic localization of FOXO3 correlated with poor patient survival (Hu *et al*, 2004). FOXO1 phosphorylation also correlates with shorter survival of acute myeloid leukemia patients (Accili & Arden, 2004). A further role for another FOXO member, FOXO4, in breast cancer has been implicated, as the expression of a constitutively active form of FOXO4 in nude mice transplanted with cells expressing the breast cancer associated HER2 oncogene reduced tumour onset, size and progression (Yang *et al*, 2005). Furthermore, RNA interference mediated depletion of PI3K in breast cancer cell lines, activated FOXO transcription factors and induced G_1 phase cell cycle arrest and apoptosis (Regan-Shaw & Ahmad, 2006).

These studies highlight the potential of FOXO transcription factors as useful biomarkers for the identification of unfavorable clinical outcomes in certain cancers, such as AML and breast cancer. These studies also suggest that FOXO inactivation may play an important role in cellular transformation.

1.4 Aims of thesis

Pathways regulated by Phosphotidylinositide-3-kinase (PI3K) have emerged as important mediators of cell proliferation and survival. When altered, several components of this pathway have been identified to contribute towards a wide range of human malignancies. Several EBV-associated B-cell lymphomas have been associated with deregulated PI3K signalling. Although progress has been made in dissecting the pathway regulated by PI3K, the key components contributing to lymphocyte transformation have not been fully characterised. This thesis sought to investigate downstream targets of the PI3K pathway in lymphocytes in order further our understanding of the key components contributing to the transforming effects of PI3K, particularly within the context of EBV-associated malignancies.

(1) The first objective was to investigate the usefulness of employing a phospho-specific antibody, specific for the substrate consensus phospho-motif of Protein Kinase B (PKB), the main downstream effector of PI3K activation, to identify important targets of PI3K in transformed lymphocyte cell lines (Chapter 3).

(2) Following on from these experiments, the next aim was to develop the technology of two-dimensional electrophoresis with a view to study PI3K and EBV regulated proteins on a larger scale. The successful establishment of this technique would also allow the study of the post-translational modifications associated with PI3K and EBV regulated transcription factors (Chapter 4).

(3) In order to further characterise the nuclear targets of PI3K in EBV immortalized cells, the final objective of this thesis was to investigate the interplay between EBV and a pro-apoptotic transcription factor target of PI3K, FOXO1. This would further our understanding of the molecular changes occurring in lymphocytes in response to EBV infection, which have the potential to contribute towards malignancy (Chapter 5).

CHAPTER 2

MATERIALS AND METHODS

2.1 Tissue culture

2.1.1. Tissue culture media and reagents

RPMI-1640 without glutamine (Gibco BRL) was stored at 4°C

Foetal calf serum (FCS; PAA, Batch number A01229-367) was stored in 50 ml aliquots at -20°C.

Glutamine 200mM (Gibco BRL) was stored in 5 ml aliquots at -20°C and used as a 100x stock solution.

Penicillin/Streptomycin 5000U/ml and 5000µg/ml (Gibco BRL) was stored in 10ml aliquots at -20°C and used as a 50x stock solution.

1M HEPES pH7.2 (Sigma) was stored at 4°C

1x phosphate-buffered saline (PBS) was made up by dissolving 50 PBS tablets (Oxoid) in 5 litres of distilled water. 500 ml aliquots were sterilised by autoclaving and stored at room temperature.

Dimethyl sulfoxide (DMSO; Sigma) was stored at room temperature.

Mycophenolic acid (Sigma) was prepared as a 2mg/ml stock solution in analysis grade methanol (Fisher) and stored in aliquots at -20°C.

Xanthine/Hypoxanthine (Sigma) was prepared as a 160µg/ml and 10µg/ml respectively stock solution in filter sterilised 0.2M NaOH and stored in aliquots at -20°.

G418 (Sigma) was prepared as a 100mg/ml stock solution in sterile distilled water and stored in aliquots at -20°C.

Tetracycline (Boeringer Mannheim) was prepared as a 1mg/ml stock solution in sterile PBS and stored in aliquots at -20°C.

Interleukin-2 (IL-2; Chiron, Proleukin) was prepared as a 100µg/ml stock solution in sterile RPMI-1640 without glutamine and stored in aliquots at -70°C.

2.1.2 Maintenance of cell lines

The cell lines used in this study are summarized in Table 2.1. All cell lines were cultured in growth medium comprising of RPMI-1640 medium, which was supplemented with 10% FCS, 2mM L-glutamine and antibiotics (100U of penicillin/ml and 100μg streptomycin/ml). The medium used to culture the Kit225 T-cell line was supplemented with 20ng/ml IL-2. Drug selection (G418) of stable cell lines was performed as specified by the creators of specific cell lines. Tetracycline was used to silence tTA-responsive genes as specified by the creators of specific cell lines. Up to 80% of each culture was removed three times a week and replaced with fresh growth medium pre-warmed to 37°C. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The EBV status of the BL41 + B95.8 cell line was checked by immunoblotting for two EBV encoded proteins – LMP1 and LMP2A (see sections 2.3 and 2.5).

2.2 Transient transfection of lymphoid cell lines

2.2.1 Plasmids and luciferase reporters.

The plasmids and luciferase reporter used in this study are summarised in Table 2.2.

2.2.2 Transient transfection

DG75 cells were transfected with plasmid DNA by electroporation to transiently express required exogenous genes. Cells were washed in pre-warmed growth medium and re-suspended at a concentration between $1.5x10^7$ and $2x10^7$ cells per ml in growth medium. A specific amount of plasmid DNA, typically between 1μg and 10μg, was pipetted into an electroporation cuvette (Biorad, 0.4mm gap) and a 0.5ml aliquot of the cell suspension was added. Cells were then electroporated at 300V and 950μF using a Biorad Genepulser II electroporator. Cells were then re-suspended in an appropriate volume of fresh pre-warmed growth medium in a 6-well plate, and incubated at 37°C in a humidified atmosphere containing 5% CO_2.

The transfection efficiency of DG75 cells was checked by transfection of cells with green fluorescent protein and subsequent analysis by flow cytometry. The mean value for transfection efficiency from three replicate experiments was 52%.

Table 2.1 Table of cell lines used.

Cell line	Description	EBV gene expression	Citation/Provider
BL41	EBV negative BL	N/A	Rowe *et al*, 1986
BL41 + B95.8	B95.8 transformed BL	Latency II/III	Rowe *et al*, 1986
IARC-171	B95.8 transformed LCL	Latency III	Rowe *et al*, 1986
Mutu I	EBV-positive BL	Latency I	Gregory *et al*, 1990
Mutu III	EBV-positive BL	Latency III	Gregory *et al*, 1990
BJAB gpt.1	EBV-negative B cell lymphoma	N/A	Wang *et al*, 1990
BJAB gpt.2	EBV-negative B cell lymphoma	N/A	Wang *et al*, 1990
BJAB MTLM.6	LMP1 expressing stable cell line	LMP1	Wang *et al*, 1990
BJAB MTLM.17	LMP1 expressing stable cell line	LMP1	Wang *et al*, 1990
DG75	EBV-negative BL	N/A	Ben-Bassat *et al*, 1977
DG75 tTA	EBV negative BL	N/A	Floettmann *et al*, 1996
DG75 tTA LMP1	Stable cell line with inducible LMP1 expression	LMP1	Floettmann *et al*, 1996
DG75 tTA LMP2A	Stable cell line with inducible LMP2A expression	LMP2A	Floettmann *et al*, 1996
DG75 tTA EBNA2A	Stable cell line with inducible EBNA2A expression	EBNA2A	Floettmann *et al*, 1996
Akata	EBV-positive BL	Latency I	Takada & Ono, 1989
AK31	EBV negative subclone of Akata	N/A	Jenkins *et al*, 2000
IB4	B95.8 transformed LCL	Latency III	Sample & Kieff, 1990
SP	B95.8 transformed LCL	Latency III	Generated at CUSM
EB	B95.8 transformed LCL	Latency III	Generated at CUSM
Kit 225	IL-2 dependent T-cell line	N/A	Hori *et al*, 1987

2.2.3 Luciferase reporter assay on transiently-transfected cells

Luciferase reporter assays were carried out using a Dual-Luciferase Reporter Assay system (Promega) according to the manufactures instructions as described below.

Luciferase assay

Approximately $5x10^6$ of transfected cells (half of transfection) were harvested, washed once in chilled PBS, and lysed in 100µl of 1x Passive lysis Buffer (Promega) for 20 minutes. The lysate was then clarified by centrifugation at 13,000rpm for 30 seconds in a microcentrifuge. 50µl of the lysate (supernatant) was assayed for luciferase activity. Light release was measured by Berthold LB9501 luminometer following injection of lysate with 100µl of Luciferase Assay reagent II (Promega). Light release was integrated for 10 seconds.

Table 2.2 Plasmids and luciferase reporters.

Name	Description	Citation
Plasmids		
pSG5-LMP1	Wild-type LMP1	Huen *et al*, 1995
pSG5-LMP2A	Wild type LMP2A	Longnecker *et al*, 1991
pSG5-LMP1^{AAA}	CTAR1 inactivated LMP1 mutant	Eliopoulos *et al*, 1999b
Luciferase reporter		
Bcl-6ΔBcl-6 -Luc	Bcl-6 reporter construct containing a FoxO consensus binding sequence upstream of a luciferase gene. The *Bcl-6* promoter sequence was mutated to lack the consensus Bcl-6 binding site in the *Bcl-6* gene promoter.	Tang *et al*, 2002

2.3 Cellular protein analysis

2.3.1 Preparation of whole cell extracts for SDS-PAGE

Reagents

2x gel sample buffer (GSB) contained 100mM Tris-HCL pH6.8, 20% glycerol, 0.2M DTT, 4% sodium dodecyl sulphate (SDS), 0.02% bromophenol blue. Aliquots of 2x GSB were stored at -20°C.

Cells were counted on a haemocytometer and re-suspended in 50µl of 1x PBS per 10^6 cells. An equal volume of 2x GSB was then added. Cells were sonicated using a W0385 sonicator (Hearsystems-Ultrasonics Inc.) and, following sonication, samples were heated at 100°C for 5 minutes on a dry heating block.

2.3.2 Preparation of cytosolic and nuclear extracts for SDS-PAGE

Reagents

Low salt detergent lysis buffer contained 10mM HEPES pH7.9, 1.5mM $MgCl_2$, 10mM KCl and 0.1% NP40 and was stored at room temperature.
High salt buffer contained 20mM HEPES pH7.9, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA and 25% glycerol and was stored at room temperature.

Nuclear and cytosolic extracts were prepared using a method previously described (Brennan and O'Neill, 1995). Extracts were prepared on ice. Cytosolic extracts were prepared by lysis of cells for 5 minutes in 100µl of low salt detergent lysis buffer per 10^7 cells. Low salt detergent lysis buffer was supplemented with 1mM phenylmethoslfonyl fluoride (PMSF), a 1:100 dilution of Phosphatase Inhibitor Cocktail I (Sigma) and a 1:100 dilution of

Phosphatase Inhibitor Cocktail II (Sigma). These supplements were added immediately prior to use due to their short half-lives. Following centrifugation (13,000 rpm, 5 minutes, 4°C), supernatant was collected (cytosolic extract). Nuclear extracts were prepared by incubating the pellet for 15 minutes in 60µl of high salt buffer per 10^7 cells. High salt buffer was supplemented with a 1:100 dilution of Phosphatase inhibitor cocktail I and a 1:100 dilution of Phosphatase inhibitor cocktail II immediately prior to use. Following centrifugation (13, 000rpm, 5 minutes, 4°C), supernatant was collected (nuclear extract). For extracts prepared from equivalent cell numbers, 50µl of 2x GSB was added to both the cytosolic and nuclear extracts. Alternatively, the protein concentration of the cytosolic and nuclear extracts was determined by a protein determination assay (section 2.3.3), prior to addition of an equal volume of 2x GSB to the extracts. Extracts were heated at 100°C for 5 minutes on a dry heating block.

2.3.3 Protein determination assay

Protein concentration was determined using a method based on that of Bradford (Bradford, 1976). Protein concentration standards were generated using a 1mg/ml BSA (bovine serum albumin; Sigma) solution. Doubling dilutions of the 1mg/ml BSA solution were prepared in a flat-bottomed 96 well plate. For the generation of a protein standard curve, triplicate 10µl aliquots of each BSA dilution was used. Three wells containing 10µl distilled H_2O were included for the generation of the standard curve. A fixed volume (2-5µl) of the nuclear or cytosolic extract was pipetted into the 96 well plate. Protein assay reagent (Biorad, 500-0006) was diluted 1 in 5 with distilled H_2O and 200µl was added to each standard and extract. The plate was incubated at room temperature for 5 minutes. A microplate reader (Biorad, 170-6850) was used to read the absorbance of each well at 570nm. The protein concentration of each extract was determined by plotting the OD_{570nm} of a protein standard curve on an Excel spreadsheet, from which the concentration of protein in each sample extract was calculated.

2.3.4 DNA affinity precipitation of nuclear proteins

The ability of various transcription factors to bind to consensus DNA sequences was investigated by a DNA affinity precipitation (DNA-AP) protocol. This method is a way of measuring protein-DNA binding. Streptavidin-conjugated agarose beads precipitate biotinylated oligonucleotide sequences from nuclear extracts of target cells. DNA affinity precipitation experiments were performed by a modification of a method previously described (Beadling *et al*, 1996).

Reagents

Dilution buffer contained 50mM Tris-HCl pH8, 0.25mM EDTA, 10mM NaF and 10% v/v Glycerol and was prepared when required.
Tris-EDTA (TE) contained 10mM Tris-HCl pH8 and 1mM EDTA and was stored at room temperature.

2.3.4.1 Generation of double stranded oligonucleotides

The forward and reverse oligonucleotide sequences used are summarised in table 2.3. The consensus binding sites are underlined. Oligonucleotides were purchased from MWG Biotech. The forward oligonucleotides were supplied with 5'-biotinylation, whereas the reverse complementary sequence was not modified with biotinylation. Double stranded oligonucleotides were prepared using a method previously described (Brennan & Athie-Morales, 2001). Oligonucleotides were diluted to a concentration of 1μg/μl in TE and equal volumes of the forward oligonucleotide and the complimentary sequence were mixed. Preparations were incubated for 10 minutes at 95°C in a water bath. The water bath was switched off and preparations were allowed to cool to room temperature. This gave a 1μg/μl stock solution. For working dilutions, preparations were diluted to 0.1μg/μl with TE buffer. Stock solutions were stored at -20°C. Working dilutions were kept at 4°C.

Table 2.3 Oligonucleaotides used for DNA Affinity precipitation.

Transcription factor	Oligonucleotide	Oligonucleotide sequence (Forward/reverse)
CREB	CREB consensus	5'-BIO-agagattgcctgacgtcagagagctag-3' 5'-ctagctctctgacgtcaggcaatctct-3'
CREB	Cyclin D2 consensus	5'-BIO-gggaggagagctaactgcccagccagcttgcgtcaccgcttcagagcgga-3' 5'-tccgctctgaagcggtgacgcaagctggctgggcagttagctctcctccc-3'
P65 NFκB	NFκB consensus	5'-BIO-agttgaggggactttcccagggc-3' 5'-gccctgggaaagtcccctcaact-3'
FoxO1	Bim	5'-BIO-cagagttactccggtaaacacgccagggac-3' 5'-gtccctggcgtgtttaccggagtaactctg-3'

2.3.4.2 DNA affinity precipitation

Cytosolic and nuclear extracts were prepared from a maximum of 10 million cells on ice as described in section 2.3.2. 50µl of nuclear extract was mixed with 950µl of dilution buffer supplemented with a 1:200 dilution of Phosphatase inhibitor cocktail I (Sigma), a 1:200 dilution of Phosphatase inhibitor cocktail II (Sigma), 0.5mM PMSF, 0.5mM NaVO4 and 5mM DTT. Supplements were added immediately prior to use due to their short half-lives. Nuclear proteins were subsequently incubated (4°C, 60 minutes, rotating) with 1µg of 5'-biotinylated double stranded oligonucleotide and 30µl of pre-washed streptavidin-conjugated agarose beads (50% slurry in PBS; Sigma, s-1638) to allow binding of proteins to DNA. 1µg of non-specific (STAT1) non-biotinylated double stranded oligonucleotide was also included in the mixture to favour the binding of low affinity proteins to reduce non-specific binding of proteins to the 5' biotinylated oligonucleotide. Oligonucleotide conjugated beads were collected by centrifugation (4°C, 6000rpm, 5 minutes) and complexes were washed three times using dilution buffer supplemented with 0.5mM PMSF, 40mM NaCl and 5mM DTT immediately prior to use. DNA binding proteins to be analysed by 1-dimensional SDS-PAGE were eluted from the DNA by the addition of 2x GSB and heating at 100°C for 5 minutes on a dry heating block. DNA binding proteins to be analysed by 2-dimentional

(2D)-electrophoresis were eluted by the addition of 2D sample buffer (7MUrea, 2M Thiourea, 2% w/v CHAPS) and incubation at room temperature for 1 hour.

2.4 Protein analysis by 2D-electrophoresis (2DE)

2.4.1 Protein precipitation and removal of interfering substances.

Prior to isoelectric focusing (IEF) protein samples were cleaned using a 2D Clean-up kit (Amersham) according to manufactures instructions. This procedure precipitates proteins and eliminates interfering substances that may interfere with IEF such as detergents, salts, lipids, phenolics and nucleic acids.

Nuclear protein extracts were generated as described in section 2.4.2. A nuclear protein sample containing 100µg of protein was mixed with 300µl of Precipitant (Amersham) and incubated on ice for 15 minutes. 300µl of Co-precipitant (Amersham) was subsequently added to the mixture and was mixed briefly by vortexing. The sample was centrifuged at 13,000rpm for 5 minutes and the supernatant was carefully removed without disturbing the pellet. 40µl of co-precipitant was layered on top of the pellet and was left to incubate on ice for 5 minutes. The sample was centrifuged at 13,000 rpm for 5 minutes and the wash was removed and discarded. 25µl of distilled water was added to the remaining pellet and the sample was vortexed until the pellet was fully dispersed. 1 ml of pre-chilled Wash Buffer (Amersham) containing 5µl of wash additive (Amersham) was added and incubated at -20°C for 1 hour with vortexing every 10 minutes. The precipitated protein pellet was collected by centrifugation for 13,000rpm for 5 minutes. The pellet was subsequently re-suspended in an appropriate volume of 2D sample buffer that allowed complete solubilisation of the protein pellet (typically 80-100µl), and were incubated at room temperature for 1 hour before use or storage. Samples were stored at -20°C.

2.4.2 Resolution of protein samples by Isoelectric-focusing (IEF)

Isoelectric focusing (IEF) of proteins was carried out using Immobilized pH gradient (IPG) strips (Amersham). The pH gradients used in this study are summarized in Table 2.4.

Reagents

2D sample buffer contained 7M Urea (Sigma), 2M Thiourea (Sigma) and 2 or 4% w/v CHAPS (Sigma). Stocks were stored as 1 ml aliquots at -20°C for no longer than 2 months.

Dithiothreitol (DTT) (Sigma) was supplied as a 1M solution and was stored as 200µl aliquots at -20°C.

Immobline pH gradient (IPG) buffers (Amersham) were stored at 4°C.

Bromophenol blue (Sigma) was prepared as a 0.01% w/v solution in distilled water

7cm Immobiline Drystrip Gels (IPG) (Amersham) were rehydrated, for 12 hours at 20°C in Ettan IPGphor Strip Holders (Amersham), with an appropriate amount of protein sample, typically between 10-15µg, in a total volume of 125µl of 2D sample buffer supplemented with DTT (20-50mM), 1% bromophenol blue and 0.5% v/v IPG buffer (Amersham). Isoelectric focusing of the samples was performed on the Ettan IPGphor II IEF system (Amersham) using the following program: 1 hour at 500V; 2 hours at 1000V (gradient); 1 hour at 1000V, 2 hours at 8000V (gradient); 8 hours at 8000V. IPG strips were subsequently equilibrated for 15 minutes in equilibration buffer (1X NuPAGE LDS sample buffer, Invitrogen) containing 0.5 ml 10X NuPAGE sample reducing agent (Invitrogen). The focused IPG strips were then equilibrated for 15 minutes in equilibration buffer containing 125mM iodoacetamide (Sigma). Equilibrated IPG strips were transferred to the IPG well of NuPAGE 4-12% Bis-Tris Zoom gels (Invitrogen) (Table 2.5) for separation in the second dimension by SDS-PAGE as described in section 2.6.3.

Table 2.4 Immobiline Drystrip gels and IPG buffers used for IEF

Immobiline Drystrip Gel (7cm)	IPG buffer
pH 3-10 non-linear	pH 3-10 nonlinear
pH 5.6-8 linear	pH 6-11
pH 4-7 linear	pH 4-7

2.5 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and western blotting.

Protein analysis by SDS-PAGE was carried out using two methods. Gels were poured using the Mini protean II SDS-PAGE apparatus (Biorad). Alternatively, the NuPAGE^{R}-Novex Pre-cast system (Invitrogen) was used. The NuPAGE-Novex pre-cast gels used are summarised in table 2.5.

2.5.1 Solutions and Buffers

Pre-stained molecular weight markers (Invitrogen, 10748-010 and LC5925) were supplied as ready-to-use protein markers and were stored in aliquots at -20°C.

Acrylamide solution (Merck) was purchased as a ready mixed 40% stock containing acrylamide and bis-acrylamide at a ratio of 37.5:1 and stored at 4°C.

Ammonium persulphate (APS) was prepared as a 15% solution (w/v) in distilled water and stored at -20°C.

Resolving gel buffer was prepared as a 4x stock solution containing 1.5M Tris HCL pH8.8, 0.24% v/v TEMED, 0.4% w/v SDS. This 4x stock was stored for up to one month at 4°C.

Stacking gel buffer was prepared as a 2x stock solution containing 0.25M Tris-HCl pH6.8, 0.12% v/v TEMED, 0.4%w/v SDS. This 2x stock was stored for up to one month at 4°C.

Electrophoresis running buffer for poured gels was prepared as a 10x stock solution containing 0.25M Tris-base (USB), 1.92M glycine (Fisher), 1% SDS. The pH of the running buffer was pH8.3, and was not adjusted with HCl. This 10x stock solution was stored at room temperature.

Electrophoresis running buffers for pre-cast gels (Invitrogen) were diluted appropriately to 1x for use and were stored at room temperature.

Transfer buffer was prepared as a 1x working solution containing 25mM Tris-base, 192mM glycine, 20%w/v analysis grade methanol (Fisher). This 1x working solution was stored at room temperature.

1X PBS (phosphate-buffered saline) was prepared by dissolving 50 PBS tablets (Oxoid) in 5 litres of distilled water. The 1 x PBS stock solution was stored at room temperature.

PBS-Tween contained 0.1% Tween-20 detergent (v/v) in 1x PBS and was stored at room temperature.

Blocking buffer was prepared as a 1 x working solution containing 0.2% I-block (Tropix. Inc), 0.1% v/v tween-20 and 0.02% v/v sodium azide (NaN_2) in PBS. 500ml of distilled water was heated in a microwave until boiling and 5 PBS tablets were added. 1g of I-block was then dissolved in the heated PBS by stirring on a magnetic mixer. After the I-block had dissolved, the solution was cooled before 0.5ml of Tween-20 and 0.5ml of NaN_2 were added. The blocking buffer was stored for up to 1 month at 4°C.

Alkaline phosphatase assay (APA) buffer (Tropix Inc.) was prepared as a 10 x stock solution containing 1M diethanolamine pH 9.5 and 10mM $MgCl_2$. 10 x APA buffer was stored for up to one month at 4°C.

CDP-Star development reagent (Tropix Inc.) was supplied as a ready to use solution and was stored at 4°C. It is an alkaline phosphatase substrate for use in chemiluminescent detection protocols.

MESNA stripping buffer was prepared as a 1 x working solution containing 6.25mM Tris-HCl pH 6.8, 2% w/v SDS and 50mM 2-mercaptoethansulfonate (MESNA; Sigma). MESNA stripping buffer was kept for no longer than two weeks and was stored at 4°C.

2.5.2 Equipment

Polyethylene lay flat film 204mm (Jencons).
Hypobond-P polyvinylidene diflouride (PVDF) membrane (Amersham)
Chromatography paper 3MM Chr (Whatman)
P200 gel loading tips (Greiner)
Mini protean II SDS-PAGE apparatus (Biorad)
Mini protean 1mm glass plates, spacers, combs and stand (Biorad)
X-cell SureLock Mini-cell SDS-PAGE apparatus (Invitrogen)
Mini-trans-blot blotting apparatus (Biorad)
Powerpac 300 power suppliers (Biorad)
X-omat LS Kodak film 18 x 24 cm (Amersham)

2.5.3 Resolution of protein samples by SDS-PAGE

Poured gels were prepared by mixing resolving gel buffer (2.8ml), 40% acrylamide (3.4ml), 15% APS (38µl) and distilled water (5.1ml). This gave a resolving gel of 12% and the volumes given were sufficient to pour two polyacrylamide resolving gels. The mixture was poured into a mini protean gel apparatus and a layer of water- saturated butan-2-ol was pipetted over the gel to ensure an even and flat level interface and to assist setting of the polyacrylamide gel. Resolving gels were left to set at room temperature for 1 hour. Once the resolving gels was set, the layer of butan-2-ol was poured off and the surface of the gel was rinsed with distilled water with any remaining excess water absorbed with paper tissues. Sufficient stacking gel for two mini-gels was prepared with distilled water (3.1ml), stacking gel buffer (3.9ml), 40% acrylamide solution (0.8ml), 15% APS (78µl) and TEMED (8µl). The stacking gel was poured over the solidified resolving gel and a comb was inserted to form the wells of the gel. The stacking gel was left to set for 30 minutes. When the stacking gel was set, the combs were removed and the wells were rinsed with 1x electrophoresis running buffer and filled with running buffer.

Pre-cast gels (Invitrogen) were prepared by removing the gel cassette from the cassette pouch and rinsing with deionised water. The tape covering the slot at the back of the gel cassette was removed. The comb was removed carefully to expose the loading wells and the wells were rinsed with 1x electrophoresis running buffer. The gel cassette was inserted into the lower buffer chamber assembled with the gel tension wedge, adjacent to the buffer core. Wells were filled with 1x electrophoresis running buffer.

Pre-stained molecular weight markers and protein samples were then loaded onto the gels by pipetting under the running buffer using the P200 gel loading tips. Gels were run at 200V for 45 minutes to 1 hour.

Table 2.5 NuPAGE-Novex Pre-cast gels (Invitrogen) used for SDS-PAGE protein analysis

Gel type	Running Buffer	Use
4-12% Bis-Tris (1mm) 10 wells	MOPS SDS	1D-E
4-12% Bis-Tris Zoom (1mm) IPG well	MOPS SDS	2D-E
7% Tris-Acetate gel (1mm) 10 wells	Tris-Acetate SDS	1D-E
4-12% Tris-Glycine gel (1mm) 10 wells	Tris-Glycine SDS	1D-E

2.5.4 Transfer of resolved proteins onto polyvinylidene diflouride (PVDF) membranes.

PVDF membranes were soaked in analysis grade methanol before use and then equilibrated in transfer buffer. Polyacrylamide gels were placed onto the membranes between two pieces of Whatman 3MM filter paper soaked in transfer buffer in a blotting cassette. Protein transfer/blotting was carried out at 100V for 60 minutes (Mini Trans-blot apparatus, cooled with a container of ice). The blots were then washed tree times in PBS-Tween.

2.5.5 Immunostaining of western blotted membranes

Blots were blocked in a sealed polyethylene bag with 15ml of blocking buffer and was placed on a rocking apparatus either for 1 hour at room temperature or overnight at 4°C. The blocking buffer was then removed and replaced with 10-20ml of primary antibody diluted to the required concentration in blocking buffer (see Table 2.6), and blots were incubated with primary antibody for a minimum of 1 hour. Blots were then washed three times for at least 10 minutes in PBS-Tween. Subsequently, the blots were incubated for a minimum of 1 hour with 15ml of appropriate alkaline phosphatase-conjugated secondary antibody diluted to 1/10,000 with blocking buffer (see Table 2.7). Blots were then washed again three times for at least 10 minutes in PBS-Tween. This was followed by a final 10 minute wash in 1x APA buffer. Excess APA buffer was drained off and blots were incubated with CDP-Star development reagent for 10 minutes. Again, all excess liquid was removed and blots were exposed to autoradiograph film (Kodak), through a polyethylene envelope.

2.5.6 Stripping blots for repeat immunostaining

Blots were stripped, by incubating blots with 20ml of MESNA stripping buffer in a sealed polyethylene bag for 30 minutes at 50°C in a water bath. Subsequently, blots were washed in 10 ml of SDS wash buffer followed by three 10 minute washes in PBS-Tween. Blots were then blocked overnight at 4°C with rocking by placing them in a sealed polyethylene bag with 15ml of blocking buffer. Blots were then ready for repeat immunostaining as described in section 2.4.5.

All results of protein analysis by western blotting are representative of at least three independent experiments.

Table 2.6 Primary Antibodies

Specificity	Clone/ Cat. Number	Species	Working Concentration	Citation/Supplier
Actin	A-2066	Rabbit	1/1000	Sigma
Bcl-6	Sc-858	Rabbit	200 ng/ml	SCB
Calregulin	Sc-11398	Rabbit	200ng/ml	SCB
CREB1	#9192	Rabbit	1/1000	CST
Phospho CREB1 Ser133	#9191	Rabbit	1:1000	CST
Cyclin D2	Sc-593	Rabbit	200ng/ml	SCB
EBV EBNA2	PE2	Mouse	1µg/ml	Young *et al*, 1989
EBV LMP1	CS.1-4	Mouse	1µg/ml	Rowe *et al*, 1987
EBV LMP2A	14B7	Rat	1µg/ml	Fruehling *et al*, 1996
FoxO1	#9462	Rabbit	1/1000	CST
PARP	Sc-7150	Rabbit	200 ng/ml	SCB
Phospho (Ser/Thr)-PKB substrate	#9611	Rabbit	1/1000	CST
P65-NFκB	Sc-109	Rabbit	200ng/ml	SCB
S6-Ribosomal protein	#2212	Rabbit	1/1000	CST
Phospho S6 Ribosomal protein Ser235/236	#2211	Rabbit	1/1000	CST
STAT1	Sc-346	Rabbit	200 ng/ml	SCB
α-Tubulin	T9026	Mouse	1/1000	Sigma

SCB - Santa Cruz Biotechnology; CST – Cell signalling technologies

Table 2.7 Secondary Antibodies – Alkaline Phosphatase-conjugated

Specificity	Cat. number	Species	Working concentration	Supplier
Mouse IgG	170-6520	Goat	1/10,000	Biorad
Rabbit IgG	170-6518	Goat	1/10,000	Biorad
Rat IgG	Sc-2021	Goat	1,10,000	SCB

SCB - Santa Cruz Biotechnology

2.6 Staining of proteins separated on SDS-PAGE gels

2.6.1 Silver staining

Silver staining of resolved proteins on an SDS-PAGE gel was carried out with a Silver staining kit (Amersham). The protocol supplied by the manufactured was modified to allow compatibility for subsequent analysis of proteins by mass spectrometry.

Reagents

Fixing solution contained 40% v/v Ethanol (Fisher) and 10% v/v Glacial acetic acid (Fisher).
Sensitising solution contained 75 ml Ethanol (Fisher), 1.25 ml Glutaraldehyde (25% w/v)*, 10ml sodium thiosulphate (5% w/v)* and 17g Sodium acetate*, and was made up to 250ml with distilled water.
Silver solution contained 25ml silver nitrate solution (2.5 w/v)* and 0.1ml Formaldehyde (37% w/v)*, and was made up to 250 ml with distilled water.
Developing solution contained 6.26g of sodium carbonate* and was made up to 250 ml with distilled water.
Stop solution contained 3.65g of EDTA-Na_2* and was made up to 250 ml with distilled water.

* Supplied as part of the Plus-One Silver staining kit for protein (Amersham).

Gels were removed from the gel cassette after SDS-PAGE and were placed in a tray on rocking apparatus. Gels were fixed by soaking in 125 ml of fixing solution for 30 minutes. The gels were subsequently washed three times in fresh distilled water for 5 minutes each time. Gels were then soaked in 125 ml of sensitizing solution (Amersham) for 30-60 minutes. Gels were once again washed three times in fresh distilled water for 5 minutes each time before applying 125 ml silver solution (Amersham) for 20 minutes. Silver solution

was removed by washing with distilled water twice for 1 minute each time. The silver stain was developed by applying developing solution (Amersham) for a maximum of five minutes. Staining was terminated by soaking the gels in stop solution (Amersham) for 10 minutes. The stop solution was removed and gels were once again washed with distilled water three times for 5 minutes each time. All procedures were carried out at room temperature. Gels were stored in sealed polyethylene bags containing 1% v/v acetic acid.

2.6.2. Phospho-specific staining

Phosphorylated proteins resolved on an SDS-PAGE gel were stained with Pro-Q^R-Diamond phosphoprotein gel stain (Molecular Probes, Invitrogen) according to manufactures instructions as described below.

Gels were removed from the gel cassette after SDS-PAGE and were placed in a tray on rocking apparatus. Gels were fixed by soaking in 125 ml of fixing solution (50% v/v methanol, 10% v/v glacial acetic acid) for 30 minutes. The gels were subsequently washed twice in fresh distilled water for 10 minutes each time. The gels were then incubated with 50ml pf Pro-Q Diamond phosphoprotein gel stain (Molecular probes) for 100 minutes in the dark. The phosphoprotein stain was replaced with a destain solution (Molecular probes) to reduce gel background signal and signal from non-specific binding. The gels were incubated with 125ml of destain solution twice for 1 hour in the dark, for a total of 2 hours. All procedures described were carried out at room temperature. The Pro-Q Diamond phosphoprotein stain has an excitation maximum at ~555nm and an emission maximum at 580 nm. Stained proteins were visualized using a fluorescence scanner (Amersham Typhoon 9400).

2.7 2D gel analysis

Gels were scanned to a high resolution (600 dpi) with a high specification scanner (ImageScanner, Amersham Biosciences) and saved as tiff images. Detailed gel analysis was subsequently performed using Phoretix 2D

Expression software version 2004 and later version 2005 (NonLinear Dynamics Ltd., Newcastle). This software has been previously demonstrated to be an objective and accurate method of gel analysis and to be effective in this type of study (Mahon and Dupree, 2001). The software automates the identification and quantification of gel spots by normalizing spot volumes and excluding background noise spots by having a minimum area parameter. Pairs of 2D gels can be compared with the software which matches spots between gels by comparing their location (selected by determining the centre of optical density within the spot). It was additionally necessary to manually confirm that each spot had been correctly identified by the software and validate the results for spot comparison between gels. This was achieved by studying enlarged on-screen images of the gels. In this study we were interested in the absence of spots in one gel compared to the other or a marked difference in spot intensity. The marked difference in spot intensity was arbitrarily set as a > 4-fold difference following advice from the software manufacturers (Nonlinear Dynamics Ltd).

2.8 Protein identification by Mass spectrometry

Protein spots of interest were manually excised from 2D gels and were analysed by The Functional Genomics and Proteomics laboratories at the University of Birmingham using the following protocol.

2.8.1 In-gel protein digestion

In gel trypsin digestion of manually excised spots was performed using an automated 96-well plate protocol plate modified from Shevchenko *et al.* 1996. Spots were dehydrated with acetonitrile (80 µl, 5 min), de-stained (1vol: 1vol ratio of freshly made 30 mM $K_3Fe(CN)_6$, 100 mM $Na_2S_2O_3.5H_20$; 50 µl per spot, 15 min shaking occasionally), rinsed 2/3 times in with 25 mM NH_4HCO_3 (50 µl) and supernatant removed. Acetonitrile was added (50 µl, 15 min), supernatant removed, gel rehydrated in 25mM NH_4HCO_3 (50 µl, 10 min), supernatant removed, acetonitrile added (50 µl, 15 min). The samples were

dried to completeness in an oven (60 °C, 30-45 min) and 10mM DTT in 25 mM NH_4HCO_3 added (25 µl, 56 °C for 1 h). The sample was cooled to room temperature, supernatant removed and 55mM iodoacetamide in 25mM NH_4HCO_3 added (25 µl, 45 mins at room temperature in the dark). Supernatant was removed and the gel plugs washed with 25mM NH_4HCO_3 (25 µl; 10 min). Supernatant was removed and acetonitrile added (50µl, 15 min). Supernatant was removed and the plugs were rehydrated with 25mM NH_4HCO_3 (50 µl, 10 min). Following further dehydration in acetonitrile (50 µl, 15 min) and removal of the supernatant, the spots were dried to completion in an oven (60 °C, 30-45 min). Sequencing grade modified trypsin was prepared as described by the manufacturer and made to a final concentration of 6.25ng/µl in 50mM NH_4HCO_3. This trypsin solution was added to the gel plugs (10µl on ice until the gel had fully rehydrated). Once fully rehydrated (approximately 20 min), the plug was covered with a minimal volume of 25mM NH_4HCO_3 (10µl) to keep gel wet during digestion and incubated at 37°C overnight. The digestion buffer (10 µl) that surrounds the gel plug was removed and placed in a well in a clean plate. The buffer was dried to completion in an oven (60 °C) and the remaining peptides resuspended 1 % (v/v) formic acid (6 µl) for tandem MS (MS/MS) or in 0.1 % (v/v) trifluoroacetic acid (TFA; 0.5 µl) for matrix-assisted laser disorption/ionisation-time of flight (MALDI-TOF) MS.

2.8.2 Protein identification

MS/MS was performed on the sample (5 µl) using a nanoESI Q-Tof mass spectrometer (Q-Tof Ultima GLOBAL, Micromass UK Ltd, Manchester) following separation of peptides using capillary liquid chromatography (Waters Ltd, Elstree, Hertford UK) and a PepMap C18 column (75 µm i.d. x 15 cm; Cat. No. 160396, Dionex (UK) Ltd, Camberley, Surrey UK). Following MS/MS the raw data was processed using MassLynx 3.5 (Micromass). The tryptic peptide sequences were then compared with the NCBI non-identical protein sequence database using MASCOT software (Matrix Science Ltd, London UK). The criteria used for conclusive

identification of a protein was either one peptide with a peptide expectation value (p) of < 0.00001, or two or more peptides with p<0.05. A conclusive identification was also allowed for two or more peptides with peptide expectation values of <1.0 and the sum of the MOWSE scores for only these peptides was >100. Peptides matching with trypsin and keratin were disregarded as contaminants.

2.9 Molecular biology

2.9.1 Bacterial culture media and reagents

Sterilisation of bacterial culture media and other reagents was carried out by autoclaving for 40 minutes at 15psi and 121°C where indicated.

Luria-Bertani (LB) broth was prepared to a final concentration of 0.5% (w/v) yeast extract (Oxoid), 0.17M NaCl (Fisher) and 1% (w/v) tryptone (Oxoid) in distilled water, before sterilising. If, required, antibiotics were added after sterilising prior to use. LB broth was stored at 4°C.

Luria-Bertani (LB) agar was prepared by adding 15g of bactoagar (Oxoid) to 1 litre of LB broth before sterilisation. Antibiotics for selective agar plates were added when the agar had cooled sufficiently following sterilisation, immediately before pouring the agar plates. Both LB agar and agar plates were stored at 4°C.

Ampicillin (Sigma) was used as an antibiotic to isolate transformed bacteria. A 50mg/ml stock solution was prepared in 50% Ethanol. Aliquots were stored at -20°C and were used at a final concentration of 50µg/ml.

2.9.2 Transformation of competent bacterial cells

Competent bacterial cells (One shot TOP 10 chemically competent E.Coli, Invitrogen) were thawed on ice and 25µl of competent cells were incubated on ice with 50-200ng of plasmid DNA for 30 minutes. The competent bacteria

were then heat-shocked at 42°C for 90 seconds on a heating block. Subsequently, 0.5ml of LB broth pre-warmed to 37°C (without any antibiotic selection) was added and the cells were incubated at 37°C. The transformation reactions were plated onto ampicillin selective LB agar plates and incubated overnight at 37°C without agitation.

2.9.3 Preparation of bacterial cells

A 5ml volume of LB broth containing ampicillin (50μg/ml) was inoculated with a single colony of bacterial cells from a streaked LB agar plate, and incubated at 37°C overnight on a rocking incubator. 2.5ml of the overnight culture was then used to inoculate a further 250 ml pf LB broth, which was again incubated at 37°C until the optical density at λ=600nm (OD_{600nm}) reached 0.3 to 0.6 (approximately 10 to 16 hours incubation).

2.9.4. Large-scale preparation of plasmid DNA

Large-scale preparations of plasmid DNA were performed using a DNA preparation kit (Ultramobius 1000 Plasmid Kit, Novagen) according to the manufactures instructions as described below.

A starter culture was used to inoculate 250 ml of selective LB medium this was incubated overnight at 37°C in an orbital shaker. The bacteria were harvested by centrifugation and re-suspended in 8ml of Bacterial resuspension buffer (Novagen). Once the bacterial pellet was thoroughly re-suspended, 8 ml of Bacterial Lysis Buffer (Novagen) was added and the suspension was mixed gently by inverting the tube five times. This was incubated for 5 minutes at room temperature and 8ml of Neutralization Buffer (Novagen) was then added. The solution was mixed again by inversion. After a 5 minute incubation on ice, the white precipitate was removed by centrifugation at 10,000 x g for 2 minutes at 4°C in a Sorvall SS-34 rotor and filtration of the resulting supernatant into a fresh tube. This supernatant was loaded onto a Mobius 1000 Column (Novagen) pre-equilibrated with 10ml of Mobius

Equilibration Buffer (Novagen), and was centrifuged at 2000 x g for 3 minutes. The flow through was retained and was incubated with 2.4ml of Detox Agent (Novagen) for 15 minutes on ice. The lysate was subsequently centrifuged at 10,000 x g and the clarified lyaste was loaded onto the equilibrated Mobius 1000 Column (Novagen). Once the flow through had passed through the column, the column was washed with 20ml of Mobius Wash Buffer (Novagen). The plasmid DNA was subsequently eluted by adding 5ml pf Mobius Elution Buffer (Novagen) to the column. The plasmid DNA was then precipitated, by adding 3.5ml of isopropanol (Fisher) and centrifuging at 15,000 x g for 20 minutes at 4°C in a Sorvall SS-34 rotor. The pellet was washed carefully with 2ml of 70% ethanol (v/v) and re-centifuged at 15,000 x g for 10 minutes. The supernatant was decanted and the DNA pellet was re-suspended in a suitable volume of TE. An aliquot was used to quantify the yield of DNA obtained. This aliquot was diluted 1:100 in TE and the absorbance of the solution at 260nm was measured on a spectrophotometer (Pharmacia Biotech Ultrospec^{R} 3000). A 50μg/ml DNA solution has an A_{260nm} of 1.

2.10. Total RNA extraction from lymphoid cell lines.

Total RNA was extracted from lymphoid cell lines using the RNeasy system (Qiagen) according to the manufactures instructions as described below.

A total of 4 x 10^6 cells were pelleted by centrifugation at 13,000 rpm for 5 minutes and the supernatant was removed. The remaining pellet was loosened by flicking the tube before the addition of 350μl of Buffer RLT (Qiagen) supplemented with 1.43mM of β-mercaptoethanol (Sigma). The sample was homogenized by passing the lysate five times through a 20-gauge needle (0.9mm diameter, Tyco Healthcare) fitted to an RNase-free syringe. 350μl of 70% ethanol (supplier) prepared with DEPC treated water was added to the homogenized lystae before mixing well by pipetting. 700μl of the sample was applied to an RNeasy column (Qiagen) placed in a 2ml collection tube (Qiagen). The sample was subsequently centrifuged for 15 seconds at 8,000×g

and the flow-through was discarded. 350µl of Buffer RW1 (Qiagen) was pipetted into the RNeasy mini column and was centrifuged for 15 seconds at 8,000 × g to wash. The flow through was discarded. 80µl of DNase incubation mix (Qiagen) was pipetted directly onto the RNeasy silica-gel membrane and was incubated at room temperature for 15 minutes to ensure complete DNA digestion.350µl of Buffer RW1 (Qiagen) was subsequently applied onto the column and centrifuges for 15 seconds at 8,000 × g before discarding the flow-through. The column was transferred into a new 2 ml collection tube and washed by the addition of 500µl of Buffer RPE to the column before centrifuging for 15 seconds at 8,000 × g. The flow through was discarded. The RNeasy silica-gel membrane was dried by the addition of 500µl Buffer RPE to the column and centrifugation for 2 minutes at 8,000 × g. The RNA was eluted by the addition of 30µl of RNase-free water (Qiagen) directly onto the RNeasy silica-gel membrane and centrifugation for 1 minute at 8,000 × g. An aliquot was used to quantify the yield and purity of RNA obtained. This aliquot was diluted 1:50 in RNase free water (Qiagen) and the absorbance of the solution at 260nm and 280nm was measured on a spectrophotometer (Pharmacia Biotech Ultrospec^{R} 3000). A 40µg/ml solution of RNA has an A_{260nm} of 1. Pure RNA has an A_{260nm}/A_{280nm} ratio of 1.9-2.1.

CHAPTER 3

Identification of PI3K target proteins in lymphocytes.

3.1 Introduction

The main objective of this study was to further our understanding of the interplay of the Phosphotidylinositide-3-kinase (PI3K) enzyme with its downstream targets to define its contributions to lymphocyte biology. The serine/threonine kinase Protein Kinase B (PKB) is a crucial kinase in this pathway. The minimum peptide motif for PKB mediated phosphorylation was initially established as Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd, where Xaa is any amino acid, Yaa and Zaa are small residues other than glycine, and Hyd is a large hydrophobic residue such as phenylalanine or leucine (Alessi *et al*, 1996). This motif was found to be present in more than 400 different proteins (Nicholson & Andersen, 2002). Other studies using orientated peptide library approaches and motif-profile scoring algorithms have shown that additional motifs can be targets for phosphorylation by PKB, and are found in > 14,000 different sequences and in approximately 9500 vertebrate protein sequences (Obata *et al*, 2000, Yaffe *et al*, 2001, Lawlor & Alessi, 2001). These approaches have therefore identified many proteins to be potential targets for phosphorylation by PKB. Whether these are substrates of PKB *in vivo* remains to be defined, as factors in addition to primary protein sequence, such as subcellular localization and tertiary protein structure, are important for substrate recognition *in vivo*. Despite this, these studies suggest that many proteins remain unidentified as PKB targets.

Epstein - Barr virus (EBV) is an important contributory factor towards B-cell malignancies. EBV immortalization of primary human B-cells results in the generation of lymphoblastoid cell lines (LCLs), a good model for cells in the polyclonal phase of Post-transplant lymphoproliferative disease (PTLD)

(Rowe *et al*, 1998, Kieff & Rickinson, 2001). EBV contributes towards B-cell survival and proliferation by the co-operative action of many EBV genes that generate survival signals through various signalling pathways. EBV encoded proteins have been shown to activate signalling through the PI3K/PKB pathway (Swart *et al*, 2000, Scholle *et al*, 2000, Morrison *et al*, 2003, Dawson *et al*, 2003). Furthermore, PI3K has been shown to be essential for the proliferation of EBV immortalized B cells (Brennan *et al*, 2002). However, little is known about the downstream components of the PI3K pathway responsible for this proliferative advantage.

An alternative and powerful approach for the analysis of downstream components of signalling pathways mediated by protein kinases are the use of phospho-specific antibodies. These antibodies are directed against defined serine/threonine phosphorylated peptides within the consensus phosphorylation motif of a kinase of interest. This allows the detection of phosphorylated substrates of the kinase and is a useful way of investigating the substrates of serine/threonine kinases in intact cells. In this chapter, a phospho-specific antibody directed against the consensus phosphorylation motif of PKB was employed in combination with a specific inhibitor of PI3K, LY294002 to identify the key molecules targeted by PI3K in lymphocytes that may contribute towards cellular survival and proliferation.

3.2 Identification of a major target of PI3K signalling in B cells using a phospho-motif specific antibody.

Activation of the PI3K/PKB signalling pathway leads to an alteration in the phosphorylation status of several PKB target proteins involved in cellular proliferation and survival pathways. Protein phosphorylation is a key cellular regulatory mechanism of cellular signalling pathways, mediating several aspects of protein properties, including activity, function, subcellular localisation and interaction (Yaffe & Cantely, 1999). An investigation into the phosphorylation changes that occur in LCLs downstream of PI3K activation was therefore initiated to identify targets of PI3K/PKB activation in LCLs. The approach taken was the use of a phospho-(Ser/Thr)-PKB-substrate (phospho-PKB-substrate) antibody supplied by Cell Signalling Technologies, in combination with a specific inhibitor of PI3K, LY294002. The phospho-PKB-substrate antibody is specific for the substrate consensus motif of PKB (RxRxxS/T), and therefore detects phosphorylated substrates of PKB (Kane *et al*, 2002, Ly *et al*, 2003).

3.2.1 Identification of a 32 kDa phosphoprotein as a major target of PI3K signalling in lymphablastoid cell lines.

Nuclear and cytosolic extracts were generated from two LCLs. SP-LCL was generated in house by the *in vitro* infection of peripheral blood lymphocytes with the B95.8 isolate of EBV. IB4-LCL is an LCL generated from cord blood B-cells immortalized with B95.8 EBV (Sample & Kieff, 1990). The cells were either left untreated or were treated with LY294002 (20μM, 1 hour). Diluent control experiments were previously carried out to ensure that the effect of inhibitor treatment on cells was specific to LY294002. Proteins were subsequently resolved by SDS-PAGE and detected by immunoblotting with the phospho-PKB-substrate antibody. Many bands representing phosphorylated substrates of PKB remained unchanged in intensity upon treatment with LY294002 (Figure 3.1). However, the phosphorylation of a protein of 32 kDa (pp32) was dramatically inhibited following treatment with

LY294002 in both the cytosolic and nuclear fractions of both IB4-LCL and SP-LCL. These results indicted that pp32 is a major target of PI3K/PKB signalling in LCLs.

3.2.2 Identification of pp32 as S6-Ribosomal protein.

Two previous studies have used the same phospho-PKB substrate antibody to detect a 32 kDa LY294002 sensitive protein in 3T3-L1 adipocytes (Kane *et al*, 2002) and chronic myeloid leukaemia cells (Ly *et al*, 2003). These groups identified pp32 to be S6-ribosomal protein (S6), a component of the ribosome. When phosphorylated, S6 has been demonstrated to upregulate the translation of mRNA molecules bearing a 5'-terminal oligopolypyrimidine tract (TOP mRNAs) that translate components of the translational apparatus such as elongation factors, ribosomal proteins and ribosome biogenesis factors (Ruggero & Pandolfi, 2003). To investigate whether the LY294002 sensitive pp32 detected in this study was S6, antibodies specific for pan-S6 and a phosphorylated-(Ser 235/236)-S6 were utilized.

A dose response experiment was carried out in IB4-LCLs, treating cells with varying concentrations of LY294002 prior to the generation of nuclear protein extracts. Nuclear proteins were subsequently resolved by SDS-PAGE and detected by immunoblotting with phospho-PKB-substrate, phospho-(235/236)-S6, and panS6 antibodies. The phosphorylation of both pp32 and S6 were found to be constitutive in IB4-LCLs. Furthermore, LY294002 treatment inhibited the phosphorylation of pp32 and S6 in a similar dose dependent manner (Figure 3.2). The total levels of S6 protein expression were not affected by PI3K inhibition. The phosphorylation of pp32 and S6 were also dramatically reduced to a similar degree in response to treatment with rapamycin (20ng/ml, 1 hour) (lane 6, Figure 3.2). Rapamycin is an inhibitor of the mammalian target of rapamycin (mTOR) protein, which is activated in response to signalling through PI3K. When activated, mTOR phosphorylates and activates S6K (p70-S6 Kinase), which subsequently phosphorylates S6 (Ruggero & Pandolfi, 2003). The total level of S6 protein expression was not affected by rapamycin treatment.

To further characterise the response of pp32 to PI3K inhibition, time course experiments were performed. IB4-LCLs were treated with LY294002 (20μM) for varying periods of time prior to the generation of nuclear protein extracts for analysis by immunoblotting. The incubation of IB4-LCLs with LY294002 for increasing periods of time led constant abrogation of both S6 and pp32 phosphorylation (Figure 3.3). The total level of S6 expression was not altered over time in response to LY294002 treatment.

These data provided strong evidence to conclude that the 32kDa LY294002 sensitive protein detected with the phospho-PKB-substrate antibody is S6. This conclusion was based on the observations that pp32 was the same size, and had similar inhibitor responses and kinetics to S6. In addition, the phosphorylation of pp32 and S6 were both inhibited in response to inhibition of mTOR by rapamycin. To conclude, S6 was found to be constitutively phosphorylated, and is a major target for PI3K signalling, in LCLs.

3.3 Identification of S6 as a major target of PI3K signalling in Burkitt's lymphoma B cell lines.

Having established that S6 is an important target for PI3K signalling in LCLs, the phosphorylation status of S6, and the response of S6 to PI3K inhibition were investigated in other continually proliferating B cells lines derived from Burkitt's lymphoma (BL) patients. BL cells are driven into continual proliferation by the constitutive expression of c-myc, a transcription factor that promotes cell proliferation by regulating the expression of various genes involved in cell cycle progression (Dang, 1999). This is the result of a characteristic reciprocal translocation that places *c-myc* adjacent to immunoglobulin genes in BL B cells (Hecht & Aster, 2000). Signalling through PI3K has also been demonstrated to play a critical role in the survival of BL cells, as PI3K inhibition for 24 hours rapidly induces apoptosis (Brennan *et al*, 2002).

Nuclear protein extracts were therefore generated from two EBV negative Burkitt's lymphoma B cell lines, DG75 and BL41, as well as IB4 and SP-LCLs that were treated with varying doses of LY294002 for 1 hour. Proteins were subsequently analysed by SDS-PAGE and the phosphorylation status of S6 was analysed by western blotting. The phosphorylation of S6 was inhibited by LY294002 in a dose dependent manner in DG75 and BL41 cells in a similar manner to that observed in LCLs (Figure 3.4). Time course studies were also carried out in DG75 and IB4-LCL cells, treating the cells with LY294002 for increasing periods of time. The phosphorylation of S6 was completely inhibited by LY294002 (20μM) at 4 hours in DG75 cells (Figure 3.5). These results indicate that S6 is constitutively phosphorylated, and is an important target of PI3K signalling in BL B cells as well as LCLs.

3.4 LY294002 and Rapamycin inhibit S6 phosphorylation in T cells.

Signalling through PI3K has been demonstrated to have an important role in the regulation of T-lymphocyte proliferation. PI3K has been shown to be activated downstream of the IL-2 receptor, the T cell receptor and the co-stimulatory molecule CD28 (Reif *et al*, 1997, Ward & Cantrell, 2001). Studies using pharmacological inhibitors of PI3K have implicated a role for PI3K in antigen receptor-mediated and IL-2 induced proliferation of T-cells (Shi *et al*, 1997, Brennan *et al*, 1997; 1999). Mice deficient in PI3K p110 catalytic subunits have defects in T cell proliferation, development and antigen receptor signalling (Okkenhaug *et al*, 2002, Webb *et al*, 2005). IL-2 stimulation of PI3K has been demonstrated to induce PKB and P70 S6 kinase activation in T cells (Reif *et al*, 1997).

3.4.1 S6 is a target for PI3K signalling in T cells.

As in B cells, many targets of the PI3K/PKB pathway in T cells remain uncharacterised. The approach of employing the phospho-PKB substrate antibody was therefore also applied to attempt to identify phosphorylated

targets of PKB in the nuclei of Kit225 cells. Kit225 is an IL-2 dependent leukaemic T cell line derived from a patient with T cell chronic lymphocytic leukaemia (Hori *et al*, 1987). Kit225 cells have an absolute requirement for IL-2 for the induction of cell cycle progression and growth of the cells. In the absence of IL-2, these cells accumulate in the G_1 phase of the cell cycle. Upon the addition of IL-2, Kit225 cells re-enter the cell cycle.

Kit225 cells were therefore quiesced by washing out of IL-2 for 3 days. Cells were subsequently pretreated with LY294002 (20μM) for 30 minutes or were left untreated, and then stimulated with IL-2 (20ng/ml) for 30 minutes. Nuclear protein extracts generated were resolved by SDS-PAGE and analysed by immunoblotting using the phospho-PKB-substrate antibody. The majority of bands detected were unchanged in intensity in response to IL-2 or treatment with LY294002 (Figure 3.6). However, a band at 32 kDa that was detected at very low levels in quiesced cells showed a considerable increase in intenstity in response to IL-2 stimulation (Figure 3.6). Treatment with LY294002 (20μM, 1 hour) dramatically inhibited the phosphorylation of this protein. As this protein was of identical size to S6 and due to its inhibitory response to LY294002, it was likely that pp32 detected in Kit225 cells was also S6.

This was confirmed by testing the sensitivity of S6 and pp32 phosphorylation to the inhibition of PI3K by LY294002 in these cells. Quiesced Kit225 cells were pretreated with various doses of LY294002 for 30 minutes before they were stimulated with IL-2 for 30 minutes. Nuclear protein extracts generated were subsequently analyzed by SDS-PAGE and immunoblotting with the phospho-S6 and pan-S6 antibodies. Some constitutive phosphorylation of S6 was observed in quiesced cells (Figure 3.7). This could be increased considerably by stimulation with IL-2. Treatment with LY294002 inhibited the IL-2 induced phosphorylation of S6 in a similar dose responsive manner to that observed in LCLs and BL cells. Overlaying western blot films from the experiments carried out in Figure 3.6 and 3.7 confirmed that pp32 and S6 were of identical molecular weights to further support our hypothesis that pp32 detected in Kit225 cells is also S6. These results therefore demonstrate that S6 is a major target for IL-2 induced PI3K signalling in T cells.

3.4.2 LY294002 and rapamycin inhibit S6 phosphorylation in T cells.

Lymphocyte proliferation is a key feature of the immune system and relies upon entry of cells into the cell cycle. This process is tightly regulated by the activities of D type cyclins and their partner cyclin-dependant kinases (cdk) (Sherr, 1996). Cyclin D2 is the first cell cycle protein to be expressed in lymphocytes following cell activation in repose to B and T cell receptor ligation, cytokine stimulation and EBV infection (Sinclair *et al*, 1994, Banerji *et al* 2001, Martino *et al*, 2001). This is accompanied by a downregulation in cdk inhibitors (CDKI), such as $p27^{kip1}$ (Sinclair *et al*, 1994, Frost *et al*, 2001, Appleman *et al*, 2002). These events promote the formation of catalytically active complexes comprised of a D-type cyclin and a cdk molecule that induce progression through the G_1 phase of the cell cycle. PI3K activation in response to IL-2 signalling has been linked with components of the cell cycle machinery, inducing the expression of cyclin D3 and the degradation of $p27^{kip1}$ in T cells (Brennan *et al*, 1997). These observations demonstrate a role for PI3K in mediating T cell proliferation.

A study in the laboratory further investigated the role of PI3K and downstream pathways in the regulation of D-type cyclins in Kit225 cells using the pharmacological inhibitors, LY294002 and rapamycin. This study demonstrated that LY294002 inhibits cyclin D2 and cyclin D3 at both the transcriptional and post-transcriptional level, whilst rapamycin inhibited these cyclins at the post-transcriptional level only. The differing effects of the inhibitors prompted an investigation into the effect of these inhibitors alone and in combination on T cell proliferation. The results indicated that a combination of LY294002 and rapamycin was significantly more effective in inhibiting T cell proliferation than when used alone at similar doses. In addition, the study showed that the expression of both cyclin D2 and cyclin D3 were reduced in response to treatment with a combination of LY294002 and rapamycin and is likely to contribute towards the suppressive effect of these inhibitors on T cell proliferation (Breslin *et al*, 2005).

As part of this study, the phosphorylation status of S6 was analysed post treatment with a combination of LY294002 and rapamycin in Kit225 cells to characterise the effects of these inhibitors on downstream pathways. Kit225 cells were quiesced by washing out of IL-2 for 3 days. The cells were subsequently treated with LY294002 and rapamycin, either alone or in combination at various doses for 30 minutes prior to stimulation with IL-2 for 30 minutes. Total cellular protein extracts were generated and protein levels were analysed by SDS-PAGE and immunoblotting using antibodies specific for phospho-S6 and pan-S6 antibodies. Some constitutive phosphorylation of S6 could be observed in quiesced Kit225 cells (Figure 3.8). The level of S6 phosphorylation could however be increased further by stimulation of the cells with IL-2. Treatment with LY294002 at 20μM was sufficient to dramatically inhibit S6 phosphorylation, whilst a dose of 5μM brought S6 phosphorylation to similar levels to those seen in unstimulated cells. Treatment with rapamycin at both 20nM and 5nM were sufficient to completely abolish S6 phosphorylation. S6 phosphorylation was therefore also completely inhibited in response to treatment with a combination of LY294002 and rapamycin. Total levels of S6 protein expression were not affected by inhibitor treatments.

The combined effects of LY294002 and rapamycin on S6 phosphorylation were also investigated in primary T cell blasts. Primary T cells blasts were generated by activating and growing human T cells in PHA (1 μg/ml) and IL-2 (20ng/ml) for 1 week. The cells were quiesced by washing out of IL-2 for 3 days. Cells were subsequently pretreated with LY294002 and rapamycin alone, and in combination at various doses for 30 minutes. Cells were then stimulated with anti-CD3 (2.5ng/ml) and anti-CD28 (5μg/ml) for 30 minutes. Total cellular protein extracts were generated and protein levels were analysed by SDS-PAGE and immunoblotting using phospho-S6 protein and pan-S6 antibodies. The results indicated that some constitutive S6 phosphorylation is present in T cell blasts (Figure 3.9). The addition of antibodies to CD3 and CD28 led to an increase in the level of S6 phosphorylation in these cells. Treatment with both LY294002 and rapamycin, alone, and therefore also in combination led to an indistinguishable and dramatic inhibition of S6 phosphorylation. Again, total levels of S6 were not affected by inhibitor

treatment. These results suggest that LY294002 and rapamycin affect both overlapping and parallel pathways that lead to S6 phosphorylation. Along with the inhibition of cyclin D2 and D3 expression, the inhibition of S6 phosphorylation is likely to contribute towards to inhibitory effect of LY294002 and rapamycin on T cell proliferation.

This data contributed towards the publication *'LY294002 and rapamycin co-operate to inhibit T-cell proliferation'*, published in *British Journal of Pharmacology*, March 2005 (see appendix II).

3.5 Characterisation of the phosphorylation status of S6 and CREB in lymphocytes

The study described in section 3.5.2 established a role for PI3K in the regulation of the cyclin D2 cell cycle protein in T lymphocytes. In addition, inhibition of PI3K in EBV immortalized B cells has been shown to cause the downregulation of cyclin D2 protein expression (Brennan *et al*, 2002). Another study in the laboratory was initiated to characterise the molecular events that contribute towards the regulation of the *cyclin D2* promoter in lymphocytes. During this study, a direct role for PI3K in the regulation of the *cyclin D2* promoter was demonstrated with the use of an active mutant of PI3K and a series of *cyclin D2* promoter deletion constructs. A binding site for the transcription factor CREB1 (cAMP response element-binding protein) in the *cyclin D2* promoter was identified. In addition, the phosphorylation of CREB at serine 133, the critical site for transactivational activity, was found to be important for *cyclin D2* promoter activity in both T cells and LCLs (White *et al*, 2006). A role for the PI3K/PKB pathway in the phosphorylation of CREB at ser133 in response to growth and survival signals has previously been reported (Du & Montminy, 1998). It was therefore important to establish the phosphorylation status of CREB1 in these cell types, and whether PI3K activity was necessary for this phosphorylation event.

For this purpose, the phosphorylation status of CREB1 was investigated in IB4-LCLs and KIT-225 cells following treatment with varying doses of the PI3K inhibitor, LY294002. Nuclear and cytosolic extracts were generated and were resolved by SDS-PAGE followed by immunoblotting with an antibody specific for serine133 phosphorylated CREB1. CREB1 was found to be constitutively phosphorylated in the nuclei of EBV immortalized LCLs. Phospho-CREB1 is represented by the higher molecular weight band visible on the blot. The lower molecular weight band represents ATF-2, another member of the CREB family, and is visible due to cross-reactivity with the phospho-CREB1 antibody used (Figure 3.10). The phosphorylation status of CREB1 was not affected by PI3K inhibition. The effectiveness of LY294002 to inhibit PI3K was verified by its ability to inhibit S6 phosphorylation. Total protein levels of CREB and S6 remained constant irrespective of treatment in LCLs. Similar observations were made in the nuclei of KIT-225 T cells. CREB1 phosphorylation was constitutive in these cells and was refractory to inhibition by PI3K (Figure 3.11). Again, the inhibition of PI3K by LY294002 was verified by the inhibition of S6 phosphorylation following IL-2 stimulation. The total levels of CREB1 and S6 were unaffected by LY294002 treatment. These results demonstrate that the phosphorylation of CREB1 at ser133 occurs independently of PI3K activation in the EBV immortalized B cells and Kit225 T cells tested. This suggests that the regulation of the *cyclin D2* promoter by CREB1 occurs independently of PI3K in these cells.

This data contributed towards the publication ; *'Regulation of Cyclin D2 and the cyclin D2 promoter by protein kinase A and CREB in lymphocytes'* , published in *Oncogene*, April 2006 (see appendix II).

3.6 Discussion

In this chapter a phosphomotif-specific antibody directed towards the phosphorylated substrates of PKB was successfully used to identify S6-ribosomal protein (S6) as a major target of PI3K signalling in both the cytosol and the nuclei of transformed B cells and leukaemic T cells. S6 is phosphorylated on five clustered residues located within the C terminus of the protein, Ser 235, Ser236, Ser 240, Ser244 and Ser247 (Krieg *et al*, 1988) with the preferred phosphorylation sites believed to be Ser236 and Ser235 (Flotow & Thomas, 1992). S6 was found to be constitutively phosphorylated on Ser 236 and Ser 235 in EBV immortalized B cells (LCLs), Burkitt's lymphoma B cells and in an IL-2 dependent leukeamic T cell line (Kit225). The S6 protein resides at the interface between the large (60S) and small (40S) ribosomal subunits and is a component of 40S subunit. Ultraviolet crosslinking studies have demonstrated that S6 interacts with tRNA, initiation factors and mRNA (Nygard & Nilsson, 1990) and has therefore been implicated in the regulation of translation initiation.

The precise role of S6 phosphorylation in translation is currently unclear. S6 phosphorylation has been associated with an increase in the translation of a specific class of mRNAs that bear a terminal oligopolypyirmidine tract (TOP) in the 5'-untranslated region (Jefferies *et al*, 1994, 1997, Schwab *et al*, 1999, Loreni *et al*, 2000). TOP mRNA's encode components of the translation apparatus including ribosomal proteins, elongation factors 1A1 (eEF1A1) and eEF2 and proteins involved in ribosome biogenesis and translational control (Ruggero & Pandolfi, 2003). Thus S6 phosphorylation was proposed to increase the rate of protein synthesis by enhancing the production of components of the translational apparatus (Duncan & McConkey, 1982; 1984). However, more recent studies have challenged this model as TOP mRNA translation has been observed independently of S6 phosphorylation (Tang *et al*, 2001, Stolovich *et al*, 2002, Pende *et al*, 2004, Ruvinsky *et al*, 2005). The phosphorylation of S6 has however been directly implicated in the regulation of cell size (Ruvinsky *et al*, 2005), suggesting that S6 is a critical

mediator of cell growth. Furthermore, deletion of S6 in mice causes a block in ribosome biogenesis and prevents cell cycle progression (Volaveric *et al*, 2000). The constitutive phosphorylation of S6 observed in LCLs, BL cells and leukaemic T cells may therefore contribute towards the continual proliferation and oncogenic capacity of these cells by inducing cell growth, proliferation and progression through the cell cycle.

The constitutive phosphorylation of S6 in LCLs and BL B cells was found to be dependent on PI3K and mTOR. Inhibition of these pathways using specific inhibitors ablated the phosphorylation of S6. This occurred in response to a wide range of doses of the PI3K inhibitor, LY294002, in LCLs and BL B cells. The data from Kit225 and T cell blasts also demonstrated that S6 phosphorylation is inhibited by both LY294002 and rapamycin. These observations suggest that LY294002 and rapamycin target overlapping pathways that lead to S6 phosphorylation. Constitutive phosphorylation of S6 has previously been detected *in vivo* in a B cell lymphoma model derived from transgenic mice bearing the *c-myc* oncogene which constitutively express PKB (Wedel *et al*, 2004). This phosphorylation was shown to be dependent on mTOR as levels of phosphorylated S6 were significantly reduced in extracts from lymphomas treated with rapamycin, compared to untreated extracts. Constitutive phosphorylation of S6 has also been detected in chronic myeloid leukaemia (CML) cells, and was shown to be inhibited by treatment with LY294002 and rapamycin (Ly *et al*, 2003). These observations indicate that constitutive S6 phosphorylation is a feature of malignancies of lymphoid origin, and may contribute towards the enhanced proliferation of these cells. This is likely to be a consequence of overactivation of the PI3K pathway, as treatment with inhibitors targeting PI3K and mTOR abrogate S6 phosphorylation. The abrogation of S6 phophorylation on treatment with LY294002 and rapamycin also highlights the usefulness of monitoring the phosphorylation status of S6 as a readout of both PI3K and mTOR activity.

The detection of S6 with the phospho-PKB substrate antibody suggested that S6 is a direct target for phosphorylation by PKB. However, a complication arises with the use of phospho-motif-specific antibodies as several kinases

share similar consensus phosphorylation motifs. The consensus phosphorylation motif of PKB is RxRxxS/T. Phosphorylation of S6 on serine 236 by p70 S6 kinase occurs at the consensus phosphorylation motif RxRxxS. It is therefore likely that the phospho-PKB-substrate antibody recognized phosphorylated S6 on this basis. This hypothesis is corroborated by the fact that S6 phosphorylation was also inhibited by the mTOR inhibitor, rapamycin. Thus, the inhibition of S6 phosphorylation by LY294002 is therefore likely to be due to the activation of mTOR by PKB, resulting in the activation of P70 S6 Kinase and hence phosphorylation of S6.

Several previous studies have employed phospho-PKB substrate antibodies as an approach to identify substrates downstream of PI3K activation (Manning *et al*, 2002, Kane *et al* 2002, Zhang *et al*, 2002b, Astoul *et al*, 2003, Ly *et al*, 2003, Kovacina *et al*, 2003). Two of these studies utilized the same phospho-PKB substrate antibody used in this study to demonstrate that the phosphorylation of a 32kDa protein is sensitive to LY294002 and rapamycin in 3T3-L1 adiopcytes (Kane *et al*, 2002) and in chronic myeloid leukaemia (CML) cells (Ly *et al*, 2003). Both of these studies identified pp32 to be S6. Kane *et al* isolated pp32 by immunoprecipitation, and subsequent tandem mass spectrometric analysis revealed the identity of pp32 to be S6 (Kane *et al*, 2002). Ly *et al* utilized a different approach by firstly further analysing pp32 by 2-Dimensional elecrophoresis (2DE). 2DE analysis allowed the detection of multiple forms of S6 in the CML cells, which had isoelectric points ranging from pH8 to pH11. This information was subsequently used to search a motif scanning algorithm (Scansite) which allows protein sequence databases to be searched for motifs that are likely to be phosphorylated by specific protein kinases (Obata *et al*, 2000). The results of these investigations indicated that pp32 was S6, which was subsequently confirmed by repeat immunoblotting with antibodies specific for phospho-ser235/236-S6 and pan-S6 (Ly *et al*, 2003). A similar approach was taken by Zhang *et al* to identify phosphorylated S6 to be an important target for PI3K signalling in wild type but not PTEN deficient embryonic stem (ES) cells (Zhang *et al*, 2002). Interestingly, this study also detected phosphorylated S6 in wild type ES cells using an antibody directed the substrate consensus motif of PKC, suggesting that S6 may be

phosphorylated by pathways downstream of PI3K that are independent of PKB and mTOR. This is supported by several studies that demonstrate that S6 kinases are activated by PKC in various cell types (Akimoto *et al*, 1998, Romanelli *et al*, 1999, Martin *et al*, 2001, Wang *et al*, 2003, Valovka *et al*, 2003).

In several studies utilizing phopho-PKB substrate antibodies, in addition to the identification of S6, novel targets of PKB have been identified. For example, Manning *et al* identified tuberin, the gene product of the *tuberous sclerosis complex-2 (TSC2)* tumour suppressor gene, to be a target for PKB mediated phosphorylation in NIH-3T3 fibroblasts using the commercially available antibody used in this study (Manning *et al*, 2002). This antibody was also used to identify a 40kDa 14-3-3 binding protein (Kovacina *et al*, 2003) and a 160kDa protein containing a Rab GAP domain (Kane *et al*, 2002) as novel targets for PKB in hepatoma cell lines and 3T3-L1 adipocytes respectively. In a more recent study, Jiang *et al* used the same approach to identify WNK1, a negative regulator of insulin-stimulated mitogenesis as a novel target for PKB in 3T3-L1 adipocytes (Jiang *et al*, 2005). Astoul *et al* generated a distinct phospho-PKB substrate antibody with immunoreactivity towards a phospho-peptide sequence in glycogen-synthase kinase-3 (GSK-3), a known substrate for PKB (Astoul *et al*, 2003). This antibody was subsequently used to identify SLY (Src homology 3 (SH3) domain-containing protein expressed in lymphocytes) as a novel target for antigen receptor signal transduction in T cells. Together, this study and the studies described above demonstrate the value of phosphomotif-specific antibodies as tools to study the phosphoproteome of various cell types, and to identify new and important targets of protein kinases involved in signalling transduction pathways.

In summary, a phospho-PKB substrate antibody was used to identify the S6 protein as an important target of PI3K signalling in EBV transformed B cells, BL B cell lines and in T cells, in which PI3K is an important contributor towards cellular proliferation and survival. The constitutive phosphorylation of this protein is therefore likely to contribute towards the oncogenic capacity of deregulated PI3K activation in lymphocytes and may therefore be a

contributory factor towards the development of lymphoid malignancies such as EBV associated lymphomas and leukaemia.

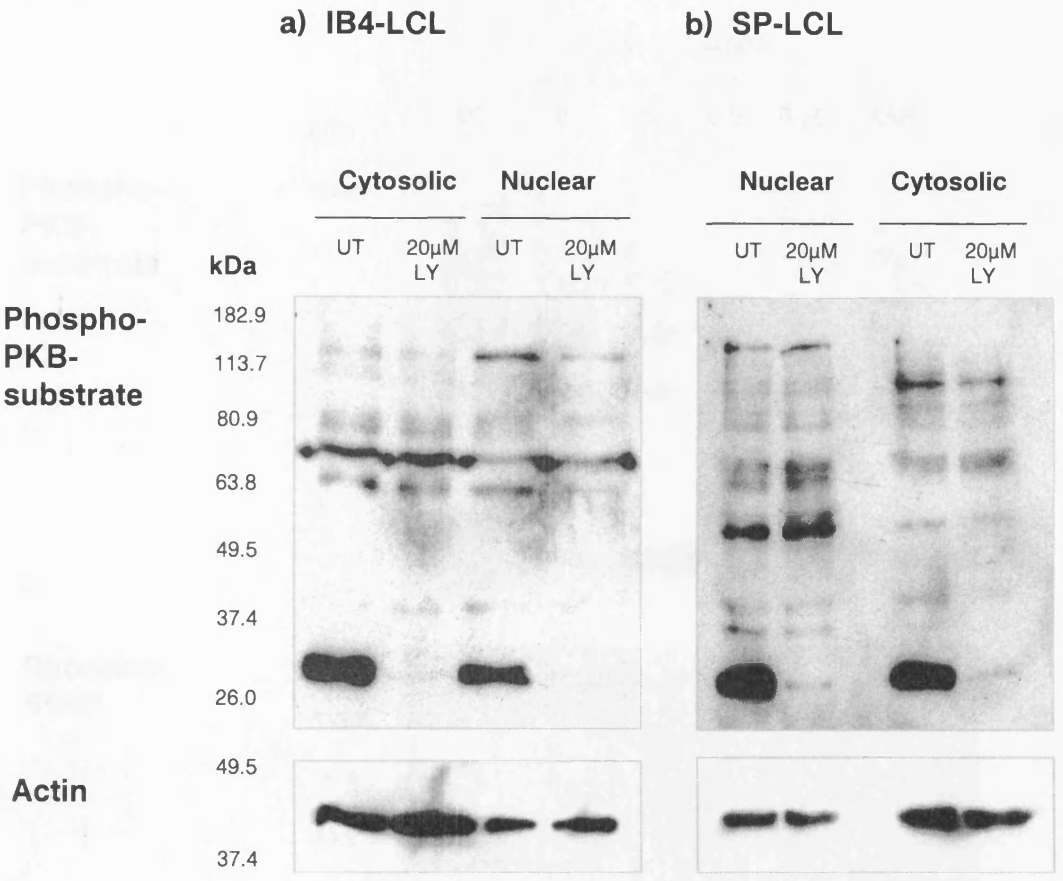


Figure 3.1 – Identification of pp32 as a major target of PI3K signalling in LCLs using a phospho-PKB substrate antibody.

Cytosolic and nuclear extracts were generated from a)IB4-LCL and b) SP-LCL that were either untreated (UT) or treated with LY294002 for 1 hour (20μM LY). Proteins were resolved by SDS-PAGE and analysed by immunoblotting using the phospho-PKB-substrate antibody. Equal protein loading was checked by immunoblotting for α-actin.

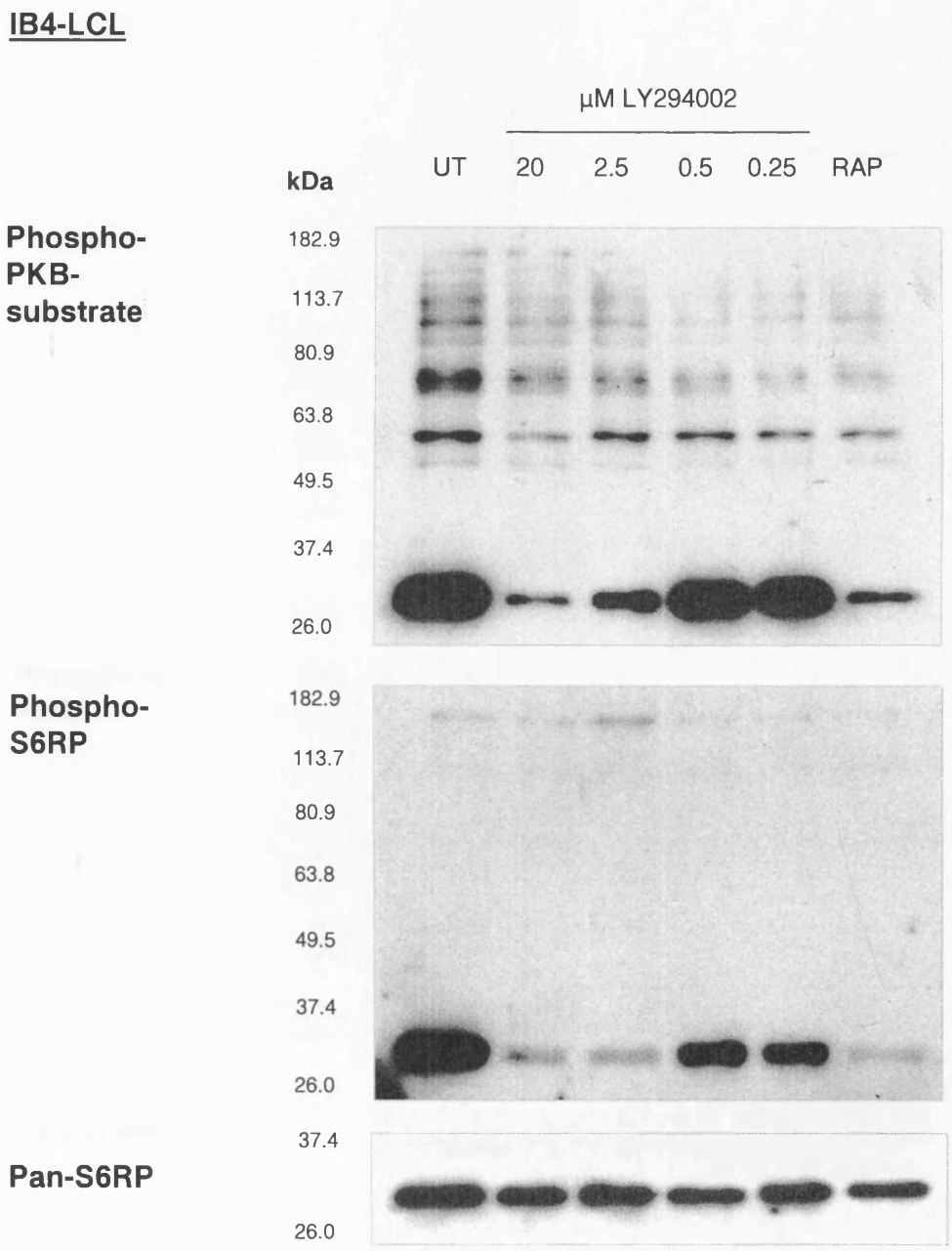


Figure 3.2 – Identification of pp32 as phospho-S6-ribosomal protein.

Nuclear extracts were generated from IB4-LCLs that were either untreated (UT), treated with a decreasing concentration of LY294002 (20, 2.5, 0.5, 0.25µM), or treated with rapamycin (RAP, 20ng/ml) for 1 hour. Proteins were resolved by SDS-PAGE, and were analysed by immunoblotting using phospho-PKB substrate, phospho-S6-ribosomal protein (S6) and pan-S6 antibodies.

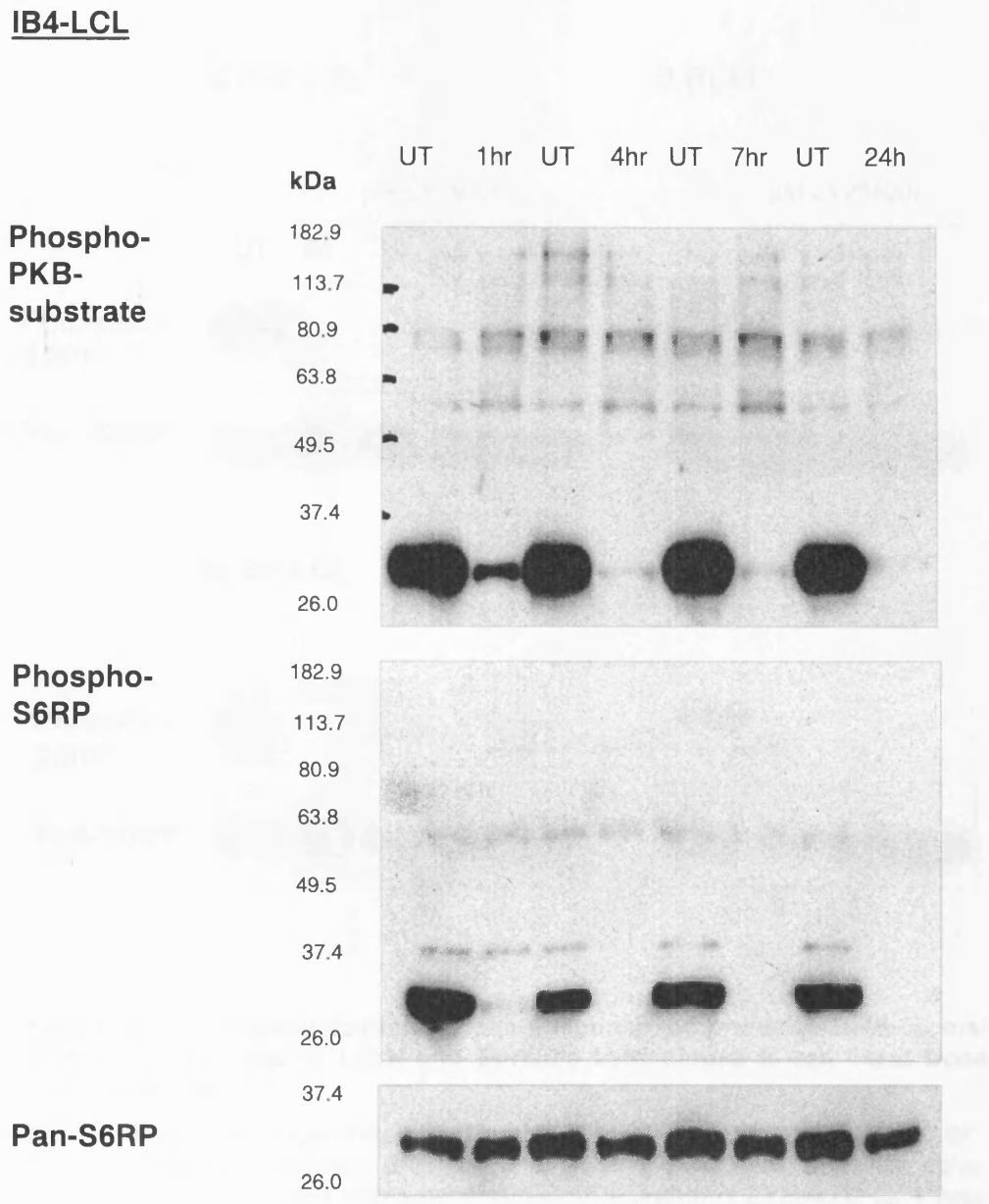


Figure 3.3 – Identification of pp32 as phospho-S6-ribosomal protein.

Nuclear extracts were generated from IB4-LCLs that were either untreated (UT) or treated with LY294002 (20μM) for increasing periods of time as indicated (1, 4, 8 or 24 hours). Proteins were resolved by SDS-PAGE, and were analysed by immunoblotting using phospho-PKB substrate, phospho-S6 and pan-S6 antibodies.

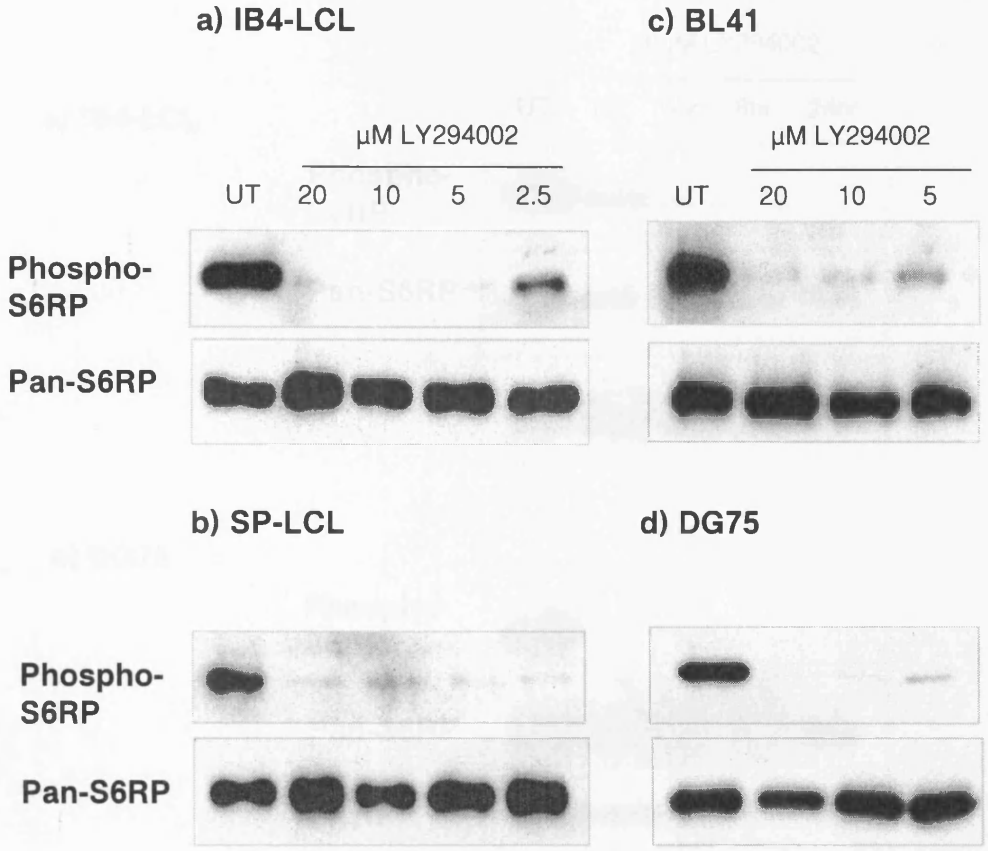


Figure 3.4 – Characterisation of the response of phospho-S6-ribosomal protein to LY294002 in LCLs and Burkitt's Lymophoma B cell lines: Dose reponse studies

Nuclear extracts were generated from lymphablastoid cell lines; a) IB4-LCL b) SP-LCL and Burkitt's Lymphoma B-cell lines; c) BL41 and d) DG75, that were either untreated (UT) or treated with a decreasing concentration of LY294002 for 1 hour (20, 10, 5 µM). Proteins were resolved by SDS-PAGE, and were analysed by immunoblotting using phospho-S6 and pan-S6 antibodies.

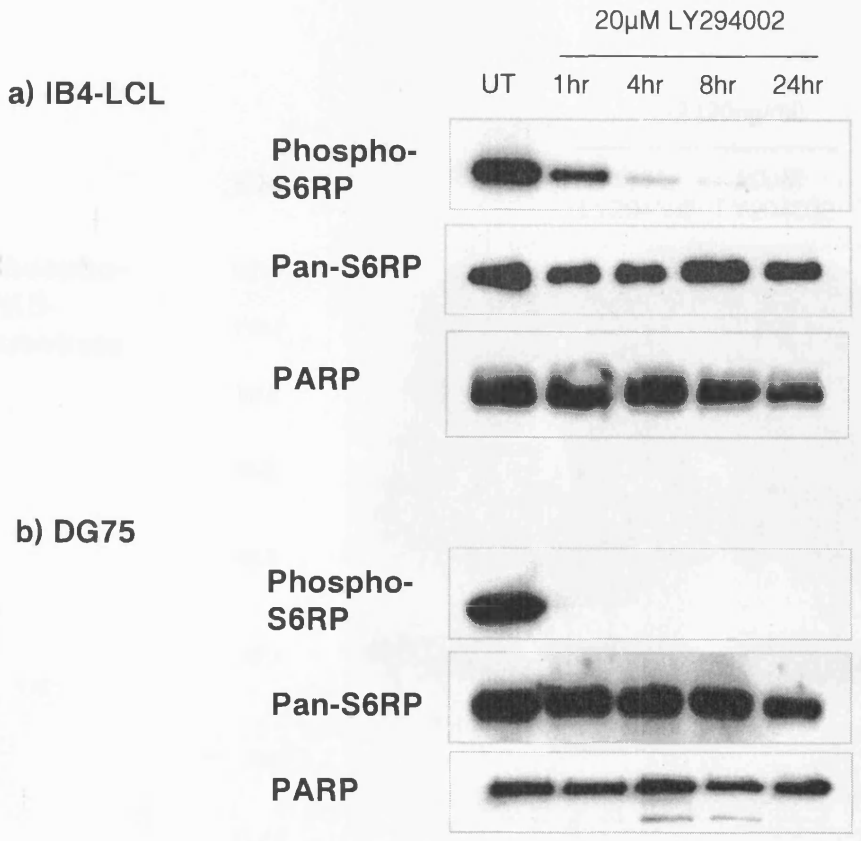


Figure 3.5 – Characterisation of the response of phospho-S6-ribosomal protein to LY294002 in LCLs and Burkitt's Lymophoma B cell lines: Time course studies

Nuclear extracts were generated from a) a lymphablastoid cell line (IB4-LCL) and b) a Burkitt's lymphoma cell line (DG75), that were either untreated (UT) or treated with LY294002 (20 μM) for increasing periods of time as indicated (1, 4, 8, 24 hours). Proteins were resolved by SDS-PAGE, and were analysed by immunoblotting using phospho-S6 and pan-S6 antibodies. Equal protein loading was checked by immunoblotting for Poly-ADP-ribose polymerase (PARP).

KIT-225

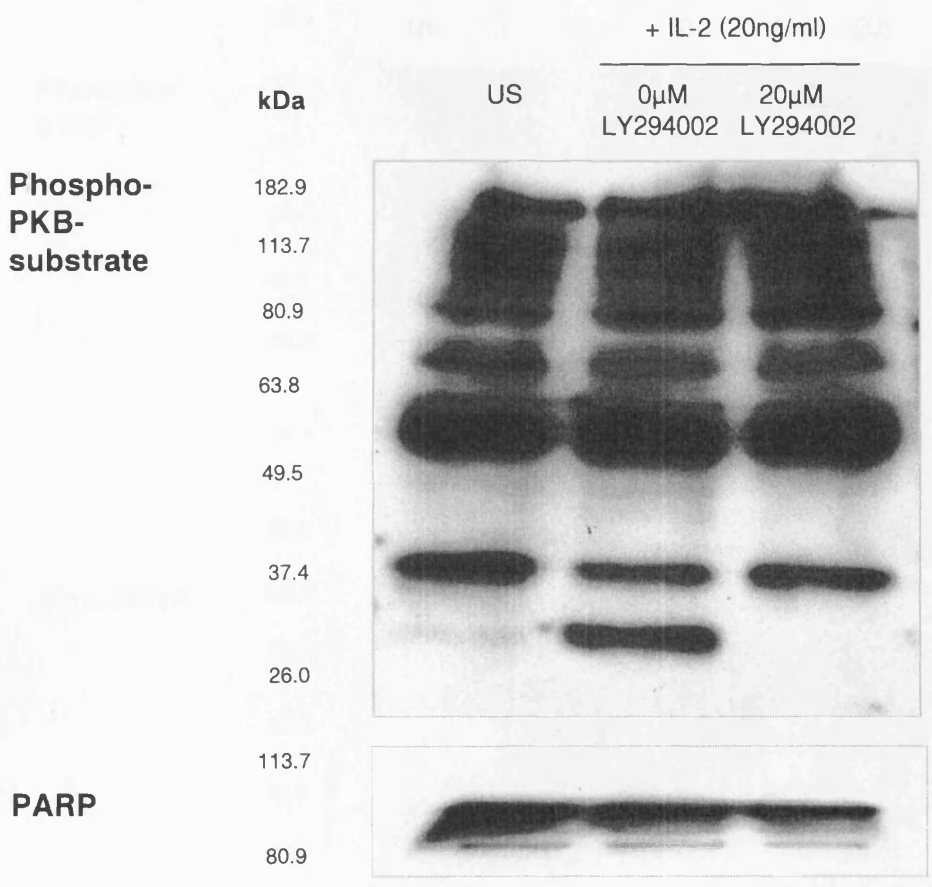


Figure 3.6 – Identification of pp32 as a major target of PI3K signalling in T-cells.

KIT225 cells were quiesced by washing out of IL-2 for 3 days. Cells were subsequently pretreated with LY294002 (20μM) for 30 minutes or were left untreated, and then stimulated with IL-2 (20ng/ml) for 30 minutes. Nuclear protein extracts generated were resolved by SDS-PAGE and analysed by immunoblotting using the phospho-PKB-substrate antibody. Equal protein loading was checked by immunoblotting for PARP.

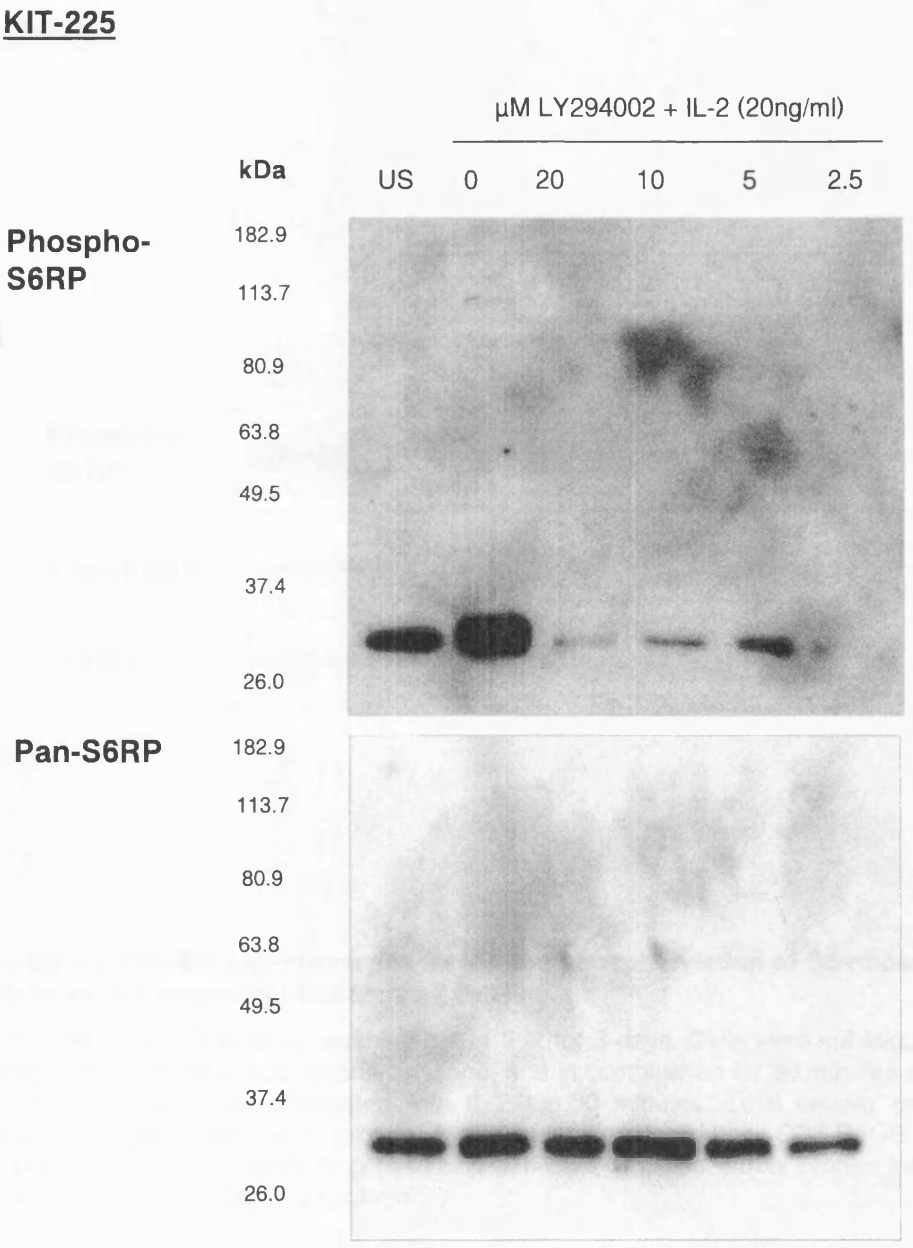


Figure 3.7 – Identification of pp32 as phospho-S6-ribosomal protein in Kit225 cells

KIT225 cells were quiesced by washing out of IL-2 for 3 days. Cells were subsequently pretreated with LY294002 at various doses as indicated for 30 minutes or were left untreated, and then stimulated with IL-2 (20ng/ml) for 30 minutes. Nuclear protein extracts generated were resolved by SDS-PAGE and analysed by immunoblotting using phospho-S6 and pan-S6 antibodies.

KIT-225

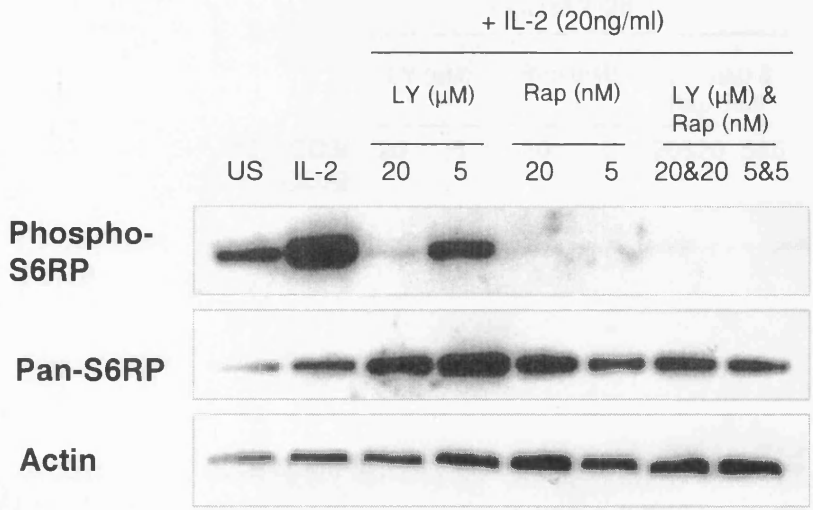


Figure 3.8 – LY294002 and rapamycin inhibit the phosphorylation of S6-ribosomal protein in an IL-2 dependent leukaemic T cell line.

KIT-225 cells were quiesced by washing out of IL-2 for 3 days. Cells were subsequently pretreated with LY294002 and rapamycin alone, and in combination for 30 minutes at the doses indicated, and then stimulated with IL-2 for 30 minutes. Total cellular protein extracts were generated and protein levels were analysed by SDS-PAGE and immunoblotting using phospho-S6 protein and pan-S6 antibodies. Equal protein loading was checked by immunoblotting for actin.

Primary T cells

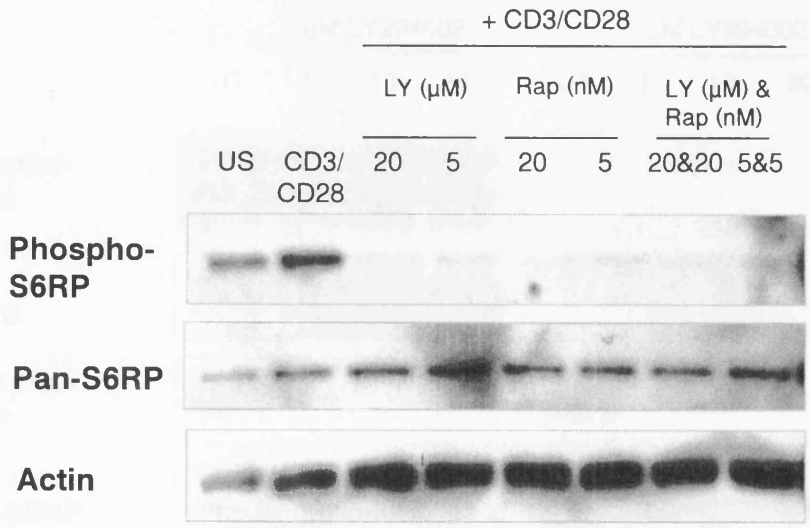


Figure 3.9 LY294002 and rapamycin inhibit the phosphorylation of S6-ribosomal protein in primary T cells.

Primary T cells blasts were generated by activating and growing human T cells in PHA (1 μg/ml) and IL-2 (20ng/ml) for 1 week. The cells were quiesced by washing out of IL-2 for 3 days. Cells were subsequently pretreated with LY294002 and rapamycin alone, and in combination for 30 minutes at the doses indicated, and then stimulated with anti-CD3 (2.5ng/ml) and CD28 (5μg/ml) for 30 minutes. Total cellular protein extracts were generated and protein levels were analysed by SDS-PAGE and immunoblotting using phospho-S6 and pan-S6 antibodies. Equal protein loading was checked by immunoblotting for actin.

IB4-LCL

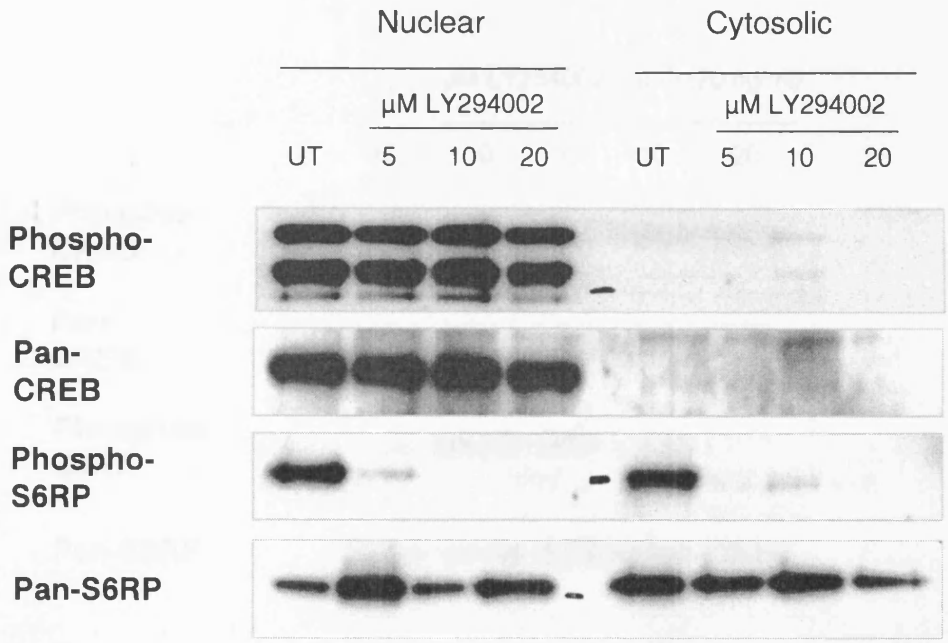


Figure 3.10 –Characterisation of the phosphorylation status of S6-ribosomal protein and CREB in response to LY294002 treatment in LCLs.

Cytosolic and nuclear extracts were generated from IB4-LCLs that were either untreated (UT) or treated with an increasing concentration of LY294002 for 1 hour (5, 10, 20μM). Proteins were resolved by SDS-PAGE, and were analysed by immunoblotting using phospho-CREB, pan-CREB, phospho-S6 and pan-S6 antibodies. Phospho-CREB is represented by the higher molecular weight band. The lower molecular weight band represents ATF-2, another member of the CREB family, visible due to cross-reactivity with the phospho-CREB antibody.

KIT-225

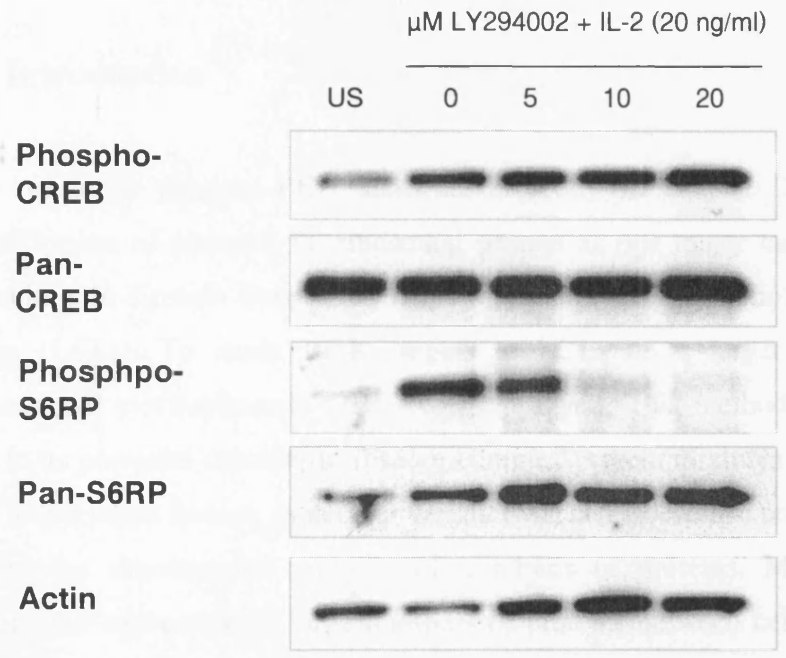


Figure 3.11 –Characterisation of the phosphorylation status of S6-ribosomal protein and CREB in response to LY294002 in T cells: Dose response studies.

KIT225 cells were quiesced by washing out of IL-2 for 3 days. Cells were subsequently pretreated with LY294002 at the doses indicated (5, 10, 20µM) for 30 minutes, or were left untreated (UT), and then stimulated with IL-2 (20ng/ml) for 30 minutes. Nuclear protein extracts generated were resolved by SDS-PAGE and analysed by immunoblotting using phospho-S6, pan-S6, phospho-CREB and pan-CREB antibodies. Equal protein loading was checked by immunoblotting for actin

CHAPTER 4

Proteomic analysis of the lymphocyte nucleus

4.1 Introduction

The use of a phospho-PKB substrate antibody in chapter 3 led to the identification of phospho-S6 ribosomal protein as one major target of PI3K signalling in Epstein-Barr virus (EBV) immortalized Lymphoblastoid Cell Lines (LCLs). To study PI3K targets in LCLs on a larger scale, two-dimensional electrophoresis (2DE) was employed. This method was chosen due to its powerful capacity to resolve complex protein mixtures according to two independent factors; molecular weight (Mr) and isoelectric point (pI). This allows the simultaneous analysis of hundreds of proteins. Moreover, the differential expression of large numbers of proteins between cell types or in response to the activation or perturbation of signalling pathways can be monitored, with a view to identify differentially expressed proteins. The resolving power of 2DE also makes it a useful technique for the study of post-translationally modified proteins (Naaby-Hansen *et al*, 2001). Other methods such as high-throughput screening of transcriptional profiles using chip technology are useful for the large scale study of gene expression at the mRNA level. However, the results of mRNA studies do not always reflect the level of transcribed proteins directly participating in cellular functions. Furthermore, these approaches cannot measure or identify post-translational modifications, such as phosphorylation or acetylation, which often influence the ultimate function or stability of a protein (Andersen *et al*, 1997, Zieske, 2006). 2DE therefore has the advantage of allowing the study of gene expression at the protein level.

Previous studies using 2DE for the proteomic analysis of LCLs have collectively resulted in proteomic maps and databases for lymphoblastoid cells

(Caron *et al*, 2002, Toda *et al*, 2003). Toda *et al* compared protein expression between pre- and post-immortal LCLs and reported alterations in several non-viral proteins (Toda *et al*, 2000, Toda *et al*, 2003). Global post-translational modifications associated with LCL proteins have also been investigated by the specific detection of methylated (Huang *et al*, 2002) and phosphorylated proteins (Caron *et al*, 2002). More than 100 methylaccepting and 400 tyrosine-phosphorylated proteins were detected in the LCL. More recent studies using a proteomic approach for the analysis of LCLs have looked at the effects of individual EBV genes on target protein expression. One study used 2DE to compare the proteomes of primary B cells before and after infection with B95.8 EBV, with a conditional immortalization system for EBV that expressed EBV nuclear antigen 2A (EBNA2A) under the control of an estrogen receptor-EBNA2 fusion protein. (Schlee *et al*, 2004). This study found that changes observed during EBNA2 reactivation reflect early events during primary B cell EBV infection. Another study analysed the effects of Latent Membrane Protein 1 (LMP1) signalling on the phosphoprotein component of the B cell by combining phosphoprotein enrichment with 2DE (Yan *et al*, 2006). This study demonstrated the capacity of EBV to increase the phosphoprotein component of the lymphocyte proteome as well as alter the phosphorylation patterns of individual proteins. In addition several novel targets of LMP1 signalling were identified, highlighting the value of this approach for the identification of molecules targeted by signalling pathways. All of these studies analysed protein expression in whole extracts of lymphoblastoid cells.

In this study, targets of the PI3K pathway were investigated specifically in the nuclei of LCLs. The nucleus plays an important role in co-ordinating cell activities, including growth, proliferation, and protein synthesis. The expression of nuclear proteins that regulate such processes are frequently altered in cancer in response to deregulated signalling through pathways activated by growth factors. The transformation of cells into diseased states is often associated with disturbances in transcription profiles (Maston *et al*, 2006). Increasing our knowledge of the expression profiles of PI3K regulated nuclear proteins will therefore lead to a greater understanding of the

mechanisms behind transcriptional regulation in cancer. LCLs are a good model for the study of PI3K as signalling through this pathway is constitutively activated by EBV encoded genes (Young & Rickinson, 2004).

The initial approach taken was the analysis of differential protein expression in the nucleus of an LCL following treatment with LY294002, a specific inhibitor of PI3K. The minigel system was utilized due to its relative speed compared with larger gel formats. Although sensitivity is greater with the use of larger gels, minigels allowed a higher-throughput of data and gave the option of being able to study specific proteins by immunoblotting after resolution by 2DE more easily. The isoelectric focusing (IEF) strips and SDS-PAGE gels used were pre-cast to improve reproducibility and reliability. This system was also employed for comparing the nuclear protein expression profiles of EBV negative and EBV positive B cells in order to further characterise the effect of the EBV genome on protein expression. Further development of the technology included a pre-fractionation protocol prior to 2DE that proved useful for the specific analysis of DNA-bound transcription factor targets of EBV. Overall, this chapter explores the use of proteomic strategies for the study of protein expression in the lymphocyte nucleus.

4.2 Separation of lymphocyte nuclear proteins for analysis

4.2.1 Partitioning of nuclear and cytosolic proteins

The objective of developing the technology of 2DE was to study the expression of nuclear lymphocyte proteins. It was therefore important to assess the efficiency of the subcellular fractionation protocol used (see section 2.3.2). Cytosolic fractions were generated by the addition of a low salt detergent lysis buffer to disrupt the plasma membrane to release cytosolic proteins, whilst keeping the nuclear membrane intact. After removal of the cytosolic fraction the remaining pellet was incubated with a high salt buffer for disruption of the nuclear proteins and release of proteins associated with nucleic acids. Partitioning of nuclear and cytosolic proteins was assessed by immunoblot analysis employing antibodies against marker proteins of the nucleus and cytosol. Protein levels of α-tubulin, a cytosolic protein, and Poly-ADP ribose polymerase (PARP) a nuclear protein, were analysed in cytosolic and nuclear extracts generated from SP-LCL and IB4-LCL (Figure 4.1a). Cells were either left untreated or were treated with LY294002 (20μM, 1 hour). Partitioning of PARP to the nuclear fraction of IB4-LCL was more successful than in SP-LCL as no PARP was detected in the cytosolic fraction. Some contamination of the cytosolic fraction with nuclear proteins was evident in SP-LCL lysates. The detection of α-tubulin in both cytosolic and nuclear fractions generated from SP-LCL and IB4 LCL demonstrated contamination of nuclear protein fractions with cytosolic proteins.

The efficiency of the subcellular fractionation protocol needed to be improved to ensure that further studies were specific to the nuclear proteome. For this reason, one or more washing steps of the nuclear pellet generated following removal of the cytosolic fraction with low salt detergent lysis buffer was included in the protocol. The low salt content of the washing buffer minimised disruption of nuclear proteins whilst ensuring the removal of cytosolic proteins. Protein levels of α-tubulin and PARP were analysed prior to washing, and subsequent to one, two and three washing steps (Figure 4.1b). In

addition, protein levels of another cytosolic protein, calregulin (also known as calreticulin) were analysed. The nuclear fraction generated from the unwashed nuclear pellet demonstrated contamination with α-tubulin and calregulin. Inclusion of the washing steps in the protocol resulted in the elimination of α-tubulin from the nuclear fraction, with one washing step being sufficient observe this effect. A similar effect was observed with calregulin, although a small amount was detectable even after 3 washing steps. Calregulin is a Ca^{2+}-binding storage chaperone that resides in the endoplasmic reticulum and is therefore predominantly found in the cytosol. However a small amount of calregulin has been found to associate with nuclear proteins. This provides an explanation as to why calregulin could not be completely eliminated from the nuclear fraction. No PARP could be detected in the cytosolic fraction and hence it could be used as a loading control for the nuclear fraction. Actin levels were analysed as a loading control for the cytosolic fraction. In order to ensure that further studies were carried out on nuclear proteins only, washing of the nuclear pellet was included as a step in the extraction protocol in all further experiments. Only one washing step was included in order to minimise the loss of genuine nuclear proteins.

4.2.2 Optimisation of separation of nuclear proteins by 2D electrophoresis (2DE)

In order to study protein expression in the lymphocyte nucleus 2DE was employed. A nuclear protein fraction generated from IB4-LCL was analysed by 2DE. Samples were prepared for 2DE using a method based on trichloroacetic acid (TCA) protein precipitation (Amersham) (see section 2.4.1). This procedure was carried out to remove interfering contaminants from the sample such as salts, detergents, phenolics, or nucleic acids that may impede proper separation of proteins during isoelectric focusing (IEF). Precipitated proteins were subsequently solubilised in 2D sample buffer (7M urea, 2M thiourea, 4% CHAPS). Precipitated and solubilised IB4-LCL nuclear proteins (20μg) were then subjected to IEF on a nonlinear gradient ranging from pH 3 to pH 10, before separation by SDS-PAGE on a 4-12% gradient gel. A standard molecular weight marker was also resolved on the gel in a

separate lane to allow approximation of molecular weight. Proteins and standard markers were visualised by silver staining. The proper displacement of proteins in the two dimensions should result in the formation of discrete protein spots. The first attempt at generating a 2D gel was unsuccessful as horizontal streaking of proteins was observed by silver staining, with no formation of discrete protein spots (Figure 4.2). The appearance of horizontal streaks of proteins was evidence that protein separation in the second dimension was successful but the process of IEF required optimization.

Efficient separation of proteins by IEF requires the cleavage of disulphide bonds within and between proteins to allow unfolding and complete denaturation. This is undertaken by the inclusion of a reductant in the sample buffer prior to IEF. Dithothreitol (DTT) was the reductant of choice. The concentration of DTT included in the original sample was 20mM. To investigate whether increasing the concentration of DTT would improve protein separation by IEF, a dose response experiment was carried out. In addition, the amount of protein loaded on the gel was reduced from 20μg to 10μg. Prepared nuclear samples from IB4-LCL were incubated with increasing doses of DTT (40mM, 50mM, 60mM) and were separated by 2DE. Proteins were subsequently visualized by silver staining (Figure 4.3). The increased concentration of DTT greatly improved the resolution of proteins by IEF, with the optimal concentration being 50mM. This was evidenced by the visualization of discrete protein spots being formed on the gel. In addition, horizontal tracks of protein spots were visible, demonstrating that distinct protein isoforms could be distinguished using this method.

Although good protein separation was achieved, this was mostly confined to higher molecular weight proteins, between 49.5kDa and 113.7kDa. The visualization of proteins located at the lower molecular weight end of the gel was obscured by a shadowing effect that occurred between pI 4.8 and pI 6.5 (Figure 4.4- upper panel). This issue needed to be resolved as proteins of interest were potentially lower molecular weight effectors of signalling pathways. It was possible that SDS precipitation during PAGE was causing this effect. SDS is a reducing agent and would therefore cause a staining effect

if precipitated out of solution during electrophoresis. One factor that has the potential to enhance this effect is a reaction between excess detergent micelles and SDS micelles during electrophoresis. A detergent is an essential component of the sample buffer in order to solubilise hydrophobic proteins and to minimize protein aggregation. CHAPS was included as a detergent in the sample buffer at a concentration of 4% (w/v). To determine if micelle formation due to excess CHAPS was the cause of the shadowing effect, a repeat experiment using IB4-LCL nuclear proteins was performed using 2% (w/v) CHAPS (Figure 4.4). Reducing the concentration of CHAPS from 4% (w/v) to 2% (w/v) eliminated the appearance of the shadow at the lower central end of the gel. This improved visualization of lower molecular weight proteins that could potentially be important for the study.

4.3 Identification of protein spots by tandem mass spectrometry (MS/MS)

The identity of protein spots on a 2D gel can be determined by mass spectrometry. To begin to construct a profile of protein expression in the lymphocyte nucleus, nuclear proteins from IB4-LCL were separated by 2DE (pH 3-10 NL gradient) and silver stained for visualisation. Twenty spots of varying intensities and location on the gel were manually excised from the gel. Gel plugs were sent to The Functional Genomics and Proteomics Laboratories at the University of Birmingham. In gel trypsin digestion of the protein spots was carried out and MS/MS was performed on each sample. MS/MS raw data was processed using MassLynx (Micromass) and the tryptic peptide sequences were provided as peak list (PKL) data files. The tryptic peptide sequences were then compared with the NCBI non-identical protein sequence database using MASCOT software (Matrix Science) (see section 2.8.2). Statistically significant hits were recorded together with the number of peptides and percentage coverage of protein. The criteria used for conclusive identification of a protein was either one peptide with a peptide expectation value (p) of < 0.00001, or two or more peptides with $p<0.05$. A conclusive identification was also allowed for two or more peptides with peptide expectation values of <1.0

and the sum of the MOWSE scores for only these peptides was >100. Peptides matching with trypsin and keratin were disregarded as contaminants. The approximate experimental Mr and pI of the protein, as judged by 2DE, were compared with the theoretical Mr and pI of the identified protein were compared to further assist protein identification.

Seven proteins were identified using this method (Figure 4.5 and Table 4.1). These were β-actin, ATP synthase - β subunit, ATP synthase – α subunit, chaperonin, 2-phosphopyruvate-α-endolase, nuclear ribonuclear protein and GTP binding protein Ran/TC4.

4.4 Differential protein expression analysis of nuclear targets of PI3K signalling in lymphocytes.

One aim of developing the technology of 2DE was to utilise the technique to identify novel targets of PI3K signalling in the lymphocyte nucleus. The approach taken was to investigate differential protein expression between nuclear extracts generated from untreated IB4-LCL with those treated with LY294002, a specific PI3K inhibitor.

4.4.1 Verification of efficacy of PI3K inhibitor – LY294002.

Before analysing the effects of LY294002 on protein expression in the nucleus as a whole, it was important to verify the efficacy of the PI3K inhibitor. As demonstrated in the previous chapter, a major target of PI3K signalling in B cells is the S6-ribosomal protein (S6). Inhibition of PI3K with LY294002 leads to the inhibition of S6 phosphorylation. Inhibition of S6 phosphorylation was therefore used as a means of verification that signalling through the PI3K pathway was being inhibited by LY294002. Nuclear protein extracts were generated from IB4-LCL that were either left untreated or were treated with LY294002 (20μM, 1 hour). Prior to analysis by 2D electrophoresis, an aliquot of each prepared protein sample was resolved by standard SDS-PAGE and analysed by immunoblotting (Figure 4.6). Phosphorylation of S6 was

significantly inhibited in all four replicate samples (A-D) prepared for analysis by 2DE. These data demonstrated that LY294002 was effective in inhibiting signalling through PI3K in the four samples analysed. Total protein levels were not affected by treatment with LY294002 as total protein levels of S6 and actin were not affected by the inhibitor.

4.4.2 Comparison of protein expression between untreated and LY294002 treated nuclear lymphocyte proteins by 2D electrophoresis.

Following verification of effective inhibition of the PI3K pathway, protein expression differences were analysed by 2DE. Untreated and LY294002 (20μM, 1 hour) treated samples from IB4-LCL were prepared and proteins (10μg) were resolved by 2DE using a non-linear pH 3-10 gradient. Proteins were visualised by silver staining. Figure 4.7 shows a complete view of protein expression in both treated and untreated samples in an example of one representative experiment. Maximal resolution of proteins was achieved between pI 4.5 and 8.5. Streaking was observed at the basic end of the gel (pH 8.5-10) due to the precipitation of basic proteins out of solution during IEF, preventing analysis of these proteins. An area within this range (pI 4.5-8.5, Mr 20.5-49.5) corresponding to four replicate experiments was selected for analysis using Phoretix 2D expression software (Nonlinear Dynamics) (Figure 4.8). An overview of the workflow of analysis of protein expression using Phoretix 2D expression software is given in Figure 4.9.

The Phoreix 2D software allows two gel images to be overlaid so that differences between the gels can be visualised using complementary false colours. Warping can also be carried out, allowing the images to be aligned according to the features of the gels, compensating for differences in running and scanning conditions. The selected areas (Mr 20.5-49.5, pI 4.5-8.5) from the untreated and LY294002 treated gels from four experiments (1-4) were warped and overlaid to identify proteins that are altered in expression following treatment with the inhibitor (Figure 4.10). Spots that altered in expression at a rate of four-fold or higher appear coloured on the image, green if expressed at a higher level in the untreated sample and magenta if expressed

at a higher level in the LY294002 treated sample. Spots that do not alter significantly in expression appear black. The power of this approach is illustrated by concentrating a specific region of a gel, where clusters of spots were differentially expressed in the untreated samples when compared with LY204002 treated samples (Figure 4.10, Areas 1-3). It was possible to zoom in on these areas of the gels and create montage windows for areas 1-3 (Figures 4.11a, 4.12a, 4.13a), allowing visualisation of the same selected area of each gel in each experiment simultaneously. The montage window images for each area illustrated that protein expression differences occurred between the two compared sample sets. In order to determine whether these protein expression differences were reproducible and specific to treatment with LY294002, it was necessary to quantify the differences in the expression of specific proteins. This was achieved in the form of histograms displaying spot volume generated by the software for each individual matched spot in the experiment (Figures 4.11b, 4.12b, 4.13b).

Area 1

The overlaid and warped images demonstrated that most proteins in area 1 were not differentially expressed by a factor of four-fold or greater when comparing untreated and LY294002 treated gels (Figure 4.10). This was indicated by the black coloured spots detected using gel overlay analysis that were common to all four sets of gels. In addition, the black spots demonstrated that matching of corresponding spots between gels was successful. Some protein expression differences in this area could however be observed, as illustrated by magenta and green coloured spots. Specific spots selected for histogram analysis are circled in Figure 4.11a. Spot A appeared magenta in experiments 3 and 4 (Figure 4.10), suggesting a higher level of expression of this protein in LY294002 treated cells. Histogram analysis of selected spots in area 1 from each experiment allowed quantification of spot volume (Figure 4.11b). This data reflected that seen with overlay analysis, indicating an increase in the expression of protein A following LY294002 treatment in experiments 3 and 4. A slight increase in the expression of protein A was observed in experiments 1 and 2, but was not significant. Overlay and histogram analysis of spot B show that although a significant decrease in

expression following LY294002 treatment was observed in experiment 4, this was not reproducible and no differential expression was seen in experiments 1, 2 or 3. Overlay and histogram analysis of spot C revealed that this protein was differentially expressed when comparing the two data sets. The pattern of change of expression, however, did not reflect a specific change in expression in response to treatment with LY294002. Spot C was significantly upregulated in experiment 4, but was downregulated in experiments 2 and 3 in response to LY294002. No significant alteration in the expression of this protein was observed in experiment 1.

Area 2

Area 2 was selected for closer analysis as many proteins within this area appeared to be differentially expressed when comparing untreated and LY294002 treated samples by overlay analysis. This was indicated by several magenta and green coloured spots (Figure 4.10). Spots selected for analysis are circled in Figure 4.12a. Histogram analysis of selected spots in area 2 from each experiment allowed quantification of spot volume (Figure 4.12b). A significant downregulation of protein A was observed in two experiments, 3 and 4. Analysis of spot A in experiment 2, however, indicated an increase in expression, whilst a small decrease was observed in experiment 1. Spot B was clearly downregulated in response to LY294002 treatment in experiment 3, but this was not reproducible between experiments. Downregulation of spot C with LY294002 treatment was observed in experiments 1, 2 and 3, although not significantly in experiment 1. In contrast, a small increase in the expression of spot C was detected on analysis of experiment 4.

Area 3

The overlaid and warped images of area 3 indicated that at least two spots within this area were differentially expressed between samples in all experiments (Figure 4.10). To investigate this further, specific spots were selected for spot volume analysis (Figure 4.13a). Histogram analysis of selected spots in area 3 from each experiment quantified changes in spot volumes (Figure 4.13b). Spot A was displayed as green on overlay analysis in experiments 1 and 4, suggesting a higher level of expression in control

samples. Histogram analysis of spot A in experiments 1 and 4 corroborate these observations with a significant decrease in spot volumes in both experiments. Data from experiments 2 and 3 however did not reproduce these results, with a contradictory increase in the expression of spot A being observed in the LY294002 treated sample in experiment 3. The expression of spot B fluctuated greatly between experiments. Overlay and analysis of spot B suggested a high level of expression in the untreated sample in experiment 1. In contrast, clear downregulation in response to LY294002 was observed in experiments 2 and 3. Expression of spot B was at a low level in both samples in experiment 4. Data from histogram analyses of spot B were in agreement with that observed by gel overlay, demonstrating great variability in differential expression between experiments. Histogram analysis of spot C demonstrated that although a downregulation of this protein following LY294002 treatment in all four experiments, this only occurred significantly in experiment 3.

All spots within the range of pI 4.5-8.5, Mr 20.5 – 113.7 kDa from experiments 1-4 were analysed by overlay and histogram analysis as described above. Many constitutive proteins expressed in the lymphocyte nucleus produced reproducible patterns detected on all gels. However, from the information gathered using the Phoretix analysis software the conclusion could be made that no proteins could be detected that were specifically upregulated or downregulated in response to inhibition of PI3K using this method. Although differential protein expression was observed between samples, these changes were not reproducible between experiments. This suggested that these changes were due to biological fluctuations in protein expression between samples.

4.4.3. Comparison of protein expression between untreated and LY294002 treated nuclear lymphocyte proteins by 2D electrophoresis using a narrow pH gradient.

Analysis of differential protein expression using a wide range pH gradient between pH 3 and pH 10 did not allow the identification of PI3K regulated

proteins. The majority of proteins detected within this gradient range were located within central areas of the gel, between pH 5 and pH 8 and with molecular weights above 49.5kDa. The density of proteins within this region therefore posed a problem in efficiently analysing the expression of individual proteins. For this reason, a narrower pH gradient was chosen to simplify the analysis of protein expression within this region. Untreated and LY294002 treated (20μM, 1 hour) samples were prepared and proteins (10μg) were resolved by 2DE using a linear pH 5.6-8 gradient. Proteins were visualised by silver staining. A typical example of gel sets produced by this method are shown in Figure 4.14. Efficient resolution of proteins was observed between pH 5.6 and 7.4. A streaking effect was again apparent between pH 7.4 and 8 due to the precipitation of basic proteins out of solution during IEF. Few proteins below Mr 39kDa could be detected. All spots detected in three replicate experiments were subsequently analysed using the Phoretix software.

Although differential protein expression was observed between untreated and LY294002 treated samples, again, these differences were not reproducible. This is illustrated by selecting a specific area (pI 6.8-7.4, Mr 55-95 kDa) for analysis (Figure 4.15). Two smaller areas from this region (1 and 2) were selected and the spot volumes of proteins that were differentially expressed when comparing untreated and LY294002 (20μM, 1 hour) were quantified by histogram analysis. The selected spot in area 1 was downregulated in experiments 2 and 3, but was upregulated in experiment 1 in response to LY294002 treatment (Figure 4.16a). Similar observations were made for the spot selected in area 2. A significant increase in expression was observed with LY294002 treatment in experiments 1 and 3, but this protein was downregulated under the same conditions in experiment 2 (Figure 4.16b). In conclusion, the use of a narrower range pH gradient improved the resolution of higher molecular weight proteins of pI 5.6-8, but did not allow the identification of specific alterations in protein expression in response to PI3K inhibition.

4.5 Comparison of lymphocyte nuclear phosphoproteomes before and after treatment with LY294002.

Many mediators of signalling pathways and gene expression are modified and regulated by phosphorylation. To investigate the phosphoproteome of the lymphocyte nucleus and phosphorylation changes that occur in response to signalling through PI3K, a specific phosphoprotein gel stain (Pro-Q-Diamond, Molecular Probes, Invitrogen) was employed. Pro-Q-Diamond is a fluorescent stain that sensitively and quantitatively detects proteins phosphorylated at tyrosine, serine and threonine residues. The intensity of staining is proportional to the number of phosphate groups associated with a protein.

Untreated and LY294002 (20μM, 1 hour) treated samples from IB4-LCL were prepared and proteins (10μg) were resolved by 2DE using a non-linear pH 3-10 gradient. Phosphorylated proteins were visualised by staining with Pro-Q-Diamond phosphoprotein stain (Figures 4.17a). Gels were subsequently silver stained to compare phosphoprotein expression with total protein expression (Figures 4.17b). Differential protein expression analysis of phospho-protein stained gels using Phoretix software could not be carried out as repeated staining of multiple gels to gain reproducible results was not successful. However, the central area of the gel (pI 5.2-7.6, Mr 23-90 kDa), where maximum separation of proteins had occurred was selected and the number of spots detected by the phosphoprotein stain and total protein stain in each gel could be calculated (Figure 4.18). The number of spots detected in the untreated and LY294002 treated phosphoprotein stained gels were 377 and 293 spots respectively. Following silver staining of the same gel, 888 spots were detected in the corresponding area of the untreated gel and 869 in the inhibitor treated sample. Although not definitive, these observations suggest that 30-40% of the lymphocyte nuclear proteome are phosphorylated proteins.

4.6 Proteomic analysis of cell lines of different EBV status.

The effects of inhibition of the PI3K pathway on protein expression in the lymphocyte nucleus were too subtle to be detected by the method of 2DE as used in this study. It was therefore investigated whether this method could be applied to detect protein expression differences between cell types that vary considerably in phenotype. The infection of B cells with Epstein-Barr virus (EBV) leads to the activation of a diverse range of cellular molecules that trigger B-cell growth and immortalization. These include the activation of tyrosine kinases, Jun N-terminal kinase (JNK), mitogen-activated protein kinases (MAPKs), the transcription factor, NFκB, as well as the PI3K pathway (Young & Rickinson, 2004). For this reason, nuclear protein expression was analysed in two genetically identical B cell lines that differ in their EBV status. BL41 is an EBV-negative Burkitt's Lymphoma (BL) line driven into continual proliferation by overexpression of c-Myc. BL41 cells grow as small single cells in suspension that are highly prone to apoptosis. IARC-171 is a B95.8 EBV immortalized LCL derived from the same patient as BL41. IARC-171 cells grow in large clumps characteristic of an LCL and are highly resistant to apoptosis. In addition IARC-171 cells express a variety of activation markers and adhesion molecules on their surface, whereas these are lacking in BL41 cells.

Several EBV encoded genes contribute towards the generation of survival and proliferation signals in infected cells. Although many of these pathways have been identified, the effector molecules and downstream nuclear targets of EBV induced signalling remain largely poorly characterized. To investigate the functional components of pathways activated by EBV, a proteomic approach was taken using 2DE to investigate differential protein expression between EBV negative and EBV positive cells.

Prepared nuclear protein extracts from BL41 and IARC-171 cell lines were resolved by 2DE on a pH 3-10 nonlinear gradient (Figure 4.19). The area where maximum resolution of proteins had occurred was selected for analysis

(pI 5.85-9.5, Mr 40.5-130kDa), (Figure 4.20). Overlay analysis of the selected area identified many spots that were expressed at a higher level in the BL41 cell line, and others that were expressed at a higher level in the IARC-171 cell line (Figure 4.21). These proteins were mostly located within the range pI 5.9 – 6.7, Mr 49-72 kDa. Montage windows focusing on this region were created and specific spots were selected for spot volume analysis (Figure 4.22a). The spot volumes of specific proteins were quantified by histogram analysis (Figure 4.22b).

Spot A was expressed at a higher level in IARC-171 in experiment 2, but was not significantly differentially expressed between samples in experiments 1 and 3. The protein represented by spot A is therefore not likely to be regulated by EBV. Both overlay analysis and histogram analysis of spot B showed a dramatic decrease in the expression levels of this protein in IARC-171 cells in all three replicate experiments. The molecular weight and isoelectric point of this protein were approximately 62k Da and pI 6.5 respectively. These results suggest that this protein is specifically downregulated by EBV. Spot C was also found to be expressed at a much lower level in IARC-171 cells in experiments 1 and 3. This was not however reproduced in experiment 2, where a significant increase in expression was seen in IARC-171. Focusing on a cluster of spots located at the higher molecular weight end of this region (Mr > 60 kDa), allowed the identification of two further proteins that differ in expression levels according to EBV status (Figure 4.23a). Histogram analysis of spot D demonstrated a reproducible downregulation of this protein in IARC-171 cells when compared to BL41. This was observed in all three experiments. The approximate molecular weight and isoelectric point of this protein are 70k Da and pI 6.2 respectively. In contrast, spot E, located at approximately 66 kDa, pI 6.25, was reproducibly expressed at a higher level in the nuclei of IARC-171 cells in all three replicate experiments (Figure 4.23b).

The comparison of nuclear protein expression between B cells of differing EBV status has therefore led to the detection of three proteins that are specifically regulated by the EBV. Two proteins were found to be

downregulated in the LCL. Another protein was identified that is expressed at a higher level in the LCL.

4.7 Analysis of the post-translational modifications of transcription factors.

The 2D gels generated for analysis of PI3K and EBV regulated proteins revealed that the method used was able to detect a large number of proteins expressed in the lymphocyte nucleus. These were mostly highly abundant lymphocyte nuclear proteins as few alterations in protein expression could be detected even in dramatically different cell lines in response to the activation of multiple signalling pathways. This was demonstrated by the comparison of EBV negative and positive cell lines (section 4.6). However, horizontal tracks of proteins were detected consistently in all gels produced. These tracks are likely to represent proteins that differ in their post-translational modification (PTM) status. Data obtained from the staining of 2D gels with Pro-Q diamond phopho-protein stain also indicted that many proteins within the lymphocyte nucleus display diverse phosphorylation patterns. It was therefore clear that it was possible to apply this technology to study the post-translational modifications associated with specific proteins.

The post-translational modifications (PTMs) of transcription factor proteins play an important role in regulating their transcriptional activities. The patterns of PTMs determine the subcellular localisation, conformation and macromolecular interactions of transcription factors, which ultimately govern their function (Tootle & Rebay, 2005, Seet *et al*, 2006). Many transcription factors are known to be activated in EBV immortalized B cells, including NFκB, STAT1 and CREB1. The constitutive activation of PI3K in EBV infected cells has been described to play a role in the activation of NFkB and CREB1 (Brennan, 2001, White *et al*, 2006). The next step in the study was to use 2D electrophoresis to investigate post-translational modifications associated with these transcription factors.

4.7.1. Western blot analysis of proteins separated by 2D electrophoresis.

For the analysis of specific proteins within complex protein mixtures, proteins separated by 2DE can be blotted onto a membrane and immunodetected using specific antibodies. To test the use of this approach, IB4-LCL nuclear proteins resolved by 2DE using a pH 3-10 nonlinear gradient were transferred on to a PVDF membrane. Immunodetection with an antibody towards actin revealed a large spot at approximately 45 kDa that ranged from pI 5.4-5.6 (Figure 4.24a). The theoretical molecular weight and isoelectric point of actin is 42 kDa and pI 5.2 respectively. Detection of the STAT1 transcription factor using this method with the use of a STAT1 specific antibody revealed a large train of spots at 91kDa, the theoretical molecular weight of STAT1 (circled, Figure 4.24b). Various spots spanning a large range of isoelectric points, pI 3.9 – 5.9, were detected at this molecular weight. The spots identified within this range are likely to represent distinct isoforms of STAT1. Several other spots were evident on the membrane immunoblotted with the STAT1 specific antibody. It is possible that these represent degraded forms of STAT1 as they occurred at molecular weights below that of the intact STAT1 protein. Several distinct spots at 42kDa could also be detected with the use of a specific CREB1 antibody (Figure 4.24c). The isoelectric points of CREB1 isoforms detected ranged between pI 4.8 and pI 5.45. The theoretical molecular weight and isoelectric point of CREB1 are 42 kDa and pI 5.45 respectively. These experiments demonstrated that the immunodetection of proteins from a complex mixture separated by 2DE was a useful method for the analysis of specific lymphocyte nuclear proteins. In addition, several distinct isoforms of two transcription factors, CREB1 and STAT1 were detected in the lymphocyte nucleus, demonstrating that this approach could be utilized for post-translational modification analysis.

4.7.2 Combining DNA affinity precipitation with 2D electrophoresis for the study of the post-translational modifications of EBV regulated transcription factors.

Post-translational modifications (PTMs) associated with transcription factor targets of EBV were studied by combining 2DE and immunoblotting as described in section 4.7.1. DNA binding is usually required for transcription factors to regulate target genes and downstream effects. DNA-Affinity Precipitation (AP) was therefore carried out prior to 2DE to allow the study of PTMs associated specifically with DNA bound complexes. In addition, this method enriched for the protein of interest, facilitating detection by immunoblotting. A schematic describing the procedure of DNA-AP used for PTM analysis is illustrated in Figure 4.25.

DNA-AP enrichment of CREB1 from the nuclear protein extracts of IB4-LCL and BL41 + B95.8 cells was carried out using two oligonucleotides containing the conserved cAMP-responsive element (CRE) (TGACGTCA) (Brindle & Montminy, 1992); the cyclin D2 promoter and the CREB consensus binding sequence. DNA binding of CREB1 was checked by resolving the DNA affinity precipitated protein extracts on an SDS-PAGE gel followed by immunoblotting using a CREB1 specific antibody. The binding specificity of CREB1 proteins was confirmed by incubating nuclear extracts with agarose beads alone. The amount of CREB1 bound to both the cyclin D2 and CREB consensus binding sequences were found to be of similar levels (Figure 4.26a-i). Binding was specific as indicated by the absence of CREB1 in the sample incubated with beads alone. DNA affinity precipitated protein extracts using the CREB consensus oligonucleotide were subsequently resolved by 2D electrophoresis on a pH 3-10 nonlinear gradient. Immunodetection of CREB1 revealed that five forms of DNA bound CREB1 could be detected in the nuclei of IB4-LCL and BL41 + B95.8 cells (Figure 4.26a-ii). The distinct isoforms detected in IB4-LCL and BL41 + B95.8 had equal molecular weights and isoelectric points ranging from pI 4.8-5.45 and pI 4.85-5.45 respectively. The three predominant isoforms in both cell lines were pI 5.2, pI 5.3 and pI 5.4. The isoform at pI 5.45 was also common to both cell lines, but bound to DNA

at a reduced level. The isoform with the lowest pI in the IB4-LCL was pI 4.8, compared to that of pI 4.85 in BL41 + B95.8 cells.

The PTMs of DNA bound p65, a component of the NFκB transcription factor, was also studied. DNA-AP enrichment of p65-NFκB from the nuclear protein extracts of IB4-LCL and BL41 + B95.8 cells was carried out using an oligonucleotide containing the NFκB consensus DNA binding sequence (GGACTTTCC). DNA binding of p65 was checked by resolving the DNA affinity precipitated protein extracts on as SDS-PAGE gel followed by immunoblotting using a p65 specific antibody. The binding specificity of p65 proteins was confirmed by incubating nuclear extracts with agarose beads alone. A clear band representing specific p65 binding to the oligonucleotide sequence was detected (Figure 4.26b-i). The same samples were subsequently resolved by 2DE on a pH 3-10 nonlinear gradient. Immunodetection with an antibody specific for p65 revealed that one form of DNA bound p65-NFκB could be detected at pI 5.5, in the nuclei of IB4-LCL and BL41 + B95.8 cells (Figure 4.26b-ii).

4.7.3 Investigation of the effect of kinase inhibitors of the DNA binding and post-translation modifications of CREB1.

The CREB transcription factor has been implicated in the regulation of lymphocyte proliferation and survival. This role has been demonstrated by studies showing that the expression of a dominant negative form of CREB in transgenic mice results in the impaired proliferation of thymocytes and B-cells (Barton *et al*, 1996. Zhang *et al*, 2002c). The transcriptional activity of the CREB1 transcription factor is believed to be dependent on the phosphorylation of CREB1 at the serine 133 residue. Kinase targets of various signalling pathways have been implicated in this phosphorylation event, including PKB downstream from PI3K, p90RSK downstream from ras signalling, CAM-kinase II (Ca^{2+}/calmodulin-dependent protein kinase II) in response to Ca^{2+} signalling and PKA activation by cAMP (Mayr & Montminy, 2001, Johannessen *et al*, 2004). To investigate the effect of inhibition of these pathways on the DNA binding and post-translational modifications of CREB1

a range of kinase inhibitors were employed. The inhibitors utilized were staurosporine, a broad spectrum protein kinase inhibitor, KT-5720, a PKA inhibitor, and LY294002, the specific PI3K inhibitor.

BL41 + B95.8 cells were either left untreated or were treated with staurosporine (2μM), KT-5720 (2μM) or LY294002 (20μM) for 1 hour prior to nuclear protein extraction. DNA binding of CREB1 molecules to the CREB consensus oligonucleotide was subsequently analysed by DNA-affinity precipitation, followed by SDS-PAGE and immunoblotting. A clear band representing CREB1 DNA binding was detected in the untreated cells. CREB1 DNA binding was significantly reduced, to the same extent, in response to treatment with staurosporine, KT-5720 and LY294002 (Figure 4.27a). The effects of kinase inhibitors on the DNA binding of the phosphorylated form of CREB1 was also analysed in parallel to total CREB1 (Figure 4.27b). A clear band representing DNA binding of phosphorylated CREB1 was detected. Treatment with staurosporine (2μM, 1 hour) completely abolished DNA binding of the phosphorylated form of CREB1. The DNA bound CREB1 detected in staurosporine treated cells using the pan-CREB1 antibody therefore represent unphosphorylated CREB1 molecules. Phosphorylated CREB1 binding to DNA was detectable in KT-5720 treated cells, although to a reduced level compared to untreated cells. These results agree with data that demonstrates that pathways in addition to PKA have a role in regulating the phosphorylation of CREB1.

2DE analysis of the kinase inhibitor treated samples from BL41 + B95.8 cells was carried out in parallel to 1D SDS-PAGE. DNA affinity precipitated CREB1 molecules resolved by 2DE on a pH 3-10 nonlinear gradient were immunoblotted using specific phospho-CREB1 and pan-CREB1 antibodies (Figure 4.28a). Multiple isoforms of CREB1 were detected in the untreated BL41 + B95.8 cells and their pI range reflected those previously detected (Figure 4.24), at pI 4.85-5.45. The predominant isoforms at pI 5.2, 5.3 and 5.4, and a lower abundance isoform at pI 5.45 were again detected. Treatment with KT-5720 did not alter the post-translational modifications associated with CREB1. However, the intensities of the spots detected were significantly

reduced, reflecting the reduction in the level of DNA binding of CREB1 post treatment with KT-5720. The isoforms at pI 5.45 and 5.3 were most significantly reduced in intensity in response to treatment with the PKA inhibitor. Treatment with staurosporine resulted in the appearance of a protein smear at 42kDa, with no detection of discrete protein spots. In addition, a lower molecular weight spot of increased intensity was evident at approximately 37kDa. This pattern was reproducible and may represent degraded forms of CREB1.

Immunoblotting using the antibody specific for the phosphorylated form of CREB1 in the untreated BL41 + B95.8 cells allowed the detection of a pattern of spots similar to that seen using the pan-CREB1 antibody (Figure 4.28a-ii). Although the resolution of isoforms into discrete spots was not achieved to the same degree, the isoform at pI 5.45 was clearly not detected suggesting that this isoform does not require phosphorylation for DNA binding. Phosphorylated forms of CREB1 in KT-5720 and staurosporine treated cells could not be detected by 2DE. This is likely to be due to the low amount of CREB bound to DNA post treatment with these inhibitors as demonstrated in Figure 4.27.

The use of a narrower pH gradient was used to further analyse the effect of KT-5720 treatment on the PTMs of CREB1. DNA affinity precipitated CREB1 molecules from untreated and KT-5720 (2μM, 1 hour) treated BL41 + B95.8 cells were resolved by 2DE on a linear pH 4-7 gradient. CREB1 isoforms were subsequently detected by immunoblotting with the pan-CREB1 antibody. The narrower gradient allowed the detection of additional isoforms of CREB1 to those detected using a pH 3-10 gradient (Figure 4.28b). This suggested that individual spots detected using the pH 3-10 gradient represented more than one isoform of CREB1 that could not be efficiently resolved using this gradient. Eight isoforms of CREB1 were detectable using a pH 4-7 gradient in the untreated cells, with isoelectric points ranging from 4.4 to 5.35. The isoforms of higher abundance had pI values of 4.9 and 5.0. Two lower abundance isoforms were of pI values 5.2 and 5.35. Four isoforms of lower pI values were also detectable at pI 4.4, 4.55, 4.7 and 4.8. Treatment

with KT-5720 again resulted in the reduction in the intensities of all isoforms detected. However two isoforms at pI 4.55 and 4.7 (indicated with an arrow) were undetectable following treatment with KT-5720, suggesting that these isoforms require PKA for DNA binding.

In summary, combining 2DE with DNA-AP has allowed the study of transcription factor PTMs. By utilizing this method, one isoform of DNA bound p65-NFκB, and multiple forms of DNA bound CREB1 have been detected in the nuclei of two B-cell lines. In addition, kinase inhibitors have been shown to reduce the DNA binding capabilities of CREB1 as well as altering the pattern of post-translation modifications of this transcription factor.

4.8 Discussion

In this chapter the use of 2DE for the analysis of lymphocyte nuclear proteins was explored. The use of the minigel system was a convenient and useful method for the relatively high-throughput large scale analysis of nuclear proteins, allowing the detection of up to 1500 proteins at one time. This system was successfully used in combination with several visualisation methods, including silver staining, phosphoprotein staining and the detection of specific proteins by immunoblotting. Reproducible patterns of protein expression were achieved. This was demonstrated by several clusters of proteins that were common to all nuclear lymphocyte samples analysed by 2DE. One such protein was identified as β-actin by tandem mass spectrometry. In addition, the resolution of proteins was achieved to a high enough quality to observe distinct isoforms of proteins, represented by protein tracks on the gels that are likely to alter in their post-translational modification patterns.

Despite the resolution of over a thousand LCL nuclear proteins by 2DE, the use of this approach failed to detect PI3K regulated proteins. Although protein expression changes in LCLs treated with a PI3K specific inhibitor (LY294002) were detected, these were not reproducible and are therefore unlikely to be specific to treatment with the inhibitor. This suggests that alterations in protein expression in response to treatment with LY294002 are relatively subtle, and that the technology as used in this study was not sensitive enough to detect these changes. A study by Gygi *et al* evaluated the use of 2DE for the proteome analysis of low abundance yeast proteins (Gygi *et al*, 2000). This study found that proteins with abundances of <1000 copies/cell could not be detected using larger gel formats that have a capacity for increased protein loads (up to 50μg), compared to the mini gel format used in this study. This highlights the limitations of 2DE for the detection of low abundance proteins and may explain why PI3K regulated proteins were not detected in this study.

The use of 2DE was however successfully used to detect reproducible alterations in protein expression in cells that altered more dramatically in phenotype. Three proteins were found to be differentially expressed in EBV immortalized B cells when compared to EBV negative Burkitt's lymphoma B cells that have the same genetic background. Two proteins were downregulated and one protein was upregualted in the LCL. These proteins have not been identified to date but are possibly targets of one or multiple signalling pathways that are activated by EBV infection. These include signalling through tyrosine kinases, Jun N-terminal kinase (JNK), mitogen-activated protein kinases (MAPKs), PI3K and the activation of the transcription factor, NFκB (Murray & Young, 2001). The identification of the EBV regulated proteins by tandem mass spectrometry would be the next step.

A useful tool for the analysis of protein spots on 2D gels was the Phoretix 2D expression software (Nonlinear Dynamics). Many features of this software simplified and automated the process of detecting differentially expressed proteins between two gels. This improved the detection of small differences in respective spot patterns that would be laborious and inefficient if carried out manually. The overlay analysis feature provided a useful way of gaining an overview of protein expression differences between samples by colour coding spots that deviated above background level. Histogram analysis was also valuable in quantifying protein expression differences between samples by measuring spot volume. However, the software did provide several challenges in efficiently analysing 2D gel spot patterns. An automated warping feature was part of the initial analysis step to accommodate differences in running conditions. This process was not always successful, and presented a problem when analysing whole gel images. To resolve this issue, smaller corresponding areas of gels had to be selected and cross-analysed. Improvements in the matching of spots through extensive user seeding by applying 'landmark' proteins was subsequently required before subsequent analyses were carried out. This was a time consuming process but did reduce errors in the data by reducing the mismatching of spots. A study assessing the manual editing of spot matching has demonstrated that this process does reduce quantitative

errors in the data and therefore results in a more reliable method of evaluating differences in corresponding proteins between gels (Mahon & Dupree, 2001).

Combining DNA-Affinity precipitation with 2DE led to the detection of several DNA-bound isoforms of an EBV regulated transcription factor – CREB1. Five isoforms of CREB1 were detected in both IB4-LCL and BL41 + B95.8 cells that ranged between pI 4.8 – 5.45. These isoforms did not differ in their molecular weights suggesting that the modification groups are small and do not alter the apparent molecular mass of the protein. Phosphorylation is the only small modification group (80 Da) that has been reported for CREB1 and regulates CREB1 transcriptional activity in response to multiple signalling pathways. A phosphorylation event replaces the neutral hydroxyl groups on serine, threonine and tyrosine residues with a negatively charged phosphate group. The effect of a single phosphate group on the isoelectric point of a protein with a pI below 5.5 would be the addition of a single negative charge (Halligan *et al*, 2004). This results in an acidic shift. The addition of multiple phosphorylation groups enhances this effect, resulting in the characteristic 'phosphorylation train'. It is therefore possible that the CREB 1 isoforms detected by 2DE in this study vary in their phosphorylation patterns. A correlation has been observed between the intensity of the stimulus and the stoichiometry of CREB phosphorylation (Mayr *et al*, 2001) and so the isoforms detected may be CREB molecules at various stages of activation. The detection of a similar pattern of protein spots by immunoblotting with a phospho-CREB1 antibody supports this theory, although additional small modifications such as acetylation or methylation cannot be discounted. A phosphopeptide enrichment technique such as immobilized metal-affinity chromatography (IMAC), which takes advantage of the affinity of phosphate group for Fe(III) or gallium(III), carried out prior to 2DE could prove beneficial in providing a definitive answer (Posewitz & Tempst, 1999, Stensballe *et al*, 2001). Other strategies that could be implemented include affinity based enrichment methods using phosphospecific antibodies and identification by tandem mass spectrometry (Gronborg *et al*, 2002).

Protein expression differences were detected by comparing EBV negative and EBV positive proteins with the use of 2DE in this study. However, the main limitation of using this technique for the detection of more differentially expressed proteins, particularly in the context of PI3K, was sensitivity. The wide range of protein expression levels within the samples meant that the use of the minigel system in combination with silver staining limited the ability of the 2DE approach to detect certain proteins, particularly those of low abundance. This only allowed the visualization of a fraction of the proteome. As molecular targets of signalling pathways are likely to be lower in abundance compared to housekeeping proteins, this proved to be a problem in this study.

Improvements to the sensitivity of proteome analysis to allow more efficient analysis of cell signalling components could be made by the use of alternative proteomic techniques. The use of fluorescent dyes for the labeling and detection of proteins as used in 2D-fluroescence difference gel electrophoresis (DIGE) technology allows increased sensitivity over a broad dynamic range. The use of larger gel formats in this technique further improves sensitivity as it allows the increased resolution of larger amounts of protein. In addition, protein samples are compared on a single gel by labeling with different fluorescent dyes, therefore eliminating gel to gel variation. This ensures that differences observed are biological rather than experimental (Van den Bergh & Arckens, 2004). Mass spectrometric (MS) based proteomics are valuable due to the ability of MS to detect and identify proteins of low abundance. MS based methods also provide the additional advantage of allowing the detection of proteins not amenable to 2DE, such as very large or small proteins, or proteins with very acidic or basic properties. For example, means of enriching for proteins with defined chemical characteristics, such as SELDI-ToF MS (Surface enhanced laser desorption/ionization time of flight mass spectrometry), allows the subproteome of interest to be studied, and generates characteristic proteomic patterns for distinct protein samples (Isaaq *et al*, 2002). This technique has been successfully used for biomarker discovery in prostate (Petricoin *et al*, 2002a) and ovarian cancer (Petricoin *et al*, 2002b). Other approaches involve the use of stable isotope technology for quantitative

profiling via mass spectrometry. One such approach is Isotope Coded Affinity Tags (ICAT) (Gygi *et al*, 1999). This involves the labeling of samples with chemically identical tags that differ only in isotopic composition (heavy and light), contain a thiol-reactive group which covalently links to cysteine residues, and also a biotin moiety. Following labeling, differentially labeled samples are combined, enzymatically digested, and labeled peptides are selectively enriched via biotin-avidin affinity chromatography. The difference in the mass of differentially labeled peptide fragments allows these peptides to be separated, and subsequently quantified by mass spectrometry (Gygi *et al*, 1999). However, the requirement for proteins to contain a cysteine residue for the use of ICAT reagents means that many potential proteins of interest, including those containing post-translational modifications, are not detected (Zieske, 2006).

More recent advances in the use of stable isotope technology have improved upon methods such as ICAT. For example, a similar approach allows the comparative profiling of proteins in mammalian cells by Stable Isotope Labelling by Amino Acids in Cell Culture (SILAC) (Ong *et al*, 2002). This technique allows the incorporation of isotopic labels into proteins by metabolic labeling in cell culture, as opposed to the use of covalently linked tags. Samples to be compared are cultured separately in media containing a light or a heavy form of an essential amino acid. This setting provides the advantage of eliminating the requirement for several chemical processing and purification steps, ensuring that compared samples are kept under similar conditions throughout the experiment (Ong *et al*, 2002).

In addition, the subsequent development of iTRAQ reagents (Ross *et al*, 2004), a new class of isobaric reagents has provided an alternative method of assessing differential protein expression in various systems. This method involves the labeling of samples with independent reagent of the same mass that, upon fragmentation in MS/MS give rise to unique reporter ions, which can subsequently be used to quantify the samples to be compared. The peptide reactive groups of these reagents react with all primary amines, including the N-terminus and the ε-amino group of lysine side chains, therefore allowing the

labeling of most peptides within a sample and enhancing peptide coverage for any given protein. In addition, this method allows the retention of other structural features such as post-tranlational modifications (Ross *et al*, 2004). The general workflow using iTRAQ reagents is as follows. Samples to be analyzed are each reduced, alkylated and enzymatically digested with trypsin. The resulting peptide pools are subsequently labeled with distinct iTRAQ reagents before the samples to be compared are combined. The peptide mixture is then resolved by reverse-phase column chromatography prior to sequence analysis by MS/MS. The resulting reporter ion spectra allows the detection of peptides that differ between samples. Subsequent database searching for peptide matches allows the identification of such proteins (Zieske, 2006). This approach has been used successfully by DeSouka *et al* to identify nine potential markers for endometrial cancer (DeSouka *et al*, 2005). A study by Zhang *et al*, combined the use of iTRAQ reagents with phospho-tyrosine immunoprecipitation, IMAC and MS/MS to identify signalling molecules in the epidermal growth factor-receptor-signalling cascade (Zhang *et al*, 2005).

The use of the approaches described above may be useful in meeting the objectives of this study; that is to identify signalling molecules downstream of PI3K that may be important for the effects of this pathway on lymphocyte proliferation and survival, particularly within the context of EBV immortalized LCLs. In summary, a combination of the approaches used in this study and the application of additional approaches may aid further characterization of the molecular changes occurring in the lymphocyte nucleus in response to EBV infection.

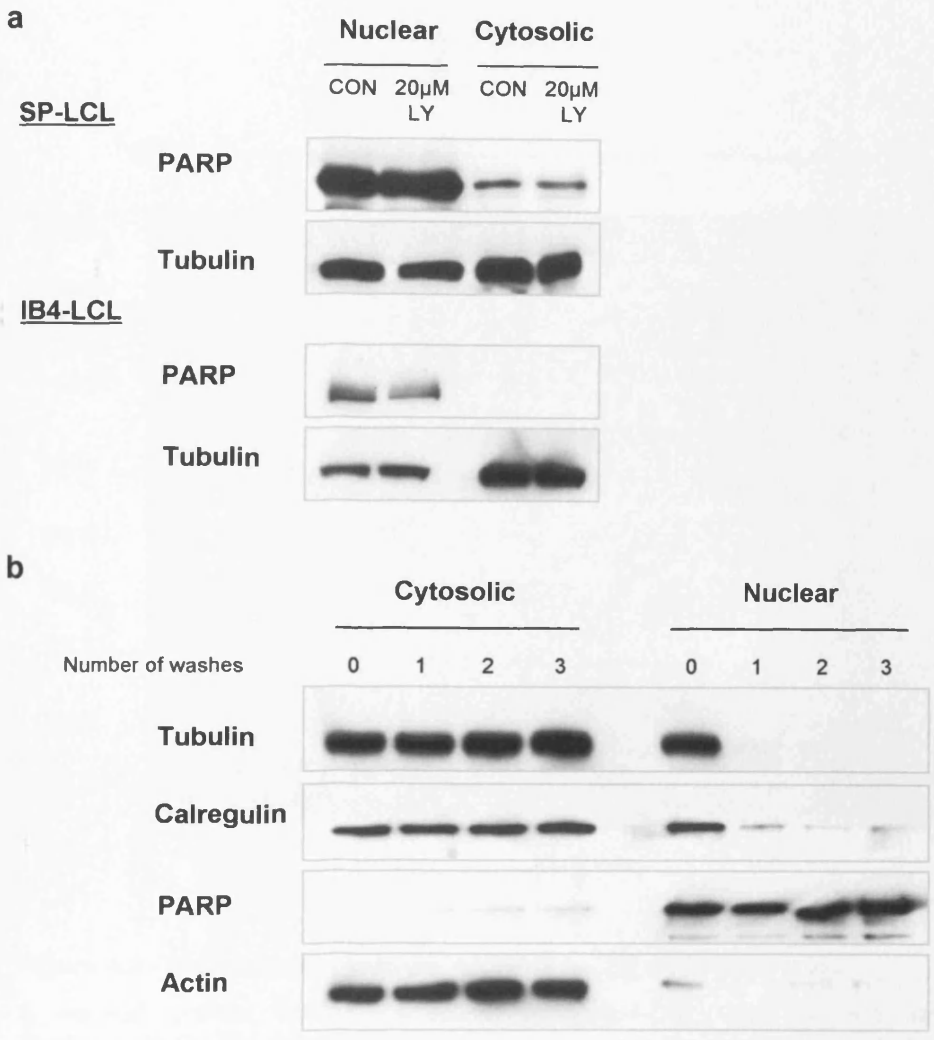


Figure 4.1 – Separation of nuclear and cytosolic fractions

a) Nuclear and cytosolic extracts were generated from SP-LCL and IB4-LCL that were either untreated or treated with LY294002 (20μM, 1 hour). Resolved proteins were analysed by immunoblotting using specific antibodies to α-tubulin and PARP.

b) Cytosolic and nuclear extracts were generated from IB4-LCL. Nuclear pellets were either unwashed (0) or washed with low salt detergent lysis buffer a number of times as indicated (1, 2 or 3). Resolved proteins were analysed by immunoblotting using antibodies specific to α-tubulin, calregulin, PARP and actin.

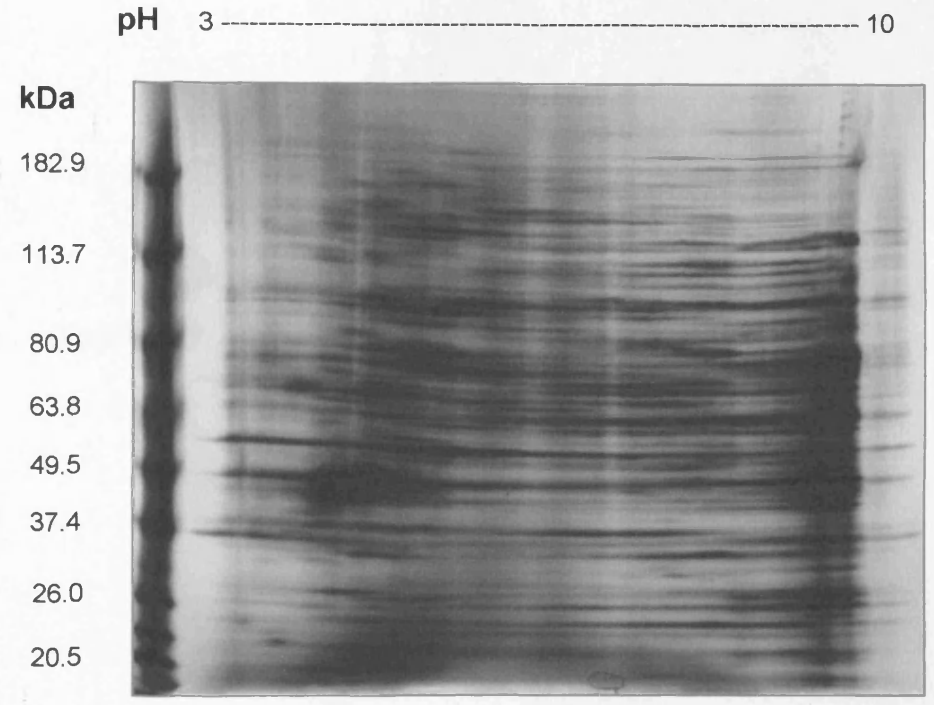


Figure 4.2 - Separation of nuclear proteins by 2D electrophoresis.

A nuclear protein extract generated from IB4-LCL was cleaned for 2D-electrophoresis. The concentration of DTT included in the sample buffer prior to IEF was 20mM. The proteins in the mixture were separated by 2D electrophoresis using a pH 3-10 nonlinear gradient. Proteins were subsequently visualized by silver staining.

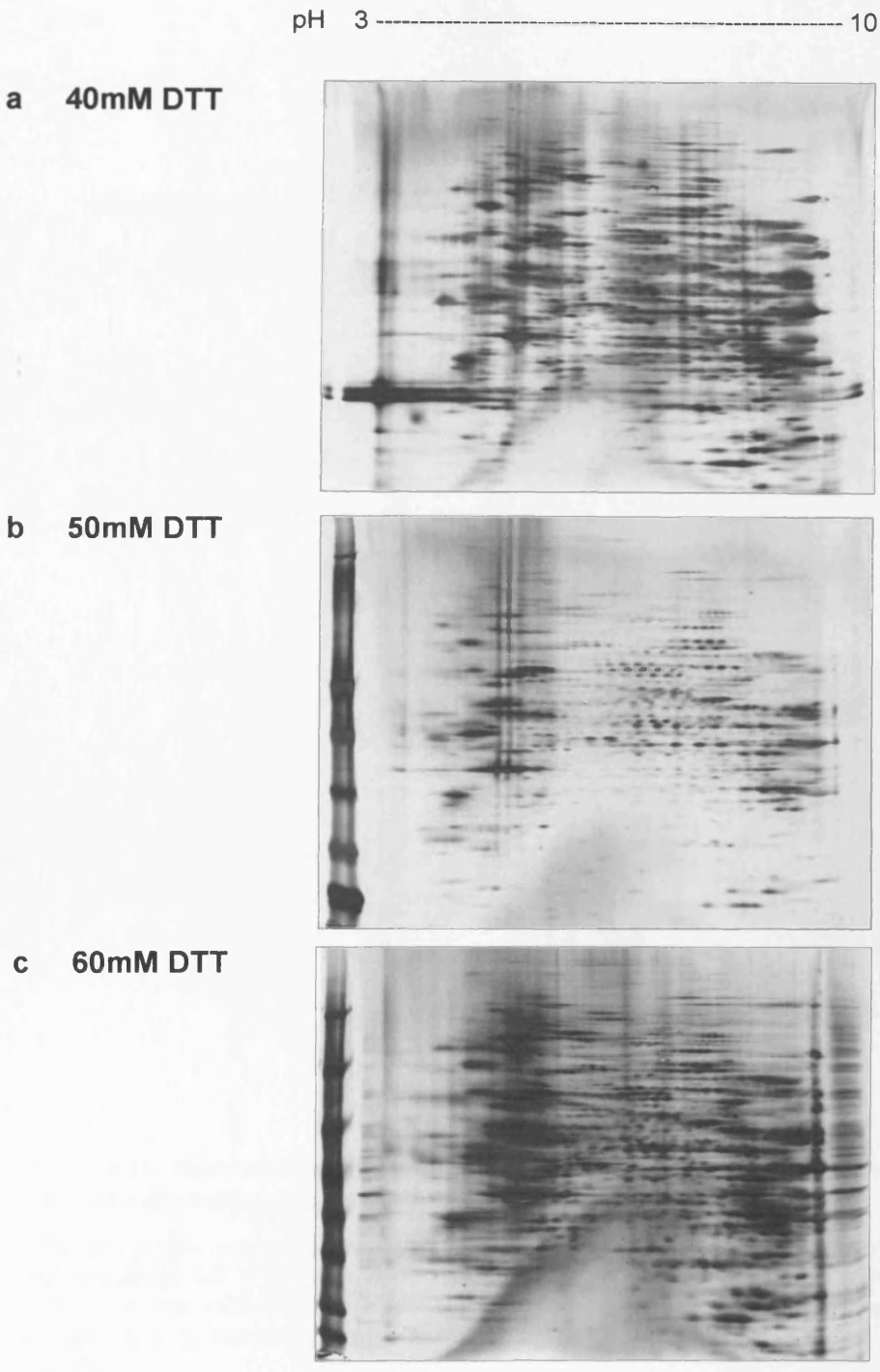


Figure 4.3 – Optimisation of concentration of DTT included in the sample buffer prior to IEF

A nuclear protein extract generated from IB4-LCL was cleaned for 2D-electrophoresis. Three samples were subsequently incubated with an increasing concentration of DTT; a) 40mM b) 50mM c) 60mM. The proteins in the mixture from each sample were separated by 2D electrophoresis using a pH 3-10 nonlinear gradient. Proteins were subsequently visualized by silver staining.

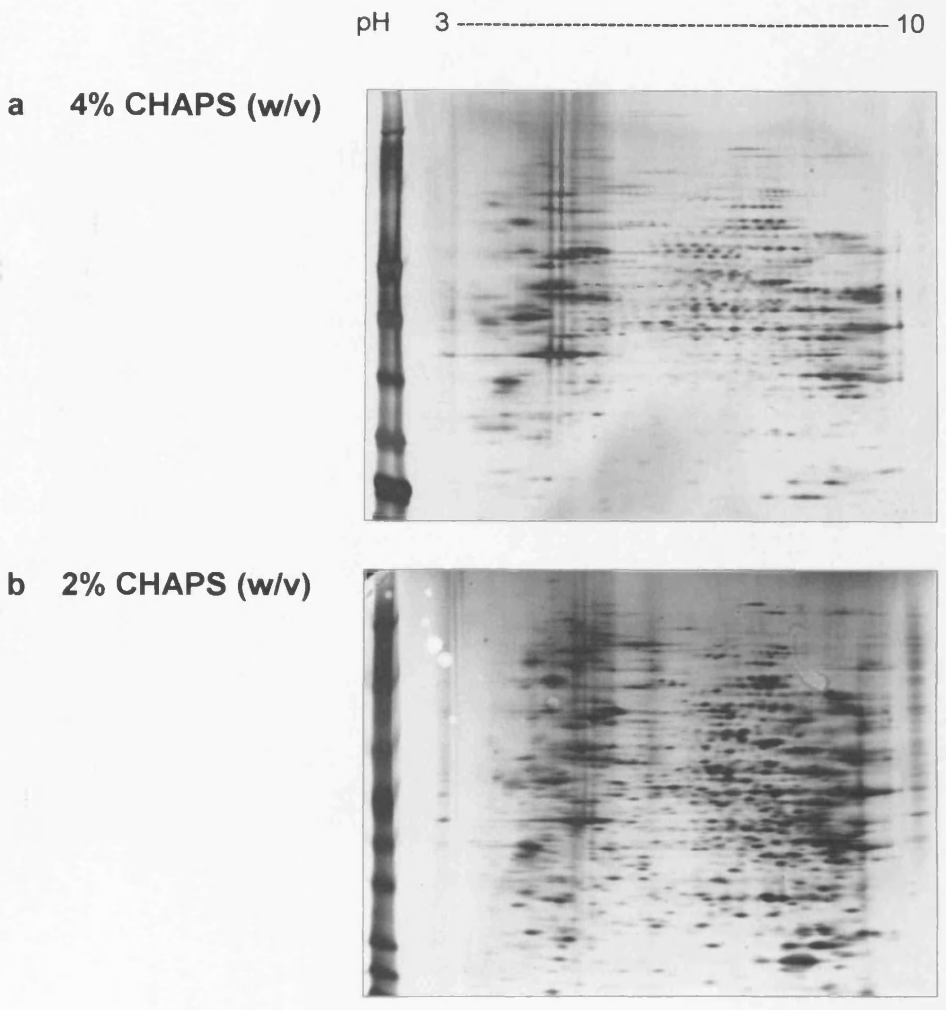


Figure 4.4 – Optimisation of the concentration of detergent (CHAPS) included in the sample buffer prior to isoelectric focusing.

A nuclear protein extract generated from IB4-LCL was cleaned for 2D-electrophoresis and re-suspended in sample buffer containing a) 4% CHAPS b) 2% CHAPS. The proteins in the mixture from each sample were separated by 2D electrophoresis using a pH 3-10 nonlinear gradient. Proteins were subsequently visualized by silver staining.

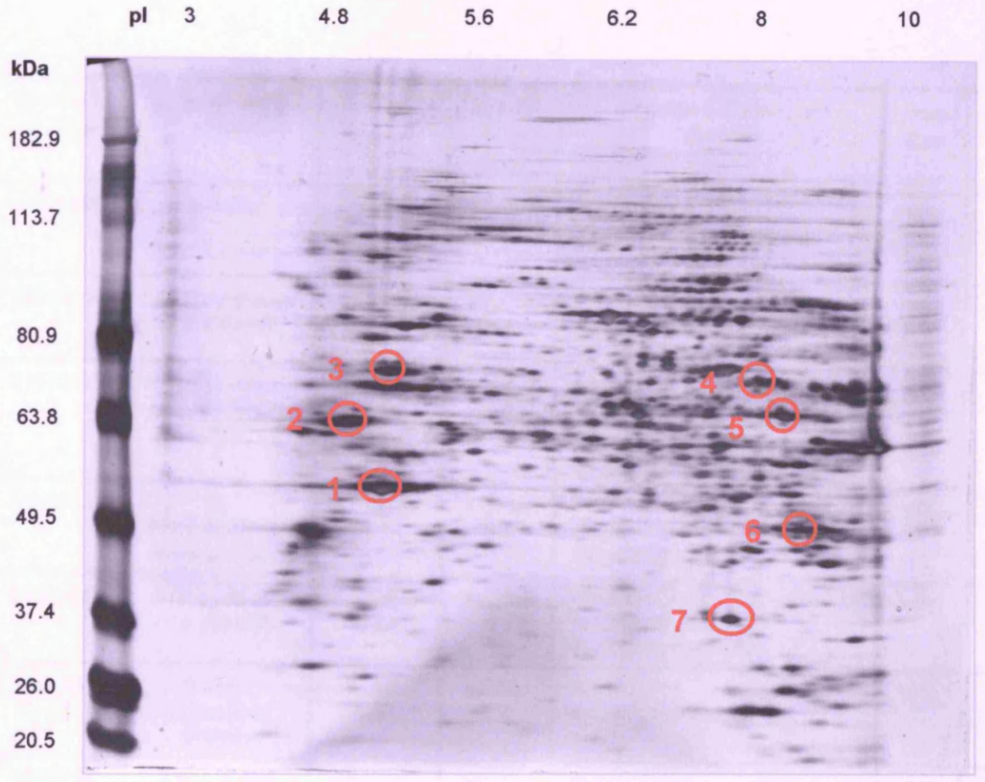


Figure 4.5 - Identification of lymphocyte nuclear proteins by mass spectrometry.

IB4-LCL nuclear proteins were separated by 2D-electrophoresis on a pH 3-10 nonlinear gradient. Protein spots were manually exised from the gels and were analysed by tandem mass spectrometry after trypsin digestion of peptides. Proteins were identified by comparing tryptic peptide sequences with the NCBI non-identical protein sequence database using MASCOT software (Matrix Science). The identified proteins corresponding with the circled spots (1-7) are listed in Table 4.1.

Table 4.1 Lymphocyte nuclear proteins identified by mass spectrometry

Spot no:	Accession number	Identified protein	Th Mr/pI	Approx Exp. Mr/pI	Sequence of identified peptides	Seq. Cov	MOWSE score
1	4501887	β-Actin	42.1kDa/ 5.2	53kDa/ 5.1	AGFAGDDAPR VAPEEHPVLLTEAPLNPK	16%	199
2	32189394	ATP synthase - β subunit	56.5kDa/ 5.26	64kDa/ 4.8	TIAMDGTEGLVR FTQAGSEVSALLGR	16%	326
3	31542947	Chaperonin	61.2kDa/ 5.7	78kDa/ 5.2	VTDALNATR TVIIEQSWGSPK CIPALDSLTPANEDQK LVQDVANNTNEEAGDGTTTATVLAR	17%	362
4	693933	2-Phosphopyruv-ate-α-endolase	47.4kDa/ 7.0	77kDa/ 8.0	EGLELLK EEELGSK GNPTVEVDLFTSK	16%	297
5	24660110	ATP synthase - α subunit	61.0kDa/ 9.14	65kDa/ 8.3	VVDALGNAIDGK SIAIDTIINQK TGAIVDVPVGEELLGR	14%	318
6	4504447	Nuclear ribonuclear protein	36.0kDa/ 8.67	49kDa/ 8.4	IDTIEIITDR GGGGNFGPGPGSNFR	10%	135
7	4092054	GTP binding protein Ran/TC4	24.6kDa/ 7.0	38kDa/ 7.8	NLQYYDIASAK VCENIPIVLCGNK	15%	117

Th. Mr/pI – Theoretical molecular weight and isoelectric point

Approx. Exp. Mr/pI – Approximate molecular weight (kDa) and isoelectric point as judged by 2DE

Seq. Cov – Sequence coverage

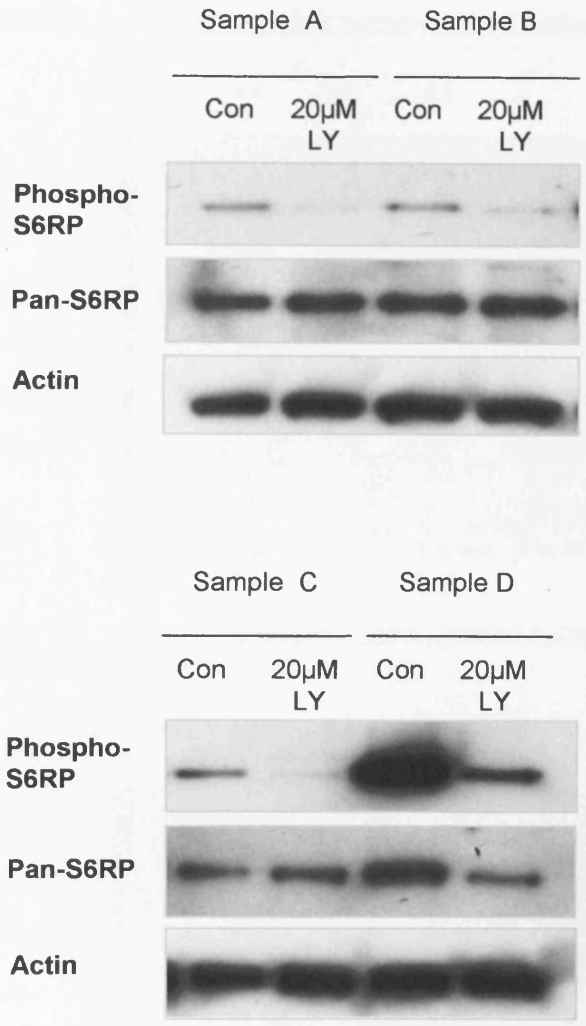
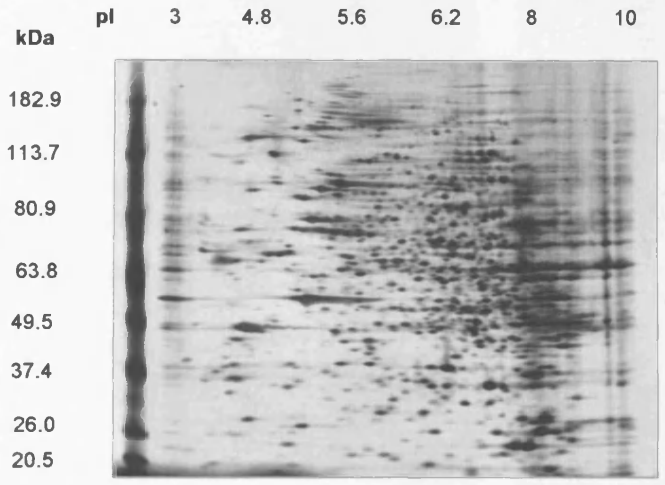


Figure 4.6 - Verification of efficacy of PI3K inhibitor – LY294002.

Nuclear protein extracts from IB4-LCL that were either left untreated or treated with LY294002 (20μM, 1 hour) were cleaned for electrophoresis in four replicate experiments (samples A-D) . An aliquot of each protein sample was resolved by SDS-PAGE. Verification of the efficacy of LY294002 was carried out by immunoblotting for the phosphorylated-S6 ribosomal protein. Protein levels of total S6 ribosomal protein and actin were also determined by immunoblotting using specific antibodies.

a) IB4-LCL – Untreated nuclear sample – Total protein stain (silver).



b) IB4-LCL – LY294002 (20μM, 1 hour) treated nuclear sample – Total protein stain (silver).

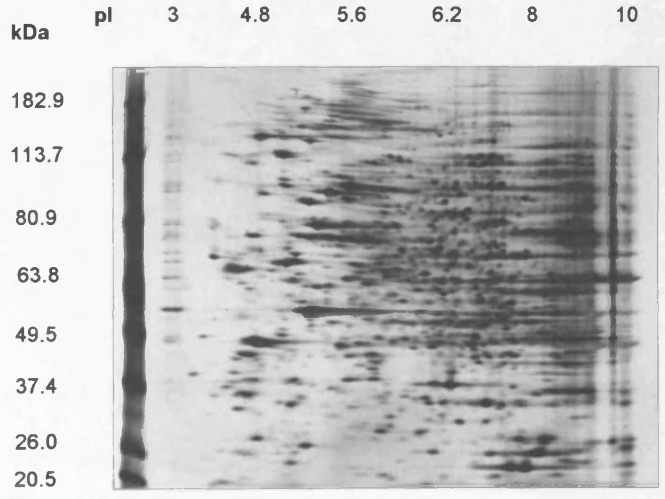


Figure 4.7 – Comparison of lymphocyte nuclear proteomes before and after treatment with LY294002.

Nuclear protein extracts generated from IB4-LCL that were either a) untreated or b) treated with LY294002 (20μM, 1 hour) were cleaned and resolved by 2D electrophoresis on a pH 3-10 nonlinear gradient. Proteins were visualised by silver staining.

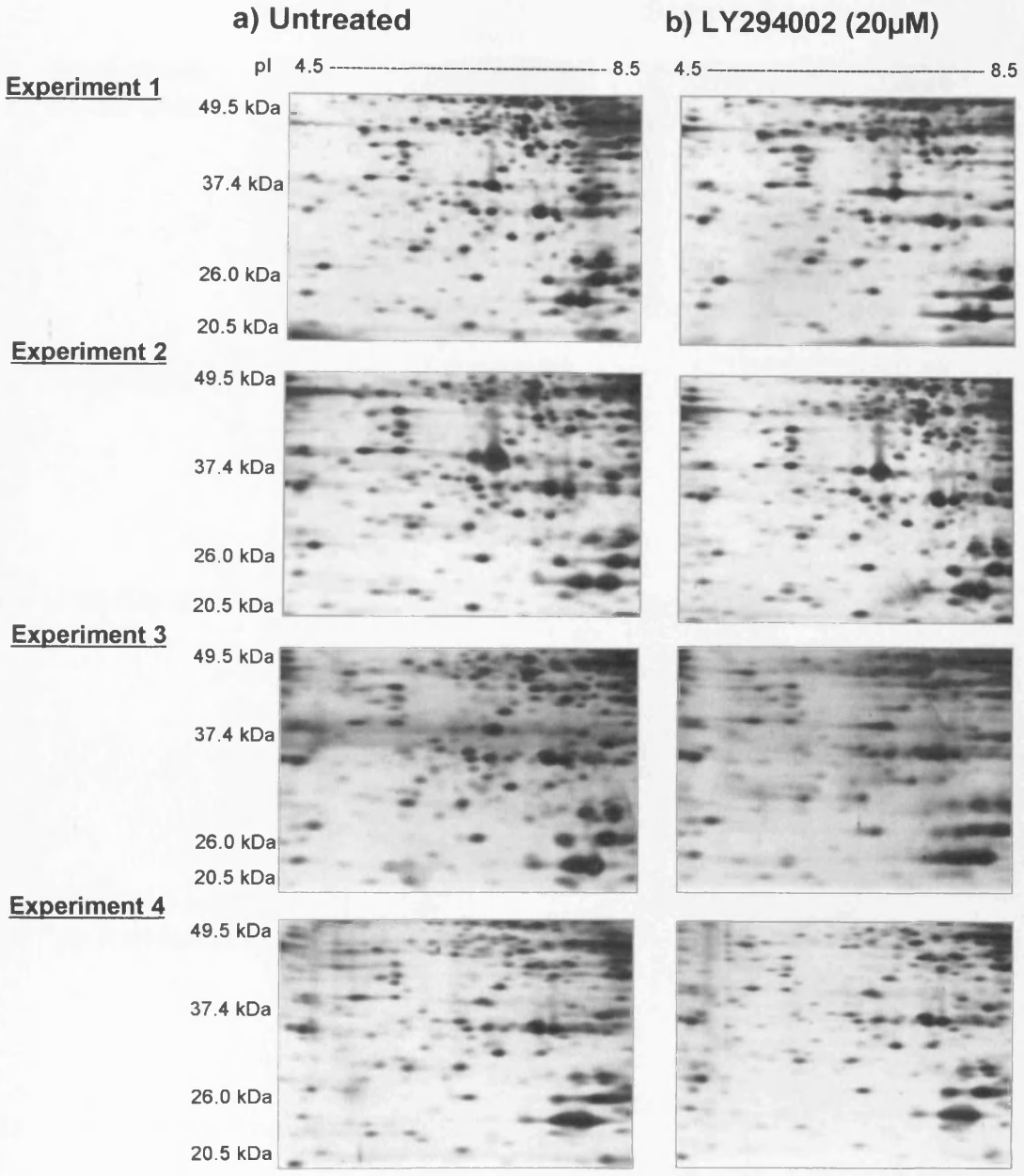


Figure 4.8 – Comparison of lymphocyte nuclear proteomes before and after treatment with LY294002.

Nuclear protein extracts generated from IB4-LCL that were either a) untreated or b) treated with LY294002 (20μM, 1 hour) were cleaned and resolved by 2D electrophoresis on a pH 3-10 nonlinear gradient in four replicate experiments (Experiments 1-4). Proteins were visualised by silver staining. The corresponding areas from each gel where maximum resolution of proteins had occurred (pI 4.5-8.5, Mr 20.5-49.5 kDa) were selected for further analysis.

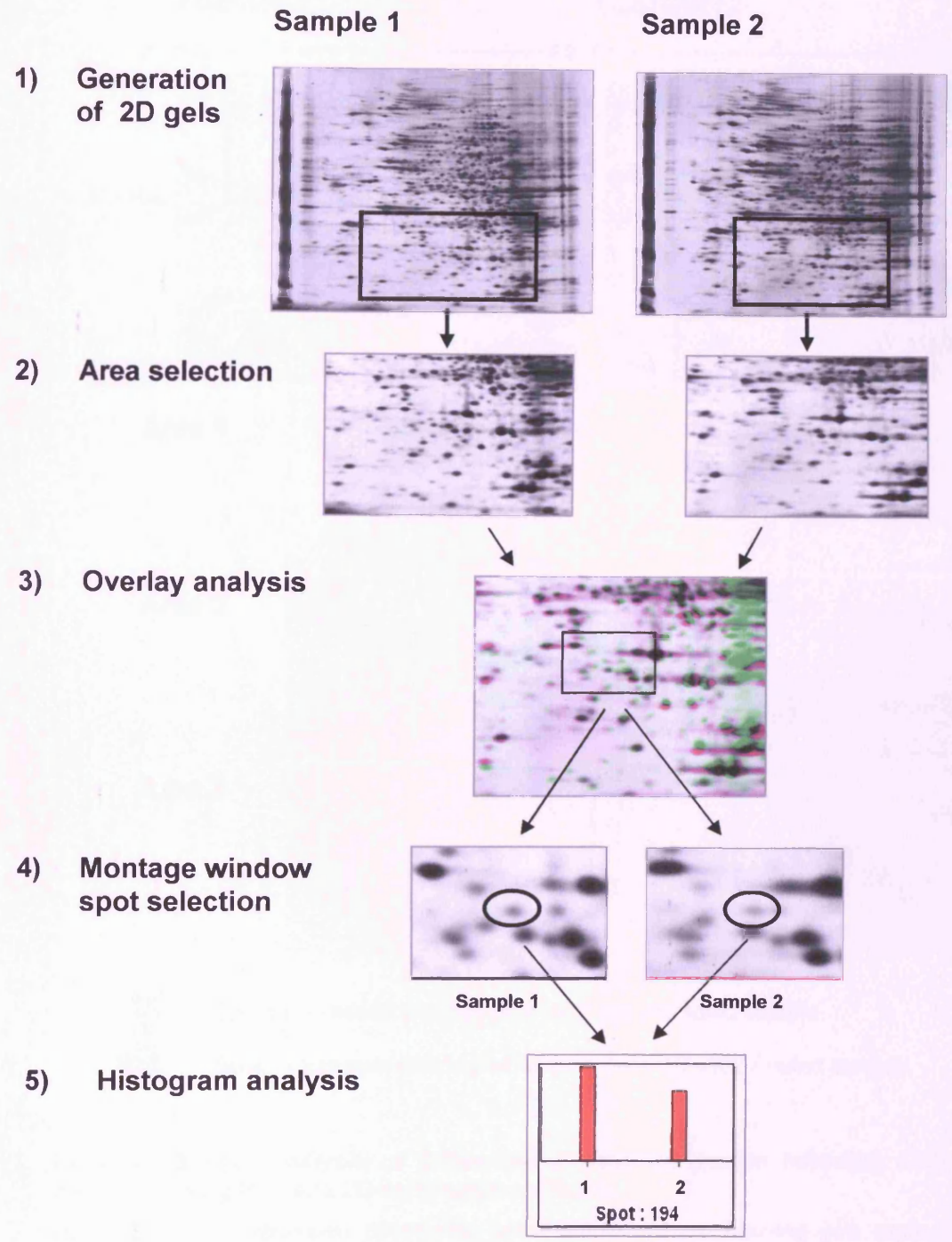


Figure 4.9 Workflow of analysis of 2D gels using Phoretix 2D expression software.

1) 2D gels were generated to compare protein expression between two samples. 2) An area where maximum separation of proteins had occurred was selected for analysis. 3) Two gel images were overlaid using Phoretix 2D expression software so that differences between the gels were visualised using complimentary colours. 4) Montage windows of areas containing proteins differentially expressed between samples were created allowing visualisation of the same selected area in the two gels. 5) Differences in protein expression levels between samples were quantified by histogram analysis of spot volume.

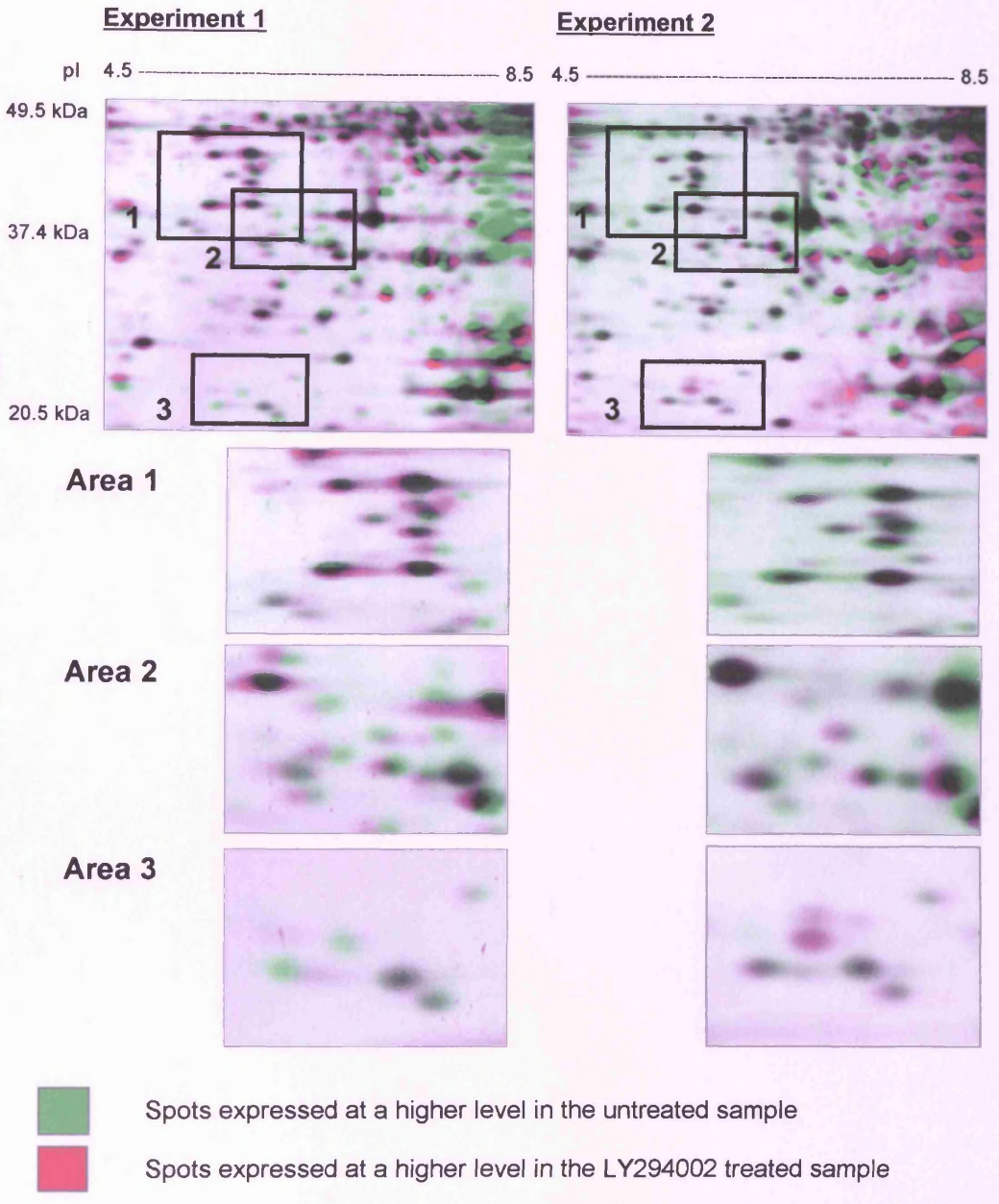


Fig 4.10- Overlay analysis of differential protein expression following LY294002 treatment using Phoretix 2D expression software.

Nuclear protein expression differences were detected by overlaying gels representing protein expression from untreated and LY294002 treated samples. This was carried out using Phoretix 2D expression software. Spots coloured green represent proteins detected to be expressed at a higher level in untreated samples. Spots coloured magenta represent proteins detected to be expressed at a higher level in LY294002 treated samples. Black spots indicate no significant difference in protein expression between samples. The areas selected for analysis (pI 4.5-8.5, Mr 20.5-49.5 kDa) from experiments 1 and 2 are shown. Smaller areas from within this region where many spots appeared to differentially expressed were selected for closer analysis (areas 1-3). Continued on next page.

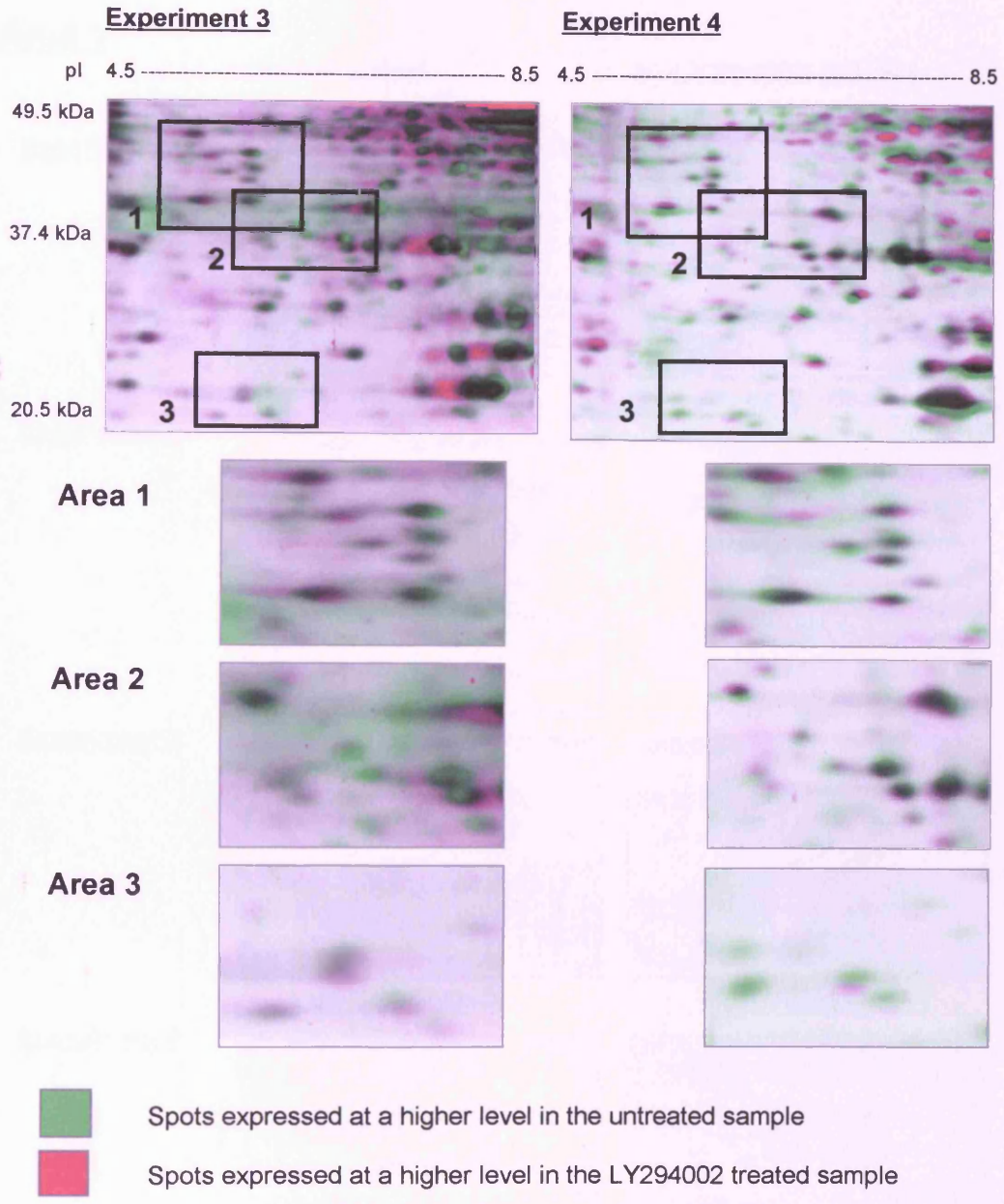


Fig 4.10 (continued). Overlay analysis of differential protein expression following LY294002 treatment using Phoretix 2D expression software.

Nuclear protein expression differences were detected by overlaying gels representing protein expression from untreated and LY294002 treated samples. This was carried out using Phoretix 2D expression software. Spots coloured green represent proteins detected to be expressed at a higher level in untreated samples. Spots coloured magenta represent proteins detected to be expressed at a higher level in LY294002 treated samples. Black spots indicate no significant difference in protein expression between samples. The areas selected for analysis (pI 4.5-8.5, Mr 20.5-49.5 kDa) from experiments 3 and 4 are shown. Smaller areas from within this region where many spots appeared to differentially expressed were selected for closer analysis (areas 1-3).

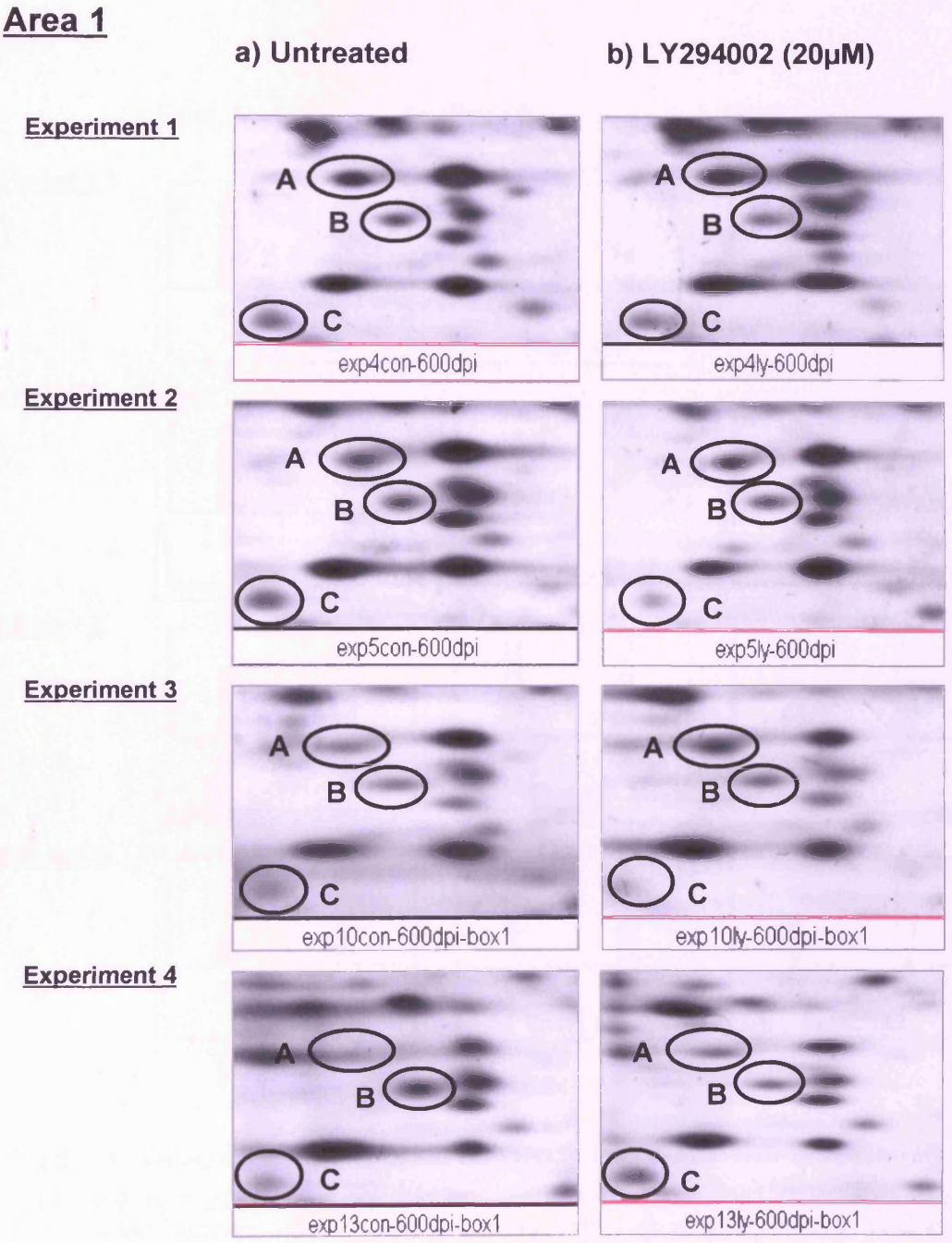


Fig 4.11a- Comparison of protein expression levels between untreated and LY294002 treated samples – montage windows of area 1.

Montage windows of area 1 are shown comparing nuclear protein expression from corresponding areas of a) untreated and b) LY294002 treated sample gels from four replicate experiments (1-4). Three spots (A, B, C) from this area were selected for histogram analysis.

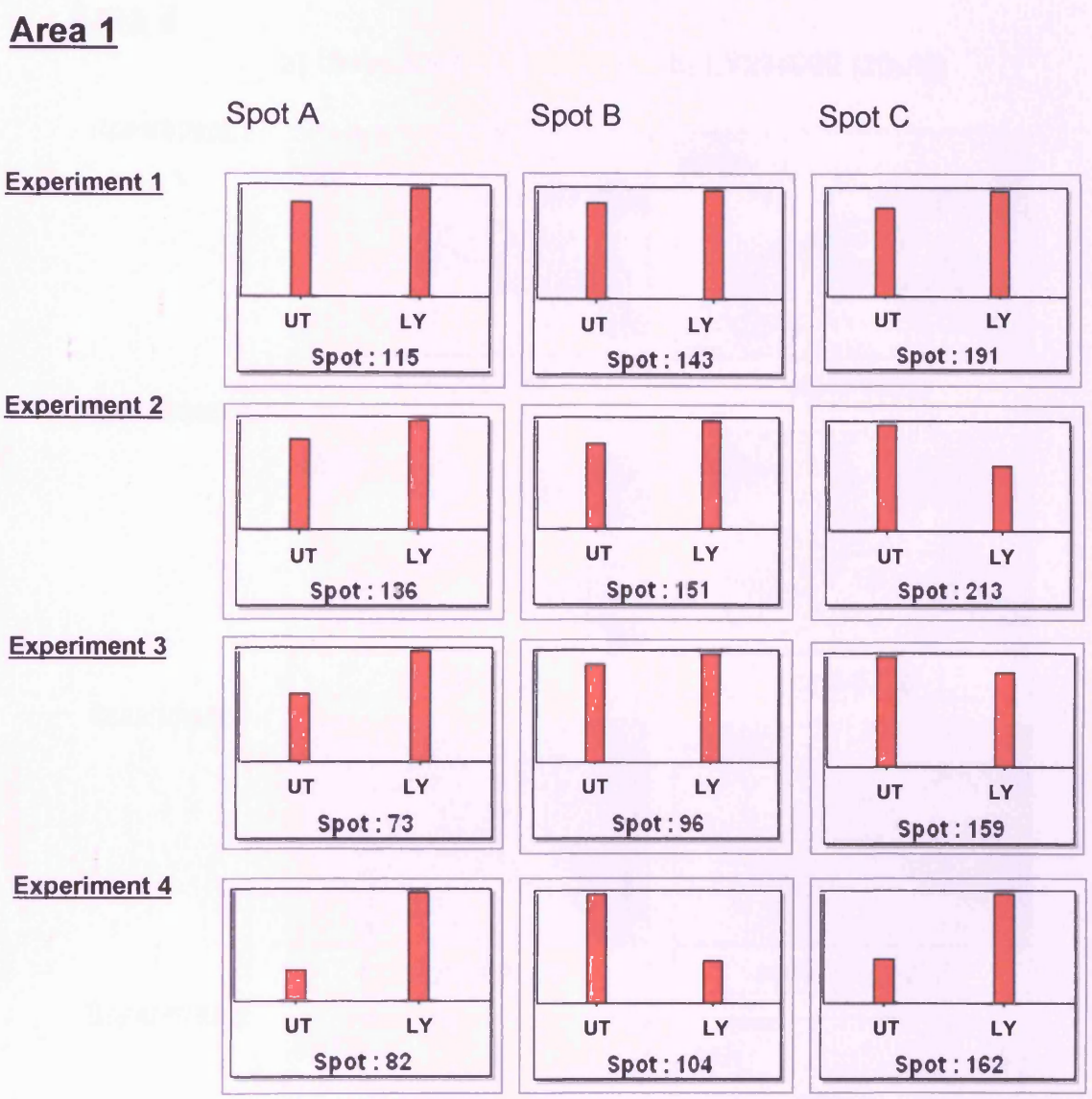


Fig 4.11b- Histogram analysis of spot volumes in area 1.

Quantification of differential expression between untreated (UT) and LY294002 treated (LY) nuclear samples were carried out using histogram analysis of spot volume. Histogram analyses of spots A, B and C in area 1 from experiments 1-4 are shown. The vertical axes represent the ratio of spot volume when comparing two matched spots.

Area 2

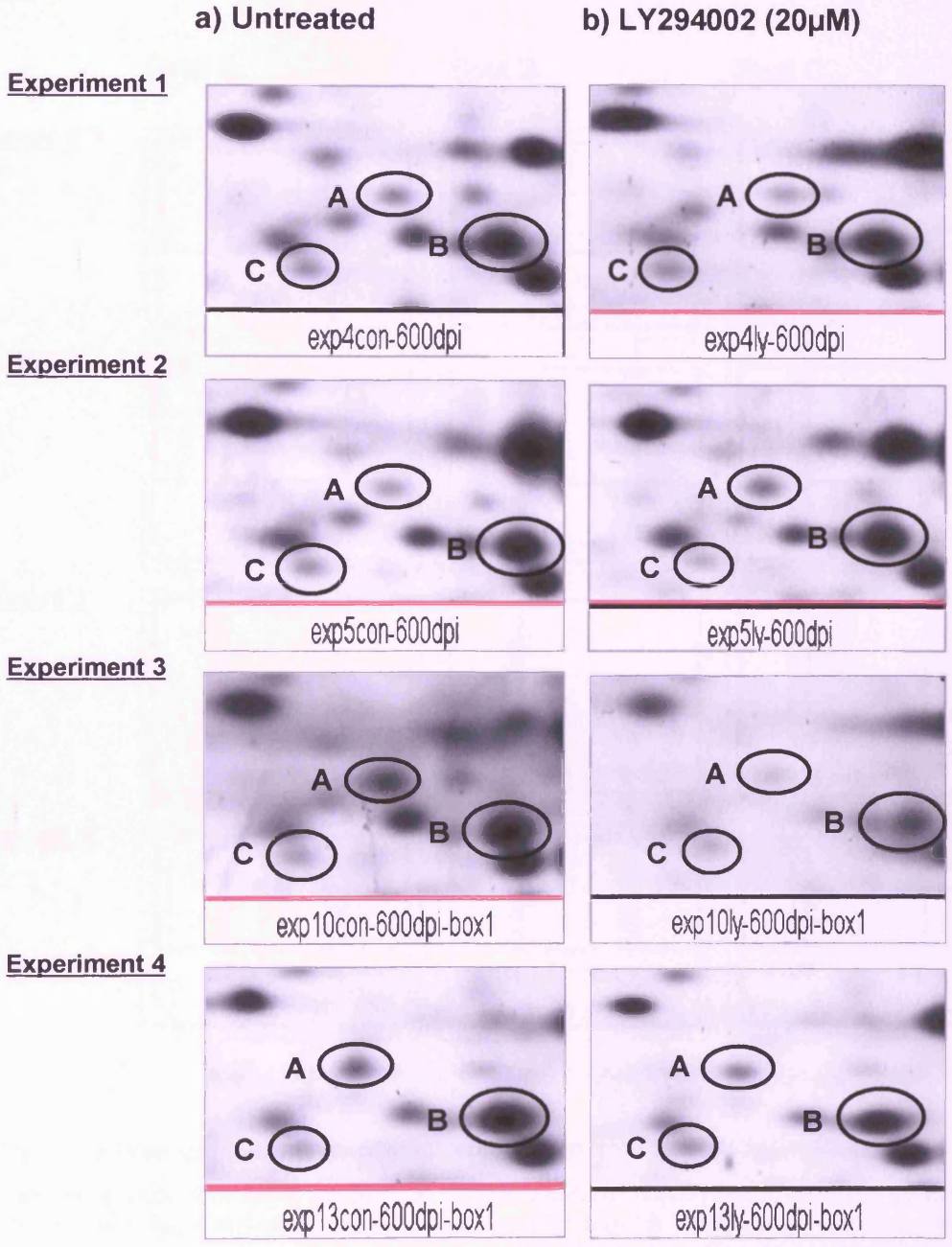


Fig 4.12a- Comparison of protein expression levels between untreated and LY294002 treated samples – montage windows of area 2.

Montage windows of area 2 are shown comparing nuclear protein expression from corresponding areas of a) untreated and b) LY294002 treated sample gels from four replicate experiments (1-4). Three spots (A, B, C) from this area were selected for histogram analysis.

Area 2

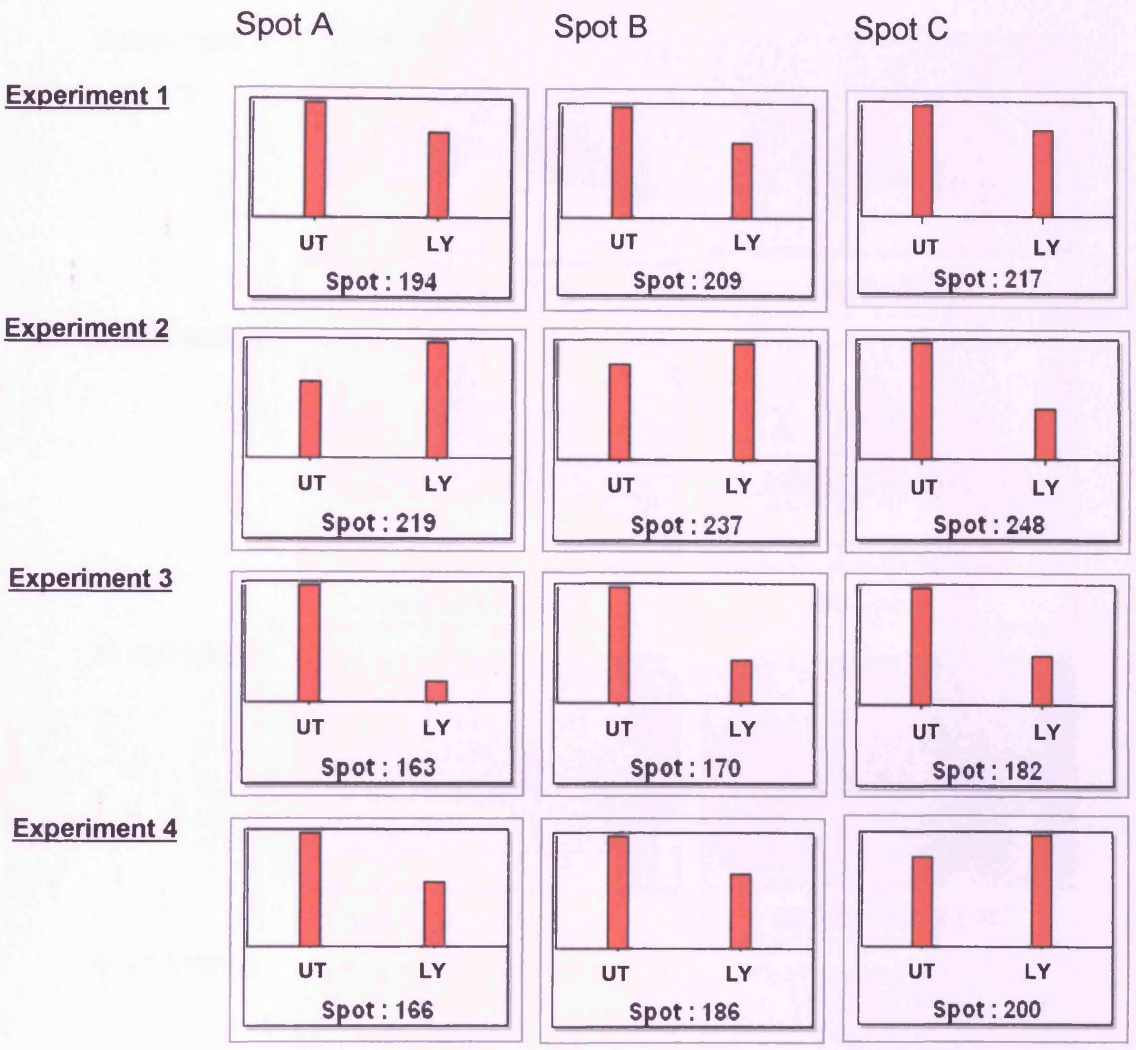


Fig 4.12b- Histogram analysis of spot volumes in area 2.

Quantification of differential expression between untreated (UT) and LY294002 treated (LY) nuclear samples were carried out using histogram analysis of spot volume. Histogram analyses of spots A, B and C in area 2 from experiments 1-4 are shown. The vertical axes represent the ratio of spot volume when comparing two matched spots.

Area 3

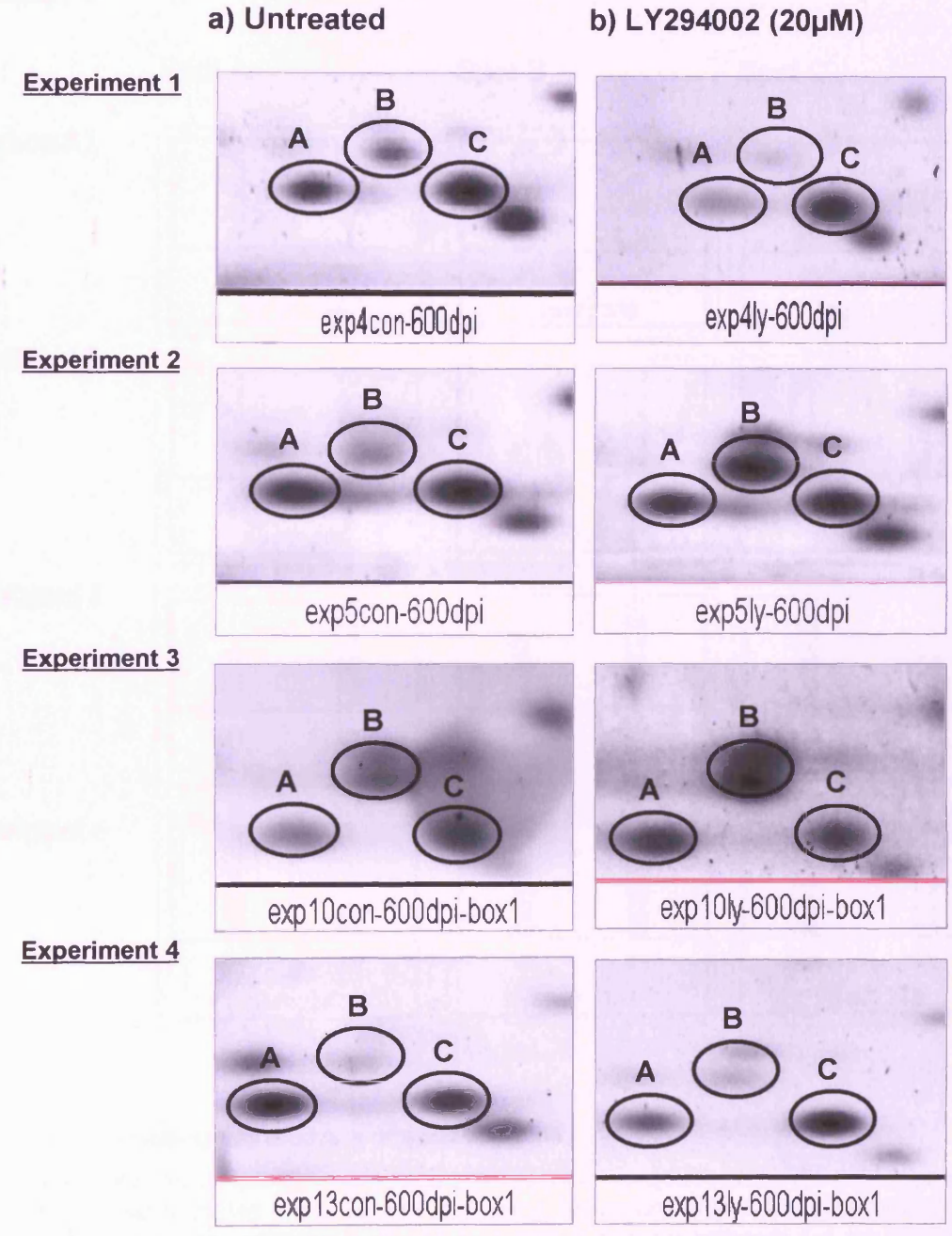


Fig 4.13a- Comparison of protein expression levels between untreated and LY294002 treated samples – montage windows of area 3.

Montage windows of area 3 are shown comparing nuclear protein expression from corresponding areas of a) untreated and b) LY294002 treated sample gels from four replicate experiments 1-4). Three spots (A, B, C) from this area were selected for histogram analysis.

Area 3

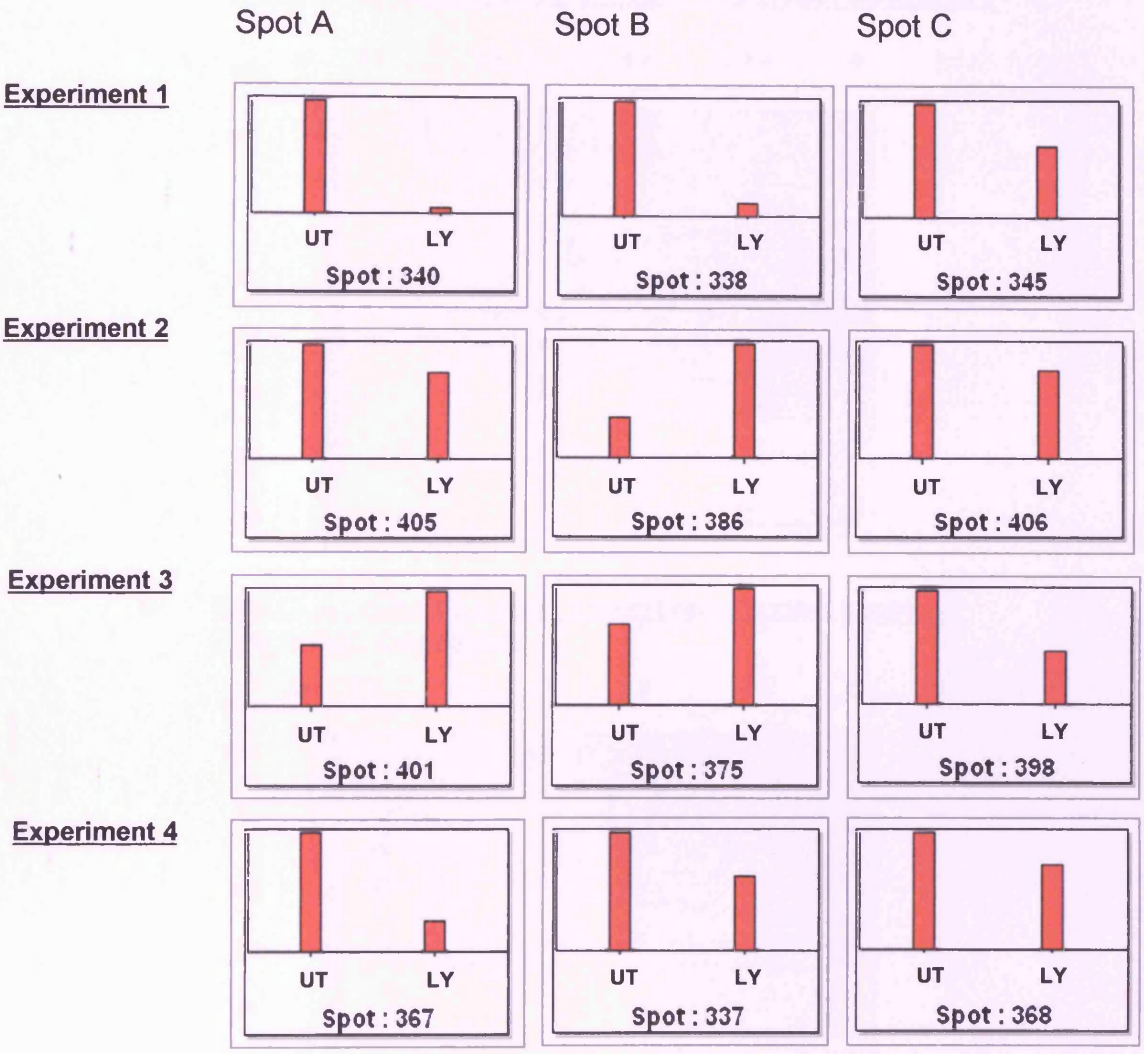
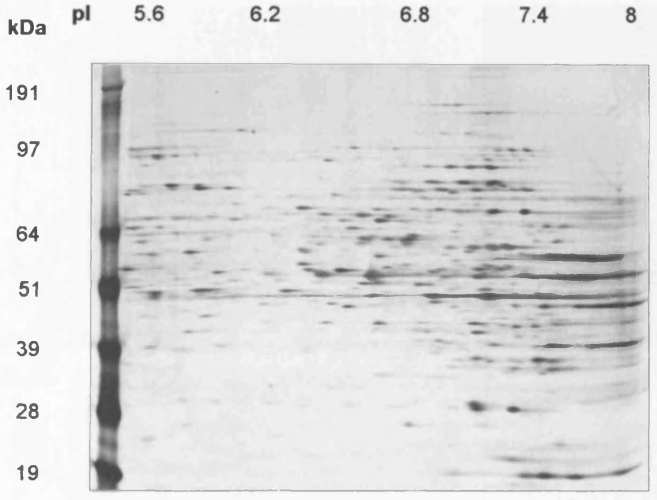


Fig 4.13b- Histogram analysis of spot volumes in area 3.

Quantification of differential expression between untreated (UT) and LY294002 treated (LY) nuclear samples were carried out using histogram analysis of spot volume. Histogram analyses of spots A, B and C in area 3 from experiments 1-4 are shown. The vertical axes represent the ratio of spot volume when comparing two matched spots.

a IB4-LCL – Untreated nuclear sample – Total protein stain (silver).



b IB4-LCL – LY294002 (20µM, 1 hour) treated nuclear sample – Total protein stain (silver).

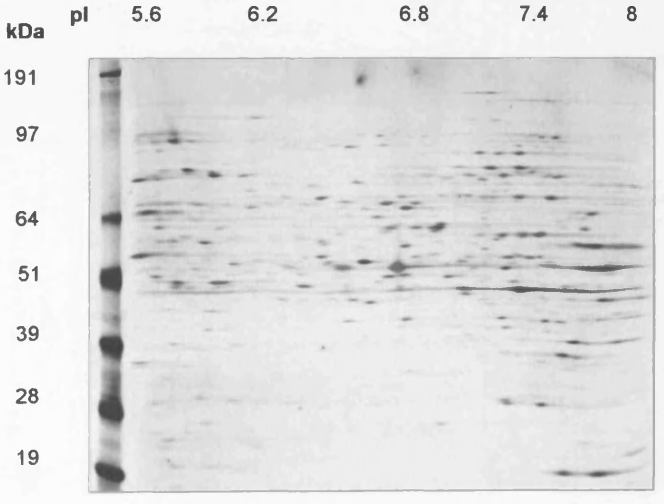


Figure 4.14 – Comparison of lymphocyte nuclear proteomes before and after treatment with LY294002 – use of a narrower isoelectric focusing range.

Nuclear protein extracts generated from IB4-LCL that were either a) untreated or b) treated with LY294002 (20µM, 1 hour) were cleaned and resolved by 2D electrophoresis on a pH 5.6-8 linear gradient. Proteins were visualised by silver staining.

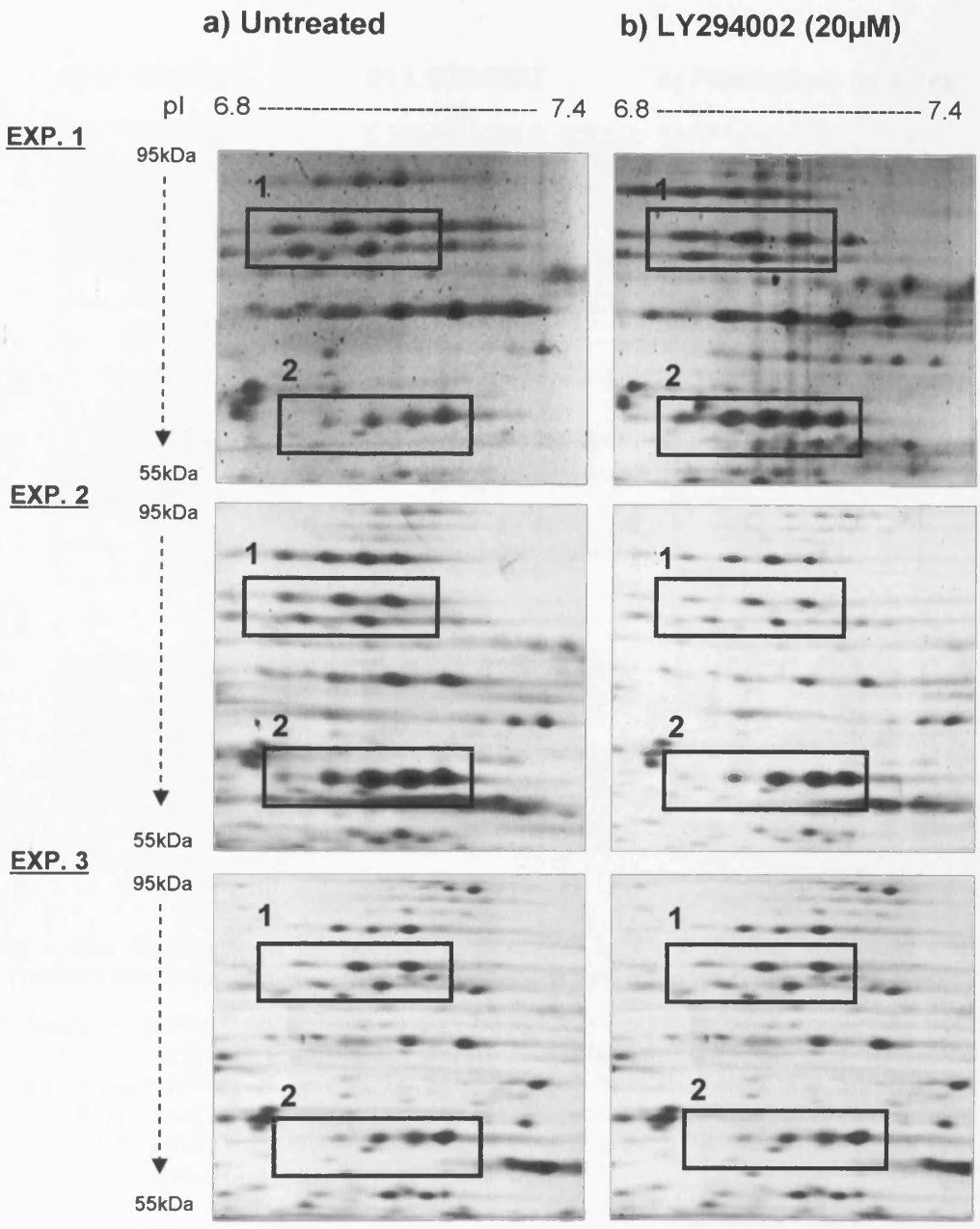


Figure 4.15 – Comparison of lymphocyte nuclear proteomes before and after treatment with LY294002- use of a narrower isoelectric focusing range.

Nuclear protein extracts generated from IB4-LCL that were either a) untreated or b) treated with LY294002 (20µM, 1 hour) were cleaned and resolved by 2D electrophoresis on a pH 5.6-8 linear gradient in three replicate experiments (EXP. 1-3). Proteins were visualised by silver staining. The corresponding areas from each gel where maximum resolution of proteins had occurred (pI 6.8-7.4, Mr 55-95 kDa) are shown. Smaller areas from within this region were chosen for closer analysis (areas 1 and 2).

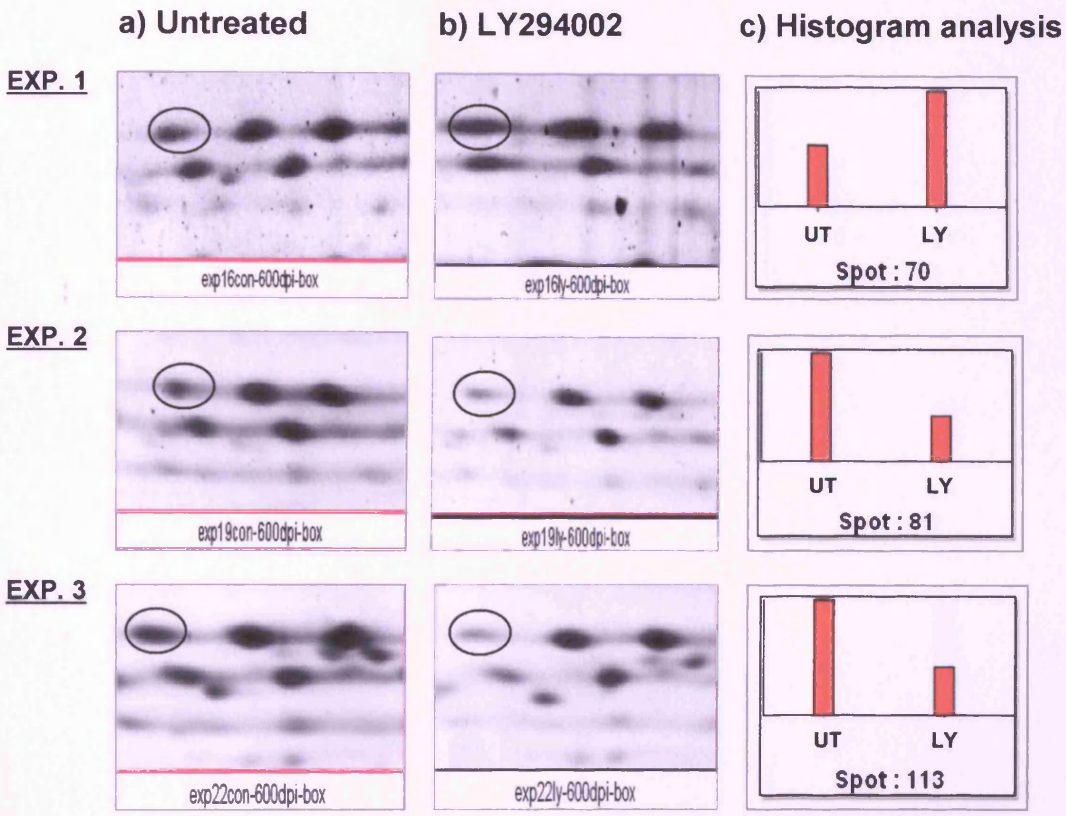


Fig 4.16a- Comparison of protein expression levels between untreated and LY294002 treated samples – montage windows and histogram analysis of area 1.

Montage windows of area 1 are shown comparing nuclear protein expression from corresponding areas of a) untreated and b) LY294002 treated sample gels from three replicate experiments (1-3) c) One spot (circled) from this area was selected for histogram analysis of spot volume to quantify differential expression between untreated (UT) and LY294002 treated (LY) samples. The vertical axes represent the ratio of spot volume when comparing two matched spots.

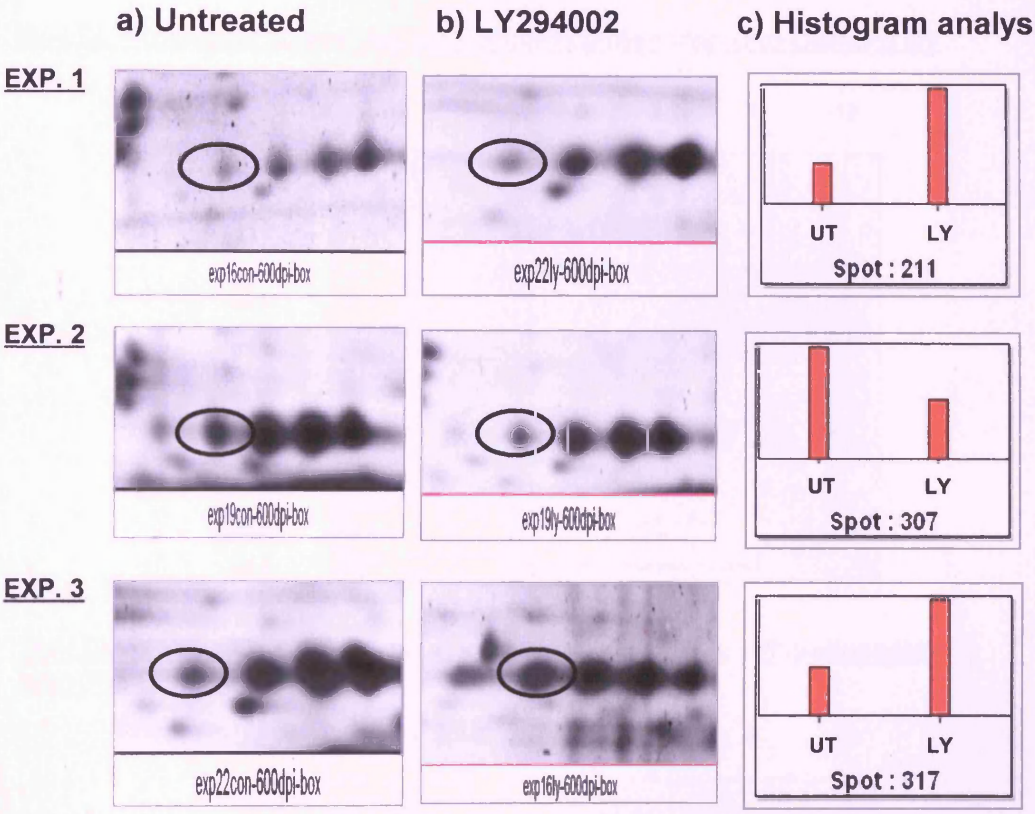
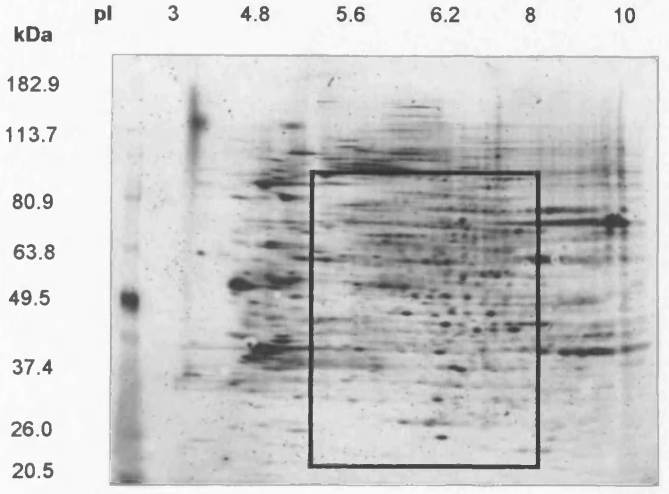


Fig 4.16b- Comparison of protein expression levels between untreated and LY294002 treated samples – montage windows and histogram analysis of area 2.

Montage windows of area 2 are shown comparing nuclear protein expression from corresponding areas of a) untreated and b) LY294002 treated sample gels from three replicate experiments (1-3) c) One spot (circled) from this area was selected for histogram analysis of spot volume to quantify differential expression between untreated (UT) and LY294002 treated (LY) samples. The vertical axes represent the ratio of spot volume when comparing two matched spots.

IB4-LCL – Untreated nuclear sample – Phosphoprotein stain (Pro-Diamond Q)



IB4-LCL – LY294002 (20μM, 1 hour) treated nuclear sample - Phosphoprotein stain (Pro-Diamond Q)

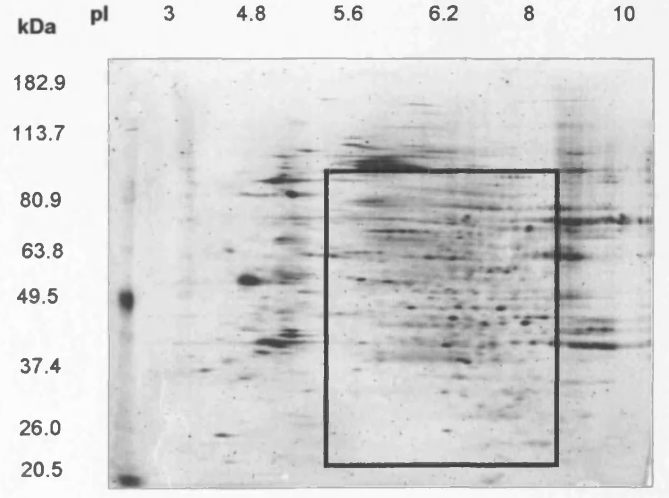
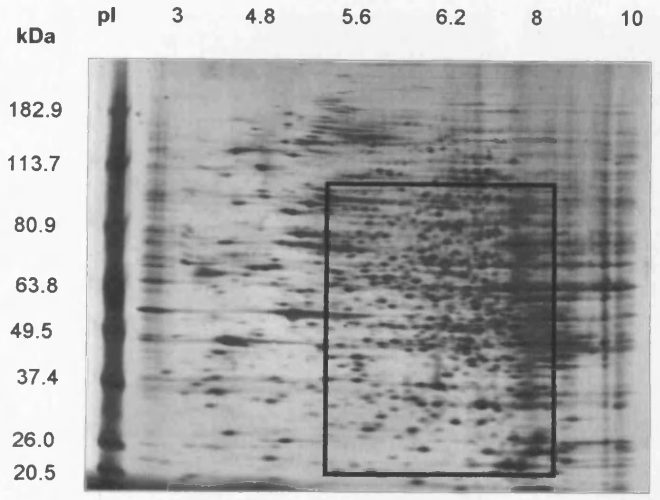


Figure 4.17a – Comparison of lymphocyte nuclear phosphoproteomes before and after treatment with LY294002.

Nuclear protein extracts generated from IB4-LCL that were either a) untreated or b) treated with LY294002 (20μM, 1 hour) were cleaned and resolved by 2D electrophoresis on a pH 3-10 nonlinear gradient. Phosphorylated proteins were visualised by staining with Pro-Q Diamond phosphostain. Continued on next page.

IB4-LCL – Untreated nuclear sample – Total protein stain (silver)



IB4-LCL – LY294002 (20μM, 1 hour) treated nuclear sample – Total protein stain (silver)

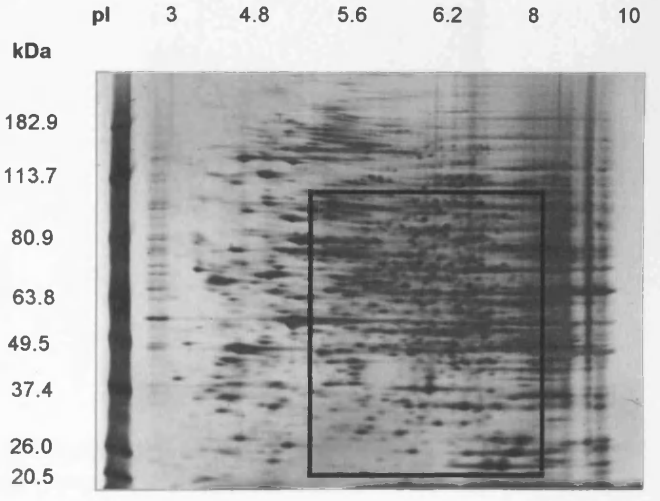


Figure 4.17b – Total protein expression in the lymphocyte nucleus before and after treatment with LY294002.

Nuclear protein extracts generated from IB4-LCL that were either a) untreated or b) treated with LY294002 (20μM, 1 hour) were cleaned and resolved by 2D electrophoresis on a pH 3-10 nonlinear gradient. Total protein expression was visualised by silver staining after staining of phosphorylated proteins with Pro-Q Diamond phosphostain.

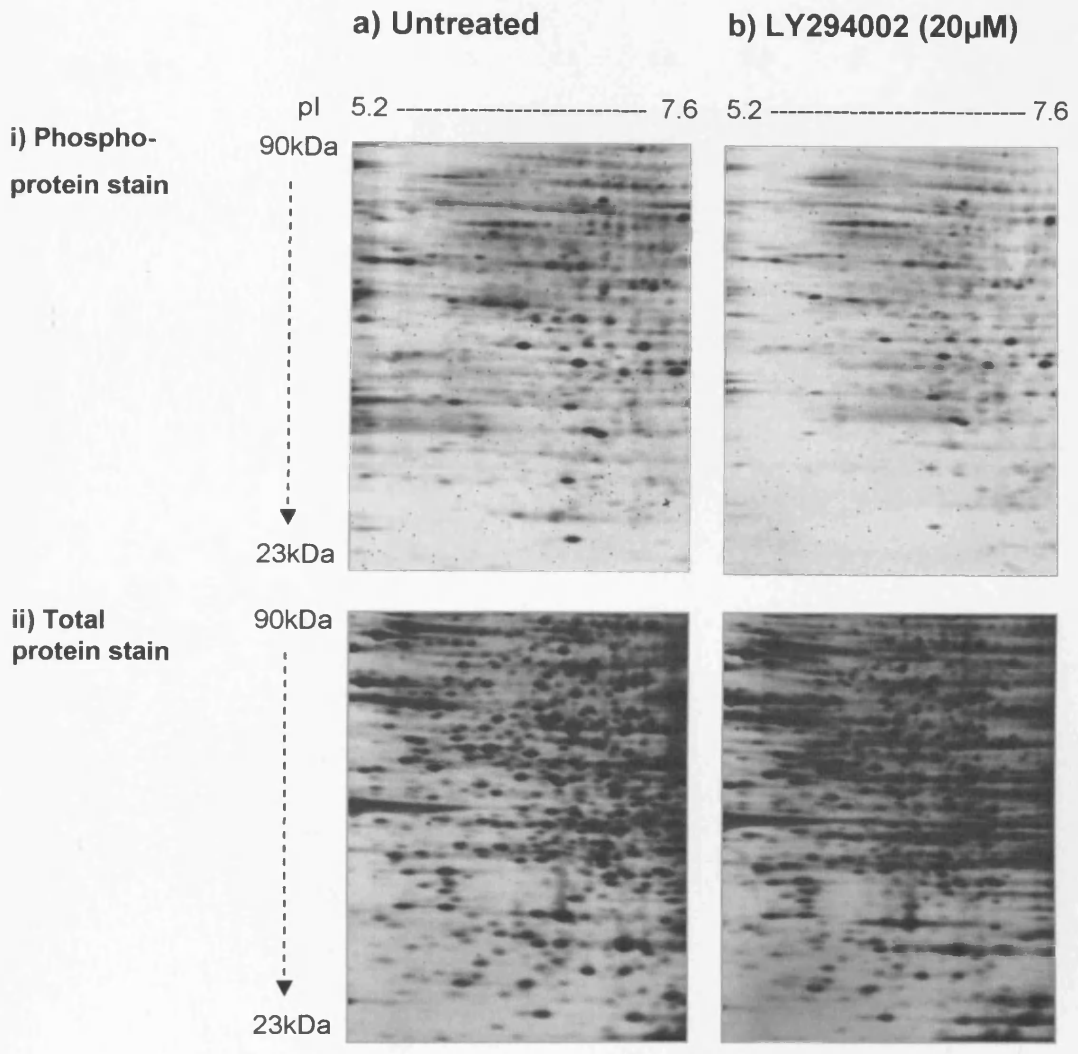


Figure 4.18 – Comparison of phospho-protein expression with total protein expression in the lymphocyte nucleus.

Nuclear protein extracts generated from IB4-LCL that were either a) untreated or b) treated with LY294002 (20μM, 1 hour) were cleaned and resolved by 2D electrophoresis on a pH 3-10 nonlinear gradient. Proteins were visualised i) by phospho-protein staining ii) by silver staining. The corresponding areas from each gel where maximum resolution of proteins had occurred (pI 5.2-7.6, Mr 23-90 kDa) are shown. The total number of spots detected in the selected area in both treated and untreated samples post phospho-protein staining and total protein staining were calculated using Phoretix 2D expression software.

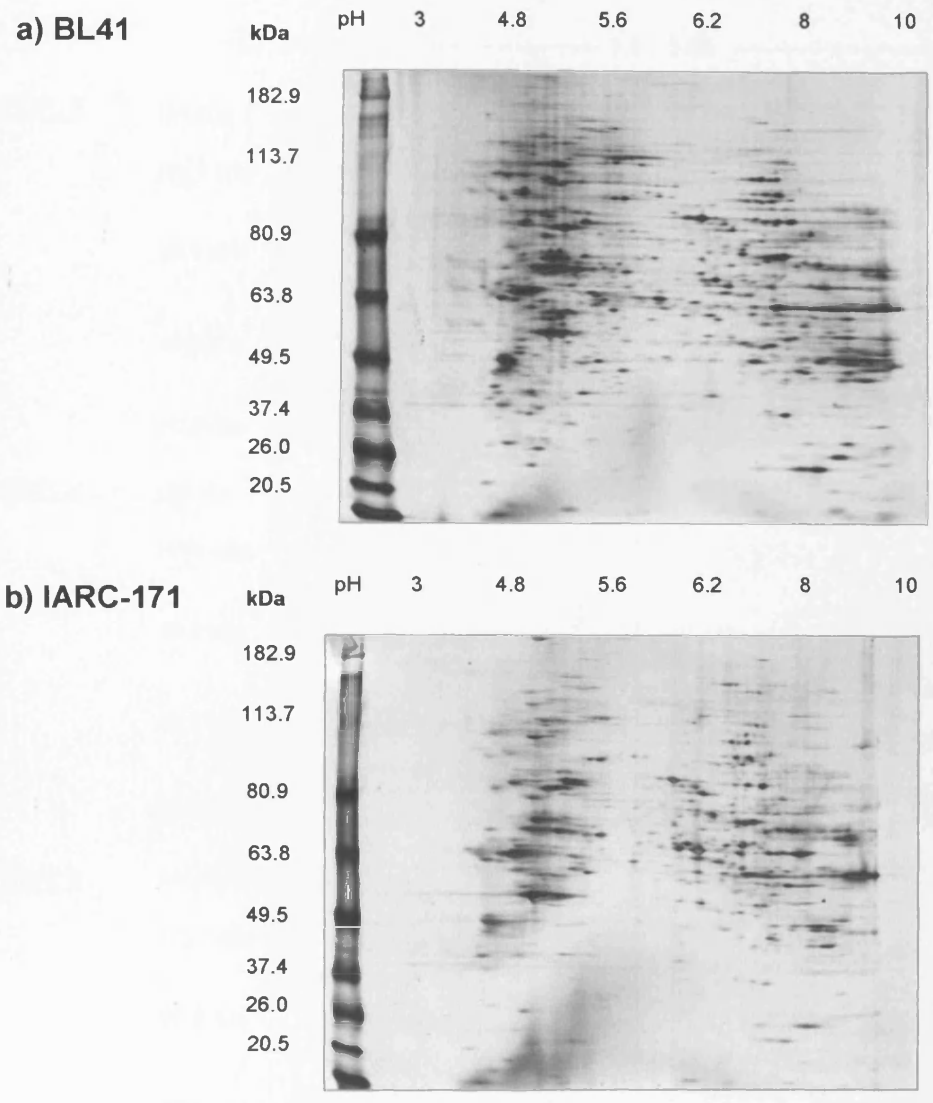


Figure 4.19 – Comparison of nuclear protein expression between EBV negative Burkitt's lymphoma B cells and EBV immortalized B cells.

Nuclear protein extracts generated from a) BL41 b) IARC-171 were cleaned and resolved by 2D electrophoresis on a pH 3-10 nonlinear gradient. Proteins were visualised by silver staining.

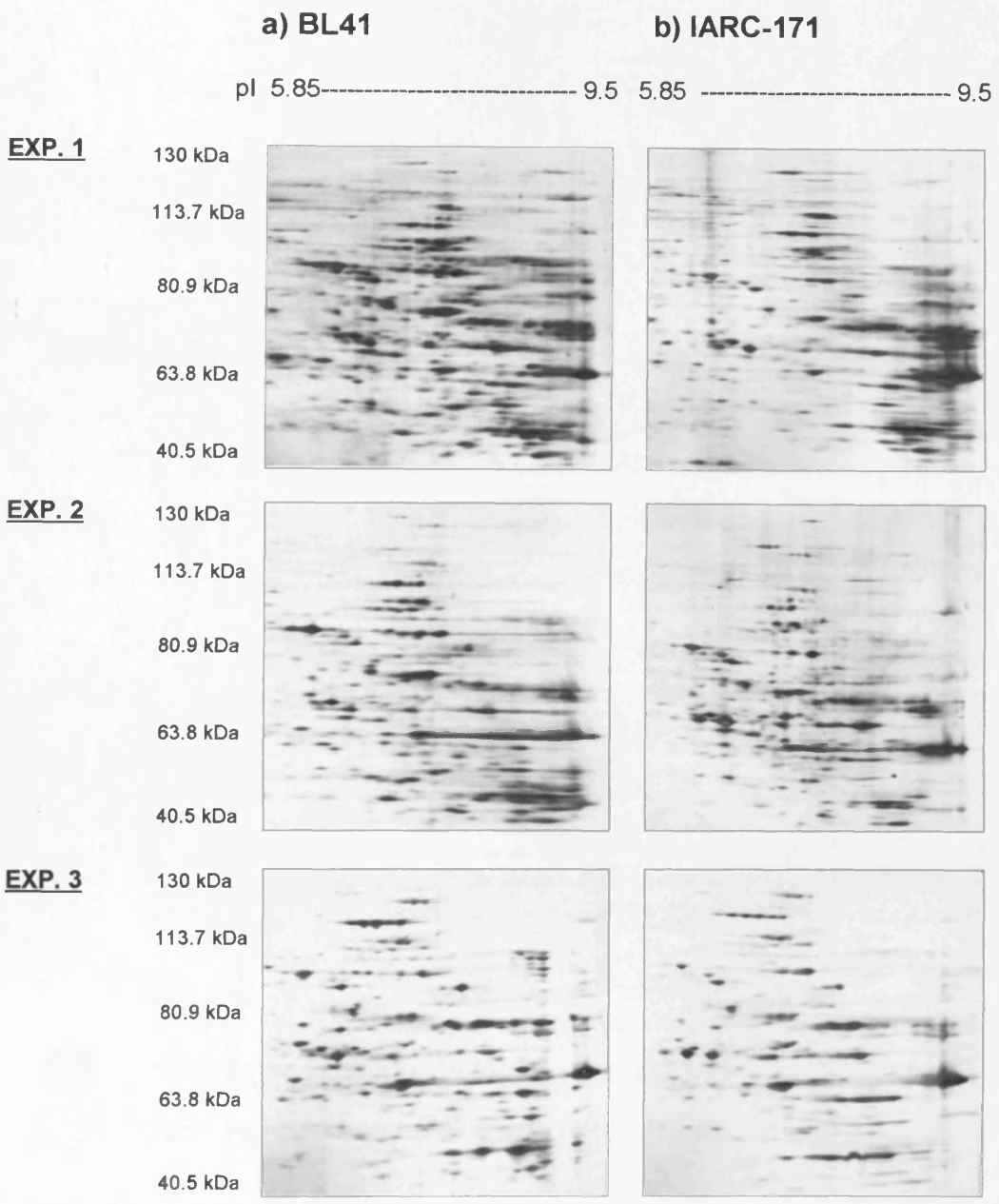


Figure 4.20 – Comparison of nuclear protein expression between EBV negative Burkitt's lymphoma B cells and EBV immortalized B cells.

Nuclear protein extracts from a) BL41 and b) IARC-171 cells were cleaned and resolved by 2D electrophoresis on a pH 3-10 nonlinear gradient in three replicate experiments (EXP. 1-3). Proteins were visualised by silver staining. Corresponding areas from each gel where maximum resolution of proteins had occurred (pI 5.85-9.5, Mr 40.5-130 kDa) are shown and were selected for further analysis.

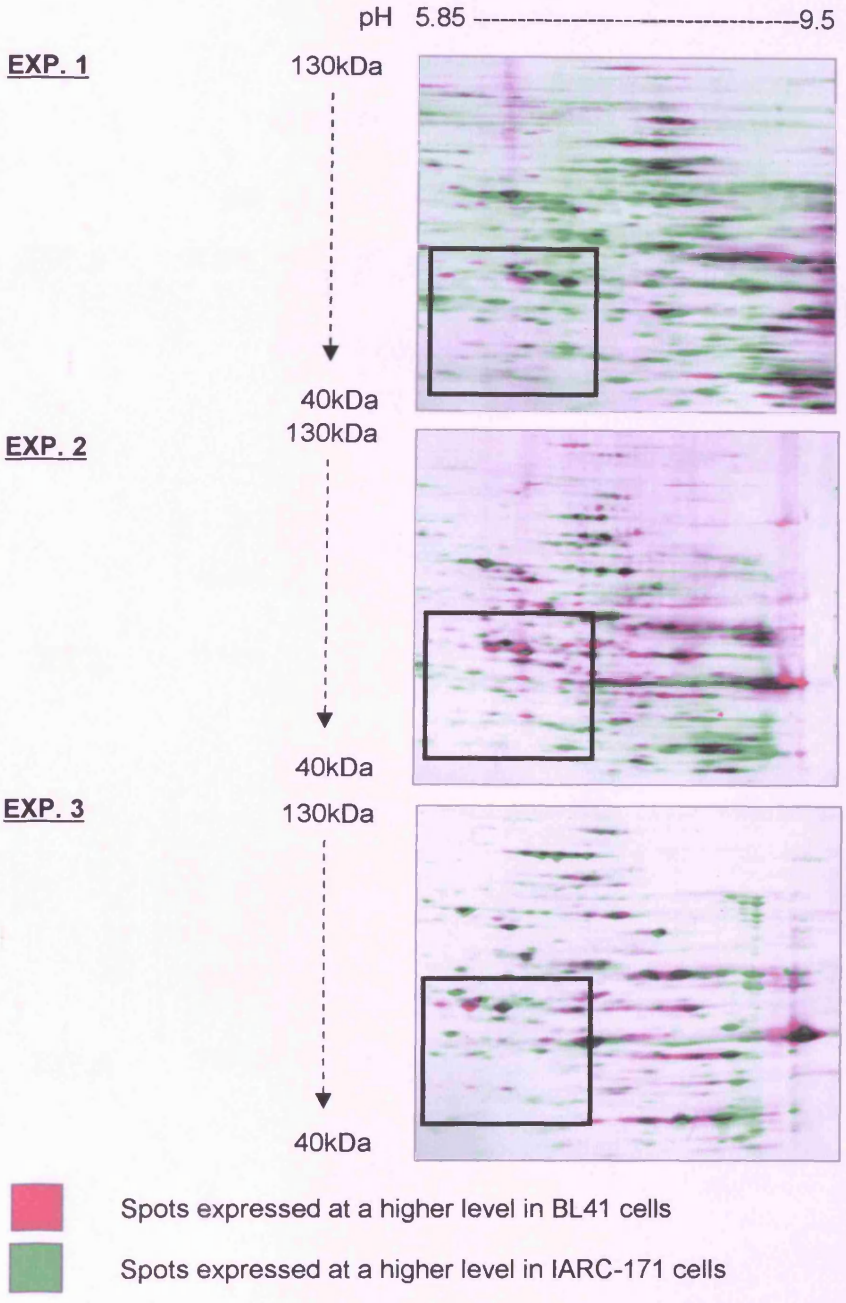


Fig 4.21- Overlay analysis of differential protein expression between EBV negative Burkitt's lymphoma B cells and EBV immortalized B cells.

Protein expression differences were detected by overlaying gels representing nuclear protein expression from BL41 and IARC-171 cells. This was carried out using Phoretix 2D expression software. Spots coloured magenta represent proteins detected to be expressed at a higher level in BL41 cells. Spots coloured green represent proteins detected to be expressed at a higher level IARC-171 cells. Black spots indicate no significant difference in protein expression between samples. The areas selected for analysis (pI 5.85-9.5, Mr 40.5-130 kDa) from experiments 1-3 are shown. A smaller area from within this region where many spots appeared to differentially expressed were selected for closer analysis (outlined).

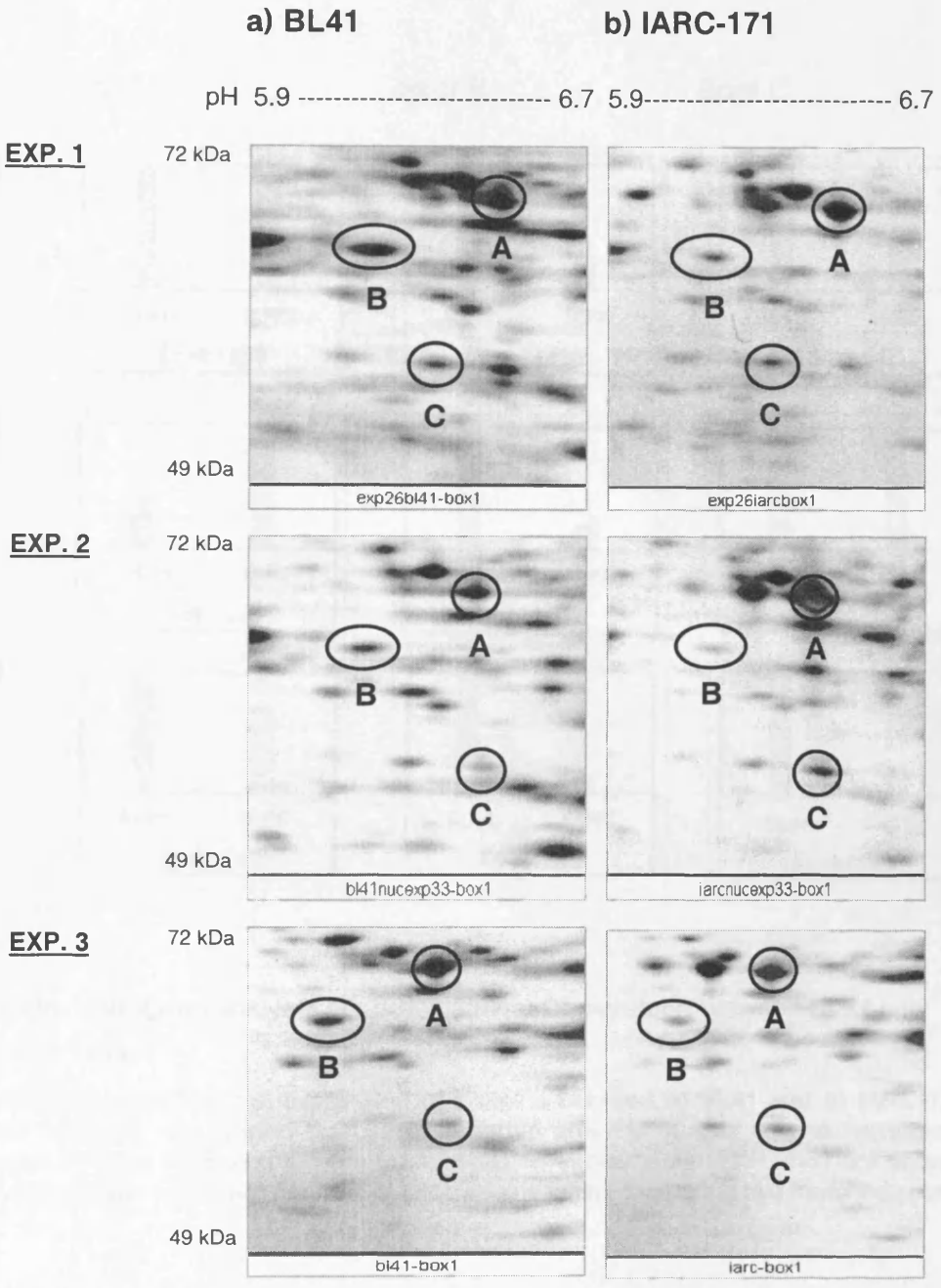


Fig 4.22a- Comparison of protein expression levels between EBV negative Burkitt's lymphoma B cells and EBV immortalized B cells; montage windows

Montage windows of a selected area (pI 5.85-9.5, Mr 40.5-72 kDa) are shown comparing nuclear protein expression from corresponding areas of a) BL41 b) IARC-171 cells from three replicate experiments (1-3). Three spots (A, B, C) from this area were selected for histogram analysis.

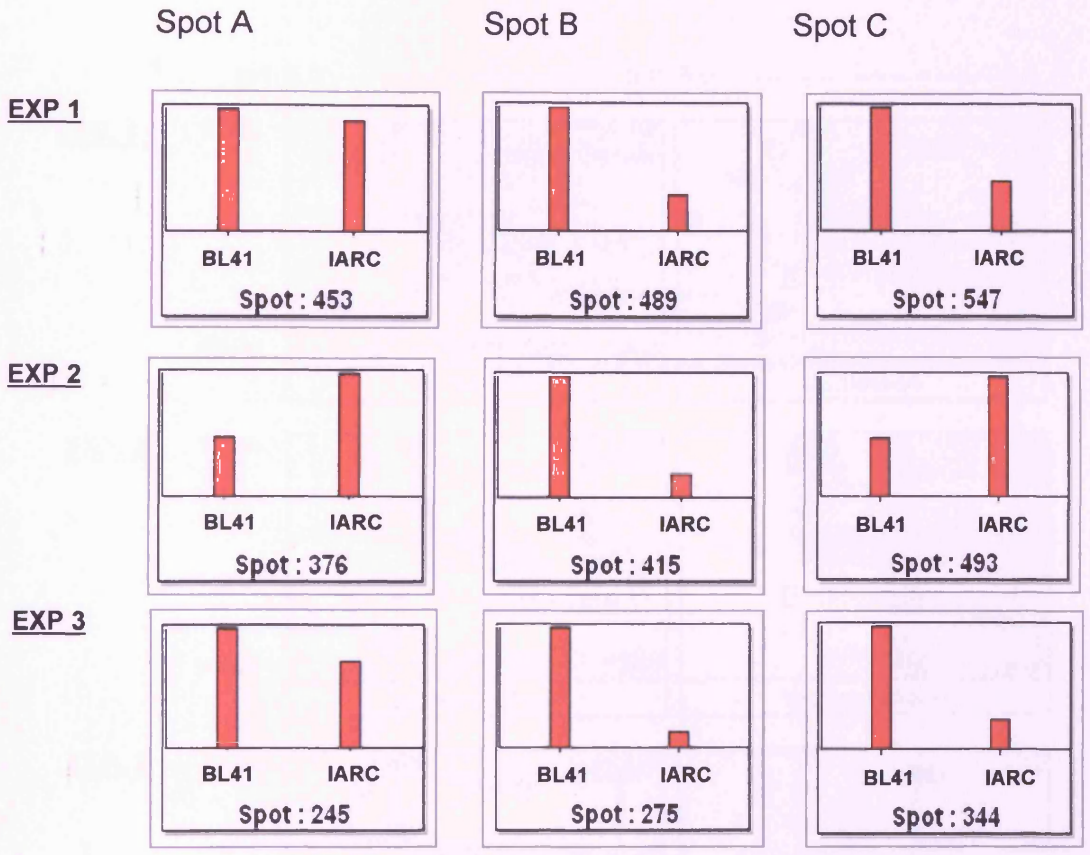


Fig 4.22b- Histogram analysis of spot volumes: Comparison between BL41 and IARC-171 cells.

Quantification of differential expression of proteins between a) BL41 and b) IARC-171 nuclear samples was carried out using histogram analysis of spot volume. Histogram analyses of spots A, B and C from three replicate experiments (EXP. 1-3) are shown. The vertical axes represent the ratio of spot volume when comparing two matched spots.

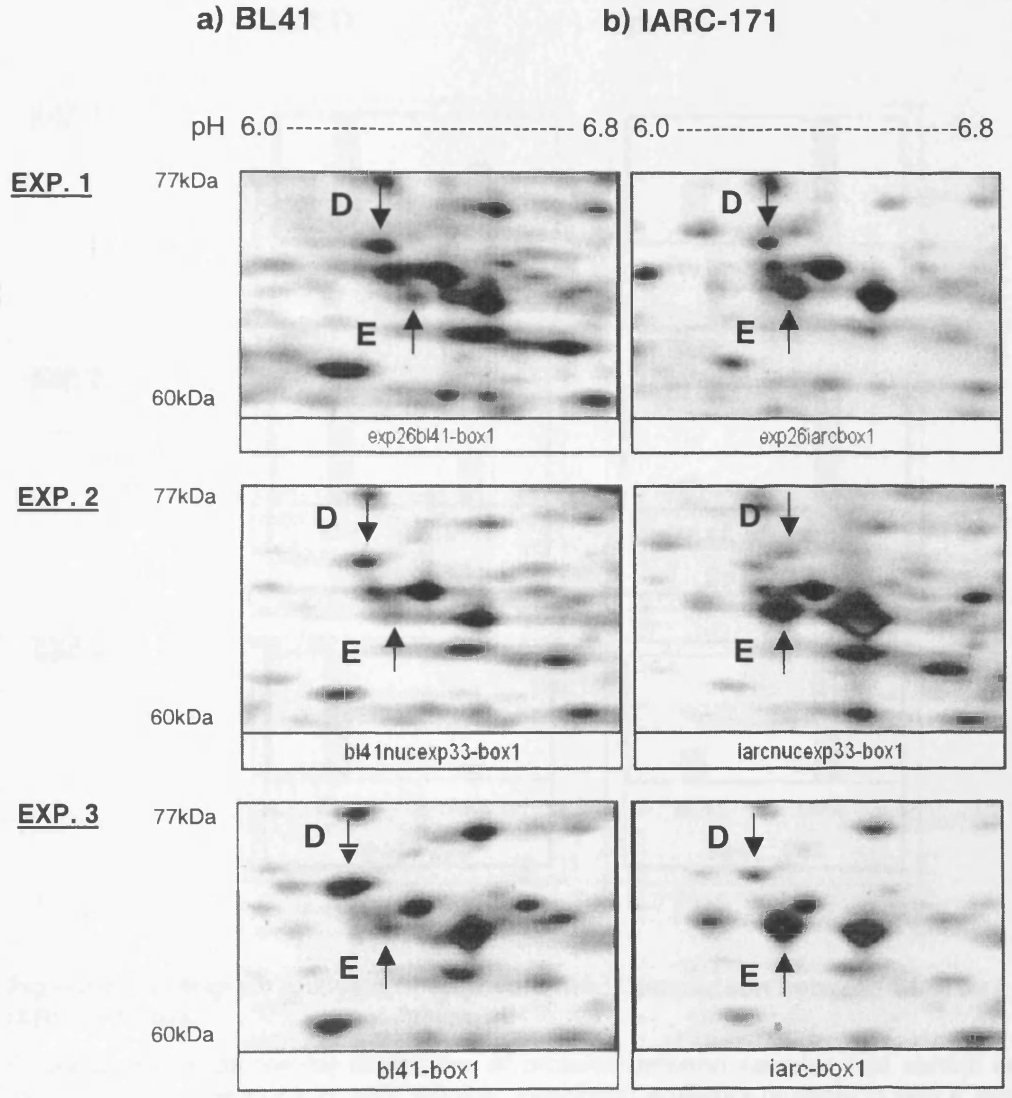


Fig 4.23a- Comparison of protein expression levels between EBV negative Burkitt's lymphoma B cells and EBV immortalized B cells; montage windows.

Montage windows of a selected area (pI 6.2-6.8, Mr 60-77 kDa) are shown comparing nuclear protein expression from corresponding areas of a) BL41 b) IARC-171 cells from three replicate experiments (1-3). Two spots (D and E) from this area were selected for histogram analysis.

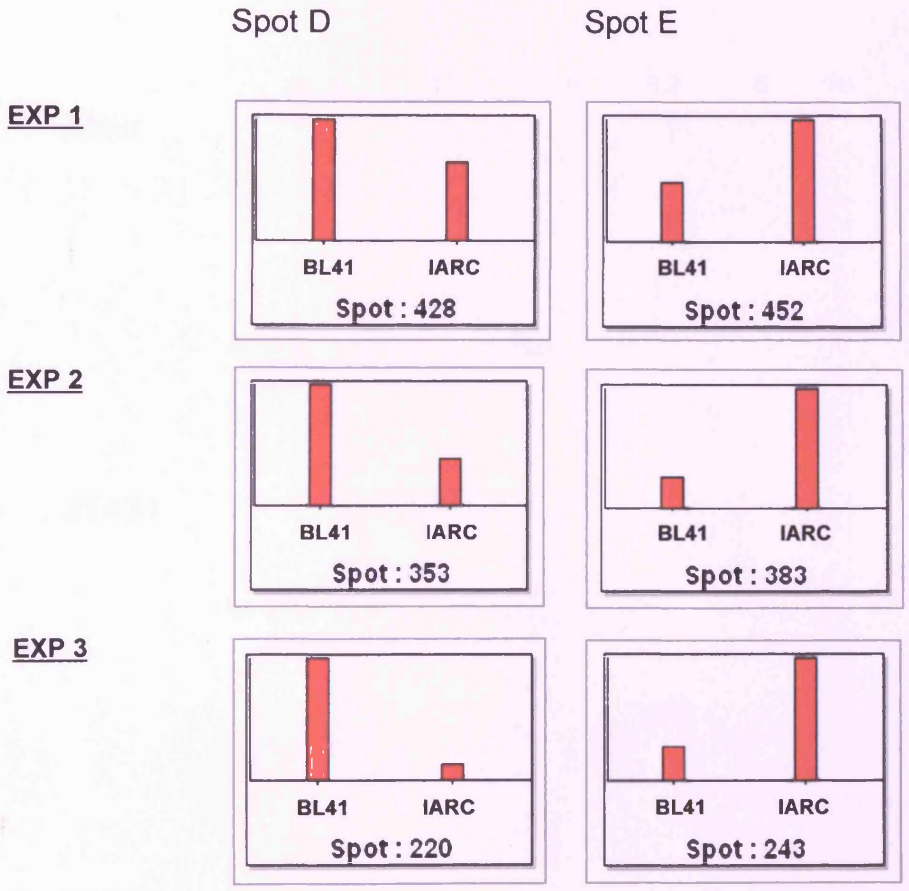


Fig 4.23b- Histogram analysis of spot volumes: Comparison between BL41 and IARC-171 cells.

Quantification of differential expression of proteins between samples was carried out using histogram analysis of spot volume. Histogram analyses of spots D and E from three replicate experiments (EXP. 1-3) are shown. The vertical axes represent the ratio of spot volume when comparing two matched spots.

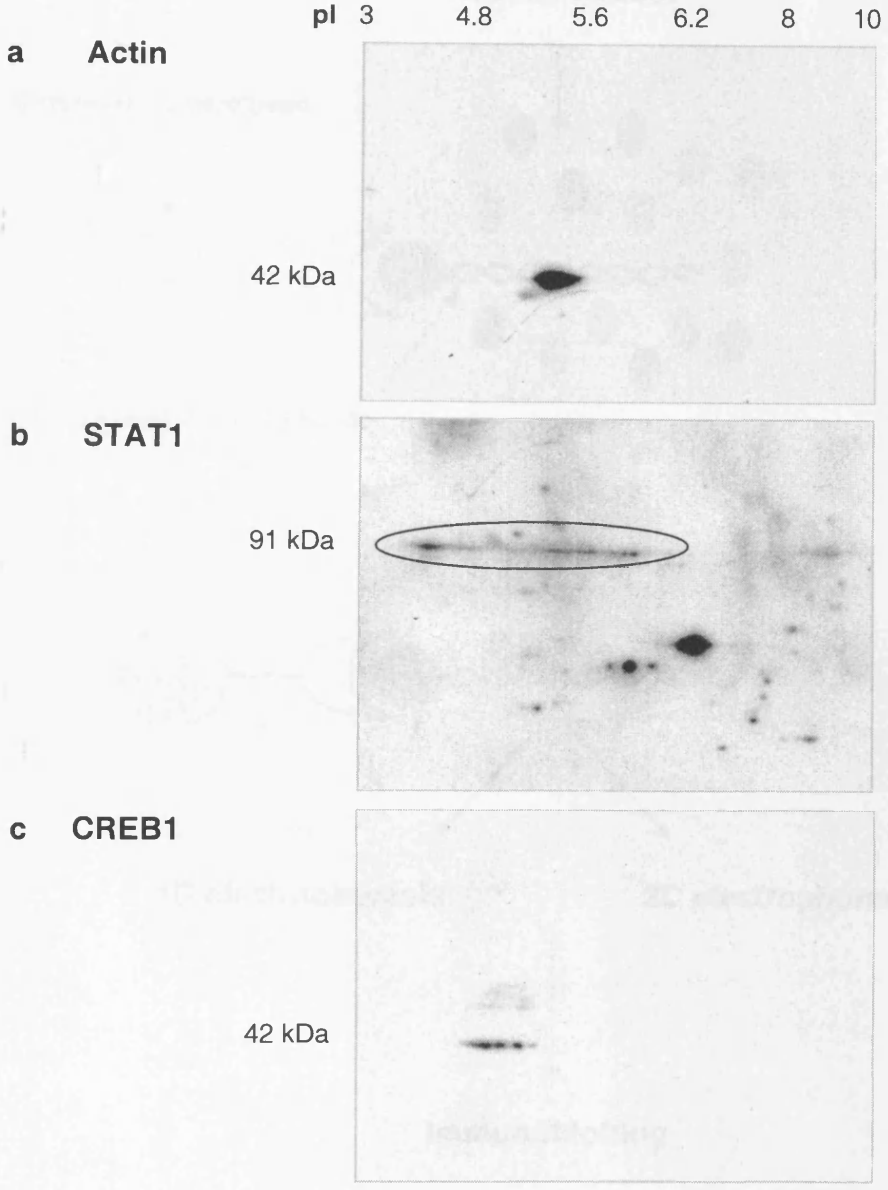


Figure 4.24 – Western blot analysis of proteins separated by 2D electrophoresis.

Nuclear protein extracts generated from IB4-LCL that were cleaned and resolved by 2D electrophoresis on a pH 3-10 nonlinear gradient. Resolved proteins were transferred on to a PVDF membrane. Protein spots corresponding to a) α-actin b) STAT1 and c) CREB1 were subsequently detected by immunoblotting with specific antibodies.

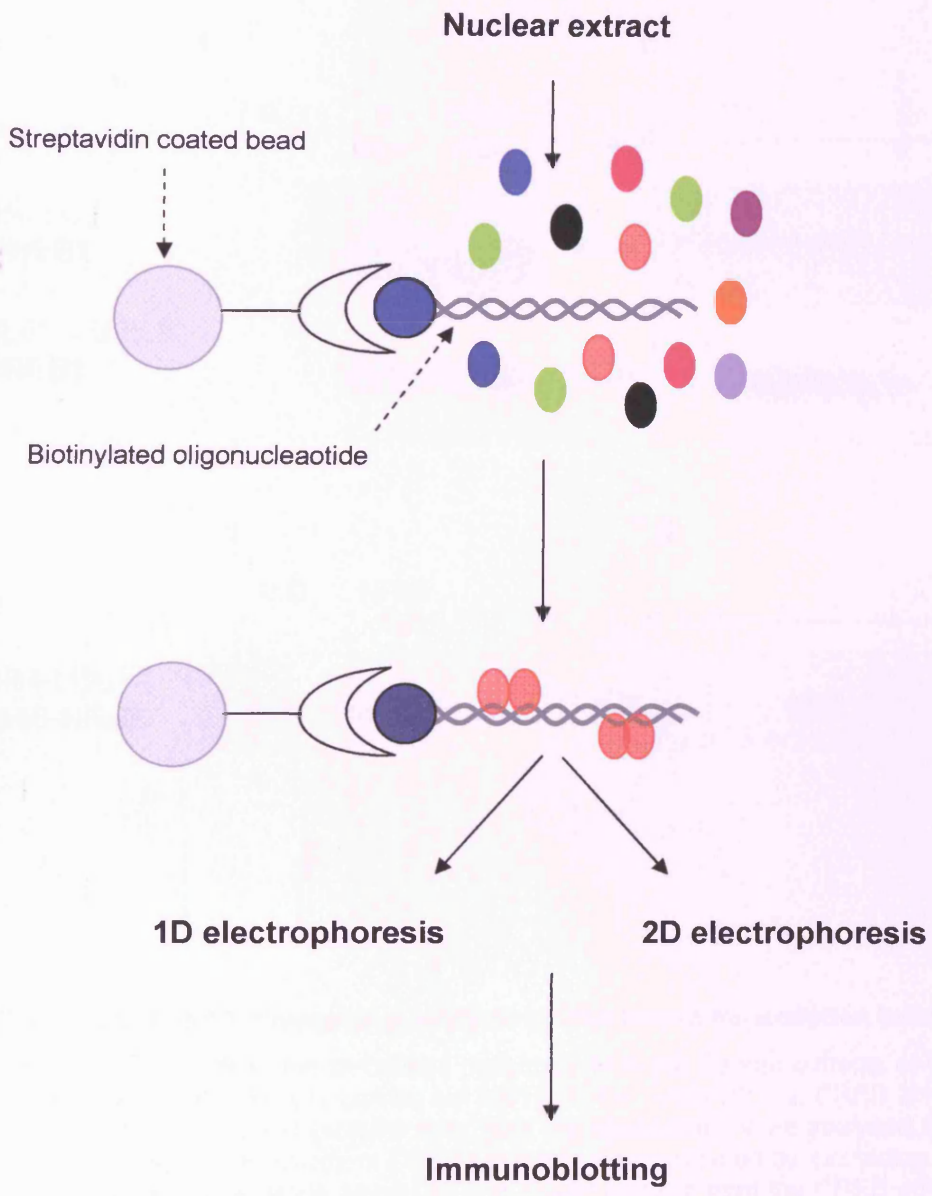


Figure 4.25 – Overview of DNA affinity precipitation

A nuclear protein extract is incubated with biotinylated oligonucleotides containing a specific DNA binding sequence for a protein of choice. Also included in the mixture are streptavidin coated agarose beads. The high affinity interaction formed between streptavidin and biotin allow proteins bound to the oligonucleotide to be isolated. DNA bound proteins (red) were subsequently eluted from the DNA and resolved by 1D SDS-PAGE or 2DE. The amount of eluted proteins were detected by immunoblotting using specific antibodies.

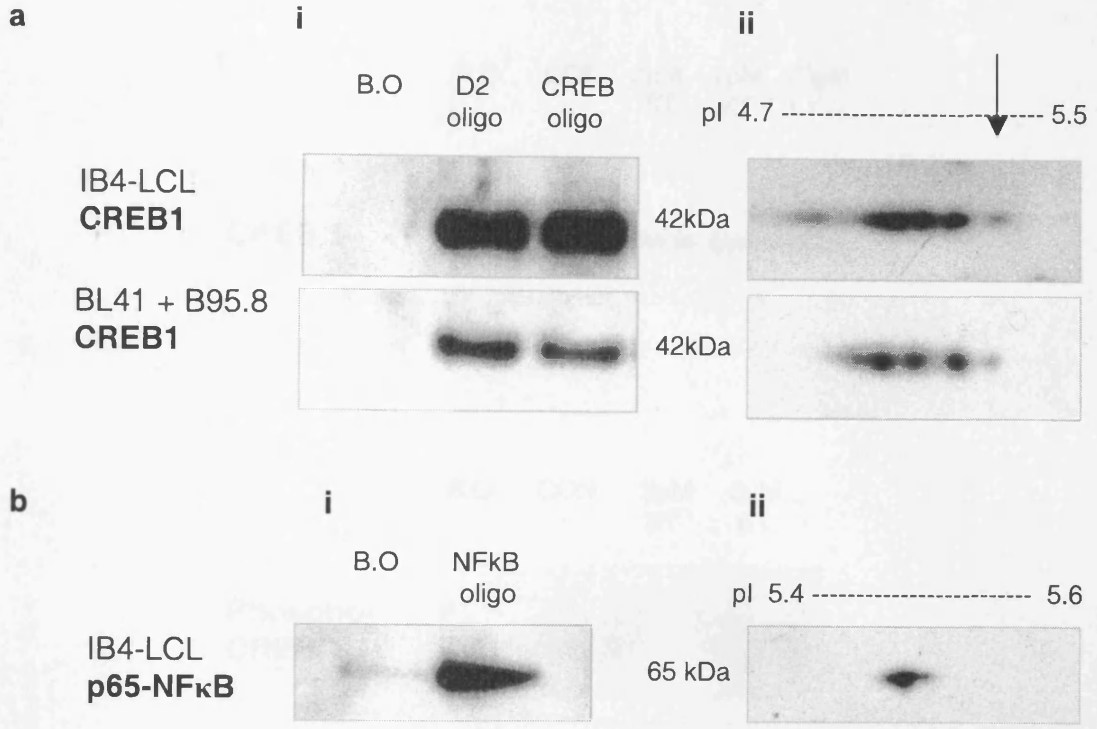


Figure 4.26 -1 and 2-dimensional analysis of DNA bound transcription factors.

a) DNA affinity precipitation of CREB1 molecules from the nuclear extracts of IB4-LCL and BL41 + B95.8 cells was carried out with both the cyclin D2 and CREB consensus oligonucleotides. i) Eluted proteins from both oligonucleotides were analysed by SDS-PAGE. The binding specificity of CREB1 proteins was confirmed by incubating nuclear extracts with agarose beads alone (B.O) ii) Eluted proteins from the CREB consensus oligonucleotides were resolved by 2D electrophoresis on a pH 3-10 nonlinear gradient. CREB1 isoforms were detected by immunoblotting with a CREB1 specific antibody. The theoretical pI of CREB is 5.45 (indicated with an arrow).

b) DNA affinity precipitation of p65-NFκB molecules from the nuclear extracts of IB4-LCL and BL41 + B95.8 cells was carried out using oligonucleotides containing the p65 specific DNA binding sequence. i) Eluted proteins were analysed by SDS-PAGE. The binding specificity of p65 proteins was confirmed by incubating nuclear extracts with agarose beads alone (B.O) ii) Eluted proteins were resolved by 2D electrophoresis on a pH 3-10 nonlinear gradient. P65-NFκB was detected by immunoblotting with a p65 specific antibody.

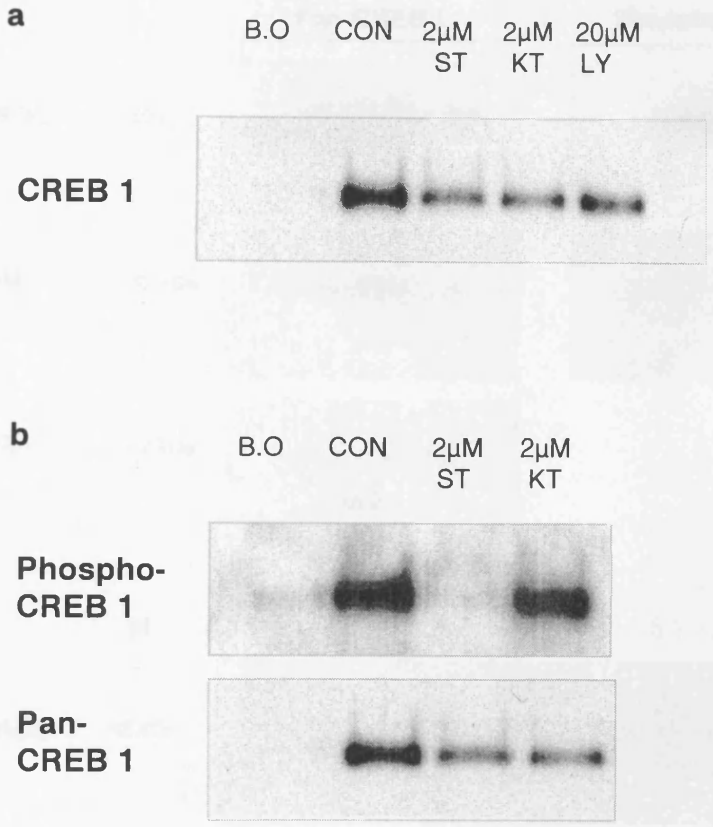


Figure 4.27 – The effect of kinase inhibitors on the DNA binding of CREB1.

Nuclear protein extracts were generated from BL41 + B95.8 cells that were either left untreated (con) or were treated with staurosporine (2μM, 1 hour) (ST), KT-5720 (2μM, 1 hour) (KT) or LY294002 (20μM, 1 hour) (LY). CREB1 molecules were subsequently DNA affinity precipitated using CREB consensus oligonucleotides. Eluted proteins were resolved by SDS-PAGE. a) CREB1 molecules were detected by immunoblotting with a CREB1 specific antibody b) The experiment described was repeated with the exception of treatment of cells with LY294002. Eluted phospho-CREB1 molecules were detected by immunoblotting with a phospho-CREB1 specific antibody. Pan-CREB1 molecules were also detected with a specific antibody. The binding specificity of CREB1 proteins was confirmed by incubating nuclear extracts with agarose beads alone (B.O).

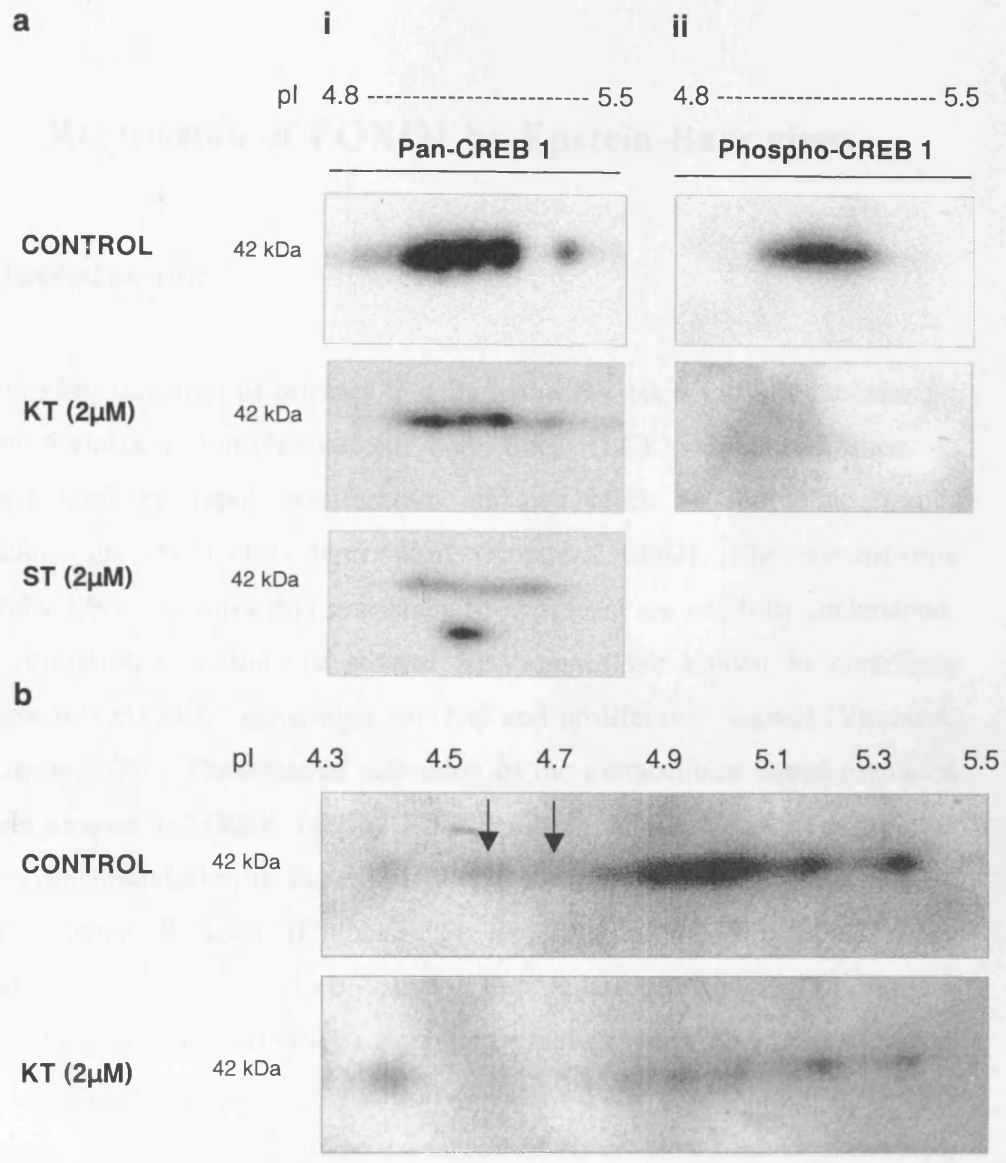


Figure 4.28 – The effect of kinase inhibitors on the post-translational modifications of CREB1.

Nuclear protein extracts were generated from BL41 + B95.8 cells that were either left untreated (control) or were treated with staurosporine (2µM, 1 hour) (ST) or KT-5720 (2µM, 1 hour) (KT). CREB1 molecules were subsequently DNA affinity precipitated using CREB consensus oligonucleotides. Eluted proteins were resolved by 2D electrophoresis on a a) pH 3-10 nonlinear gradient b) pH 4-7 linear gradient. Phospho- and pan-CREB1 isoforms were detected by immunoblotting using specific antibodies

CHAPTER 5

Regulation of FOXO1 by Epstein-Barr virus

5.1 Introduction

The *in vitro* infection of primary B cells with EBV leads to the establishment of immortalized lymphoblastoid cell lines (LCL). Immortalization is characterized by rapid proliferation and resistance to apoptotic stimuli, including growth factor deprivation (Kuppers, 2003). The mechanisms whereby EBV mediates this resistance to apoptosis are not fully understood. The co-operative actions of several EBV genes are known to contribute towards this effect by generating survival and proliferation signals (Young & Rickinson, 2004). Constitutive activation of the extracellular signal-regulated protein kinases 1/2 (ERK 1/2) by EBV has been demonstrated be associated with a downregulation in the expression of the pro-apoptotic protein, bim, in EBV infected B cells (Clybouw *et al*, 2005). The activation of the transcription factor NFκB and the phosophatidylinositol-3-kinase (PI3K)/Protein Kinase B (PKB) signalling pathways have also been shown to play important roles in the regulation of survival and proliferation of B-cells (Brennan, 2001). While many of the targets of NFκB have been characterized, the nuclear targets of PI3K are relatively poorly characterized in EBV immortalized B-cells.

Pro-apoptotic Forkhead box class O (FOXO) transcription factors are direct targets of PI3K mediated signal transduction in a variety of cell systems. Phosphorylation of members of this transcription factor family by PKB, an important downstream effector of PI3K activity, results in the nuclear exclusion and inhibition of transcriptional activity (Biggs *et al*, 1999, Brunet *et al*, 1999, Takaishi *et al*, 1999). FOXO transcription factors co-ordinate cell cycle progression and cell survival by the activation of antiproliferative genes

such as $p27^{Kip1}$ and cyclin G2 as well as pro-apoptotic genes such as Fas Ligand (FasL), bcl-6 and the bcl-2 family member bim (Brunet *et al*, 1999, Medema *et al*, 2000, Dijkers *et al*, 2002, Tang *et al*, 2002, Furukawa-Hibi *et al*, 2002, Essafi *et al*, 2005).

Initial identification of this transcription factor family in humans occurred as three members were identified at chromosomal translocations in human tumours, namely FOXO1 (FKHR) in alveolar rhabdomyosarcomas, FOXO3a (FKHR-L1) in acute myeloblastic leukaemia (AML) and FOXO4 (AFX) in acute lymphocytic leukaemias (Galili *et al*, 1993, Parry *et al*, 1994, Davies *et al*, 1994, Borkhardt *et al*, 1997, Hillion *et al*, 1997) These discoveries were the first indications that FOXO transcription factors have a role in tumour development. Recent studies have demonstrated that loss of FOXO activities due to protein degradation contributes towards cellular transformation of primary breast cancer tumours (Hu *et al*, 2004) and mouse primary lymphomas (Huang *et al*, 2005).

This part of the study was therefore initiated to investigate the interplay between FOXO and EBV, and focused on one member of the FOXO family, FOXO1.

5.2 FOXO1 expression is downregulated by EBV infection

The aim of this part of the study was to investigate the relationship between EBV infection and FOXO1 expression. To do this, nuclear FOXO1 protein levels were analyzed by immunoblotting in cells lines with different EBV status using a specific antibody. Poly-ADP ribose polymerase (PARP) protein levels were also analyzed to verify equal protein loading as PARP is found in the nucleus and PARP expression levels are not affected by EBV status. The BL41, BL41 + B95.8 and IARC-171 cell lines were generated from the same individual and thus have the same genetic background. They differ in their patterns of EBV gene expression and the presence of the myc translocation present in BL41 that is typical of Burkitt's lymphoma cells (Rowe *et al*, 1986). BL41 is an EBV-negative Burkitt's Lymphoma (BL) line. BL41 + B95.8 is the same line after infection with the B95.8 strain of EBV. IARC-171 is a B95.8 EBV immortalized LCL derived from the same patient as BL41. The results show high levels of FOXO1 protein expression in the EBV negative BL line (BL41) (Figure 5.1a). In contrast, FOXO1 expression is markedly downregulated in EBV immortalized B-cells (IARC-171). To investigate whether the presence of EBV is the cause of downregulation, FOXO1 expression was also analyzed in BL41 + B95.8 cells. Less FOXO1 was detected in BL41 + B95.9 cells when compared to BL41 cells, suggesting that EBV has a role in downregulating FOXO1 protein expression.

EBV infection of B cells can lead to the establishment of distinct latency programmes as a result of the different expression patterns of viral genes (Kuppers, 2003). To establish whether the latency programme affects FOXO1 expression, protein levels were compared between four EBV positive sublines of a BL tumour. Mutu I cells express only EBV Nuclear Antigen -1 (EBNA1), and two small polyadenylated RNA molecules (EBER1 and EBER2), a restricted pattern of viral gene expression termed Latency I. Mutu III express the full complement of EBV encoded latency genes (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA-LP, LMP1, LMP2, EBER1, EBER2 and BamHI A rightward transcripts) termed Latency III (Rowe *et al*, 1992).

FOXO1 protein levels were significantly lower in Mutu III cells compared to Mutu I cells, and were comparable to that observed in the LCL, IARC 171 (Figure 5.1b). The results therefore clearly demonstrate that a latency III pattern of viral gene expression is required for the repression of FOXO1 protein levels.

Additional EBV negative and positive lines were also tested for FOXO1 protein expression (Figure 5.1c). Three EBV negative Burkitt's lymphoma lines were analyzed. BL41 is described above. DG75 is another EBV negative BL line (Ben-Bassat *et al*, 1977). AK31 is an EBV negative subclone of Akata (Jenkins *et al*, 2000), an EBV positive BL line displaying a latency I pattern of gene expression. Again, FOXO1 protein expression was high in EBV negative tumour lines. Three LCL lines were also tested. IARC-171 is described above. IB4-LCL is an LCL generated from cord blood B-cells immortalized with B95.8 EBV (Sample & Kieff, 1990). EB-LCL is an LCL generated in house by infection of primary B cells from the blood of a healthy donor with the B95.8 strain of EBV. In accordance with previous results, FOXO1 expression was very low in EBV immortalized B cells. The level of FOXO1 expression in the BL41 + B95.8 cells was again between that observed in BL41 and IARC-171 cells.

Altogether, there results demonstrate that FOXO1 is downregulated in EBV infected cells, and that a latency III pattern of EBV gene expression is required for this repressive effect.

5.3 Analysis of the levels transcription factors in EBV negative and EBV positive cell lines

EBV has been shown to alter the expression of other transcription factors targeted downstream of cellular signalling pathways. The signal transducer and activator of transcription 1 (STAT1) transcription factor is constitutively activated in EBV immortalized B cells by the main transforming protein of EBV, Latent membrane protein 1 (LMP1) (Weber-Nordt *et al*, 1996,

Richardson *et al*, 2003). LMP1 also recruits components of the Tumour Necrosis Factor-Receptor (TNF-R) signalling pathway to activate the Nuclear Factor-κB (NFκB) transcription factor, and is one of the major signalling pathways activated by EBV (Huen *et al*, 1995, Izumi *et al*, 1997). The cAMP response element binding protein 1 (CREB1) transcription factor has been shown to be constitutively phosphorylated at the critical site for activity, serine 133, in EBV immortalized B cells (White *et al*, 2006).

The expression status of these transcription factors were investigated in the EBV negative and EBV positive B cell lines in which FOXO1 protein expression was found to be downregulated by EBV. Nuclear protein extracts were generated form BL41, BL41 + B95.8 and IARC-171 cell lines and were resolved by SDS-PAGE. The expression levels of FOXO1, STAT1, the p65 component of NFκB and CREB1 were determined by immunoblotting using specific antibodies. Again, the expression of FOXO1 was found to be significantly downregulated in the LCL (IARC-171) compared to EBV negative BL41 cells (Figure 5.2). FOXO1 levels in BL41 + B95.8 cells were again between that of BL41 and IARC-171 cells. In contrast, the expression of STAT1 was dramatically increased in IARC-171 compared to BL41. STAT1 levels in BL41 + B95.8 cells were increased compared to BL41 cells but were not at levels comparable with the LCL, reflecting the intermediate effect of EBV on FOXO1 expression in these cells. Analysis of the expression of the p65 component of NFκB showed no difference in the level of expression when comparing BL41 and BL41 + B95.8 cells, but a small increase was observed in IARC-171 cells. Total levels of CREB1 were similar in all three cell lines tested. The small decrease observed in CREB1 protein levels in IARC-171 cells is likely to be due a slight difference in loading, as demonstrated by the levels of PARP, used as a loading control. These results demonstrate that the expression of some transcription factors, namely FOXO1, STAT1 and NFκB are altered by EBV, whereas others, such as CREB1 remain unaffected.

5.4 PI3K inhibition increases nuclear FOXO1 protein levels

One mechanism whereby FOXO activity is regulated is by nuclear exclusion and subsequent degradation once in the cytoplasm. Stimulation of the PI3K pathway has a central role in this process by activating the kinase activity of PKB. FOXO transcription factors contain three consensus PKB phosphorylation sites that when phosphorylated target the proteins for nuclear exclusion, thereby increasing the survival potential of the cell (Birkenkamp & Coffer, 2003). In order to investigate the role of PI3K in regulating the subcellular localization and protein levels of FOXO1, a specific inhibitor of PI3K was employed, LY294002 (Vlahos *et al*, 1994). BL41, BL41 + B95.8 and IARC-171 cells were either left untreated or were treated with increasing doses of LY294002. Nuclear and cytosolic FOXO1 protein levels were subsequently analyzed by western blotting.

Although basal levels of FOXO1 levels are relatively high in the nuclei of BL41 and BL41 + B95.8 cells, this could be increased further following PI3K inhibition by LY294002 for 1 hour (Figure 5.3a and Figure 5.3b). In parallel to this increase, a decrease in cytosolic FOXO1 was observed following LY294002 treatment in both cell lines, although to a greater degree in the EBV negative BL41 cells. In contrast to this, no nuclear increase or cytosolic decrease in FOXO1 level could be detected in the LCL (IARC-171) after 1 hour treatment with the PI3K inhibitor. Only after treatment with LY294002 for 24 hours could an increase in FOXO1 nuclear protein be detected (Figure 5.3c). This increase was not accompanied by a decrease in cytosolic FOXO1. These results show that PI3K inhibition can increase FOXO1 in all cell types but with different kinetics, suggesting a difference in the way in which PI3K regulates FOXO1 activity in cells with different EBV status.

5.5 FOXO1 mRNA is downregulated by EBV

The slow kinetics of upregulation of FOXO1 by treatment with LY294002 in IARC-171 suggests a different mechanism of regulation in these cells

compared to EBV negative BL41 cells. For this reason, it was investigated whether FOXO1 repression by EBV could occur at the transcriptional level. Total RNA was purified from BL41, BL41 + B95.8 and IARC-171 cells. FOXO1 mRNA activity was subsequently measured relative to a housekeeping gene (L19) by real time PCR by the group of Professor Eric Lam at the Department of Oncology, Imperial College London. The results shown are the average of triplicate results from three independent experiments and demonstrate that steady state levels of FOXO1 mRNA were reduced by half in EBV infected cells compared to EBV negative cells (Figure 5.4). These results indicate that FOXO1 is downregulated at the mRNA level by EBV.

5.6 FOXO1 can bind DNA and is post-transcriptionally modified in EBV infected cells

DNA binding is required for transcription factors to regulate target genes and downstream effects. It was therefore important to determine whether the FOXO1 detected was capable of binding DNA for mediation of its effects upon cell proliferation and survival. DNA affinity precipitation experiments were therefore carried out using an oligonucleotide containing a forkhead response element (5'-TAAACAC-3') from the *bim* promoter (Essafi *et al*, 2005). FOXO1 molecules from nuclear extracts of IB4-LCLs and BL41 + B95.8 cells were therefore DNA affinity precipitated using the *bim* promoter oligonucleotide. FOXO1 DNA binding was subsequently analyzed by immunoblotting. The binding specificity of FOXO1 was tested by incubating nuclear extracts with agarose beads alone. No FOXO1 DNA binding was observed in the LCL (Figure 5.5a). This was to be expected as very little, if any, FOXO1 could be detected in the nuclei of all LCLs tested. DNA binding of FOXO1 to the *bim* oligonucleotide could however be detected in BL41 + B95.8 cells. Some of this FOXO1 DNA binding was likely to be non-specific as indicated by the presence of a small amount of FOXO1 in the sample incubated with beads alone (Figure 5.5a). However, the amount of DNA bound FOXO1 detected in the sample incubated with *bim* oligonucleotides was significantly higher, confirming that FOXO1 molecules detected are

capable of binding to target promoter sequences when present in sufficient amounts in the nucleus.

The effect of PI3K inhibition on FOXO1 DNA binding in EBV negative and EBV positive cells was also tested. FOXO1 molecules from nuclear extracts of BL41 and BL41 + B95.8 cells, either untreated or treated with LY294002 (20μM) for 1 hour, were DNA affinity precipitated using the *bim* oligonucleotide. FOXO1 DNA binding was analysed by immunoblotting. The binding specificity of FOXO1 proteins was again tested by incubating nuclear extracts with agarose beads alone. FOXO1 DNA binding was detected at comparable levels in both cell lines with no treatment (Figure 5.5b). Binding was mostly specific as indicated by only a small amount of FOXO1 DNA binding in samples incubated with beads alone. Following LY294002 treatment, the amount of DNA bound FOXO1 was significantly increased in both BL41 and BL41 + B95.8 cells to a similar degree. This suggests that a direct correlation exists between the amount of FOXO1 localized within the nucleus and DNA binding.

The role of post-translational modifications in the regulation of transcription factor activity is well established. Phosphorylation events are well known to have important effects on the activities of many transcription factors. FOXO members contain multiple PKB phosphorylation sites that mediate subcellular localization and DNA binding activity. Both ubiquitinylation and acetylation have also been reported to modulate members of the FOXO family by directing degradation and attenuation of DNA binding respectively (Huang *et al*, 2005, Matsuzaki *et al*, 2005, Perrot & Rechler, 2005) To establish how many DNA binding isoforms of FOXO1 exist in the nuclei of EBV negative and positive BL41 cells, FOXO1 was analyzed by applying the technology of 2DE, in combination with DNA-AP. This method was developed in chapter 4 of this study (section 4.7.2), and was found to be an effective method of analysing DNA bound isoforms of transcription factors. DNA affinity precipitated FOXO1 eluted from *bim* oligonucleotides was isoelectrically focused using a pH 3-10 non-linear immobilized pH gradient and subsequently separated in the second dimension by SDS-PAGE. Immunoblot detection of

FOXO1 with a specific antibody revealed that multiple isoforms of FOXO1 bind to DNA in BL41 + B95.8 cells (Figure 5.5c). The isoelectric points (pI) of the different FOXO1 isoforms range between 5.4 and 6.1 and are present is varying amounts. The most abundant isoform has a pI of approximately 5.7. In contrast, only one isoform of FOXO1 could be detected in BL41, which has a lower pI value of approximately 4.7. These results demonstrate that FOXO1 is post-translationally modified, and multiple isoforms of FOXO1 are capable of binding DNA in EBV positive cells but not in EBV negative B cells.

5.7 FOXO1 expression correlates with Bcl-6 and inversely correlates with Cyclin D2.

Having established that the FOXO1 detected is capable of binding DNA, it was investigated whether DNA binding initiated transcriptional activation, and the effect of EBV on target gene activity. To address these questions, expression levels of FOXO1 target genes were analyzed using various cell lines. Bcl-6 is a sequence specific transcriptional repressor of proteins mediating lymphocyte apoptosis and differentiation and *bcl-6* transcription is activated by FOXO1 (Tang *et al*, 2002). Nuclear bcl-6, from various cell lines, was analyzed by immunoblotting. A direct correlation between FOXO1 and bcl-6 protein levels was observed, with a high level of bcl-6 expression in cells with high FOXO1 and less bcl-6 detected in EBV transformed LCL cells (Figure 5.6). The bcl-6 levels do not correspond with EBV status, per se, as Akata, which is EBV positive but has a latency I pattern of gene expression, expresses high levels of FOXO1 and bcl-6. This agrees with the data from Mutu I cells and suggests that one, or more, of the EBV genes expressed in latency III is likely to regulate FOXO1. Cyclin D2 protein levels were also analyzed. Cyclin D2 is a cell cycle protein required for progression through the G_1 phase of the cell cycle and has been shown to be transcriptionally repressed by FOXO (Schmidt *et al*, 2002, Fernandez de Mattos *et al*, 2004). Western blot analysis showed an inverse correlation between Cyclin D2 expression and the expression of both FOXO1 and bcl-6. These data suggest

that FOXO1 is transcriptionally active, and that the repressive effect that EBV has upon FOXO1 also extends to its target genes.

5.8 PI3K activity can regulate Cyclin D2, but not Bcl-6 protein levels in EBV negative and EBV positive B cells.

Since the expression levels of FOXO1 have been shown to correlate with the expression of two FOXO1 target genes, bcl-6 and cyclin D2 (section 5.7) and nuclear FOXO1 has been demonstrated to be increased in response to PI3K inhibition (section 5.4), it was investigated whether PI3K can directly regulate the expression of cyclin D2 and bcl-6. Cyclin D2 expression has previously been shown to be important for the proliferative effects of PI3K on lymphoblastoid cells and is downregulated in response to treatment with the PI3K inhibitor, LY294002 in EBV immortalized B cells (Brennan *et al*, 2002).

Nuclear protein extracts were generated from BL41 cells treated with varying doses of LY294002 for the period of time required to induce an upregulation in FOXO1 expression in BL41 cells (1 hour). The expression of FOXO1, bcl-6 and cyclin D2 were subsequently analyzed by SDS-PAGE and immunoblotting with specific antibodies. The blot generated in section 5.4 (Figure 5.3c), where IARC-171 cells were treated with varying doses of LY294002 (24 hours), were also re-probed with these antibodies. The effectiveness of LY294002 to inhibit PI3K was demonstrated by analysing the phosphorylation status of the S6 ribosomal protein (S6), a major target of PI3K signalling in B cells as demonstrated in chapter 3 of this study. The levels of FOXO1 in the nuclei of BL41 and IARC-171 cells were shown to be increased in response to LY294002 treatment for 1 hour and 24 hours respectively (Figure 5.7). The level of bcl-6 expression was high in untreated EBV negative BL41 cells, and was low in untreated EBV immortalized IARC-171 cells, reflecting the direct correlation observed between FOXO1 and bcl-6 observed in section 5.7. Inhibition of PI3K, however, did not alter the levels of bcl-6 in BL41 or IARC-171 cells. This suggests that PI3K is not sufficient to regulate the expression of this protein. In contrast, cyclin D2 expression was

inhibited by LY294002 treatment in BL41 and IARC-171 cells for 1 hour and 24 hours respectively. These data agree with a role for PI3K in the regulation of cyclin D2 in B cells (Brennan *et al*, 2002)

5.9 Both LMP1 and LMP2A can downregulate FOXO1 expression

The establishment of EBV latency requires the expression of a repertoire of EBV encoded latent genes. LMP1 is the major transforming protein of EBV and is required for the transforming effects of EBV on primary B cells. LMP1 mimics a constitutively activated CD40, a member of the tumour necrosis receptor (TNFR) superfamily, mediating ligand independent signalling through a range of key signalling pathways essential for survival including NFκB, mitogen-activated protein kinases (MAPKs), JNK, JAK/STAT pathways as well as the PI3K pathway (Young & Rickinson, 2004). LMP2A, also encoded by EBV, is another potent activator of the PI3K pathway, and acts as a constitutively activated B cell receptor (BCR), thereby inhibiting normal signalling through the BCR (Swart *et al*, 2000, Morrison *et al*, 2003). Since the activities of both LMP1 and LMP2A have been shown to activate PI3K signalling it was reasonable to suspect that they may have a role in the repression of FOXO1.

To test this hypothesis, the effect of LMP1 on FOXO1 expression was firstly investigated by employing EBV negative B cell lymphoma lines (BJAB) stably transfected with LMP1 (Wang *et al*, 1990). Protein expression levels were compared between the nuclear extracts generated from two BJAB clones stably expressing LMP1, MTLM-17 and MTLM-6, and their corresponding vector control lines, GPT1 and GPT2 respectively, by SDS-PAGE and immunoblotting. The expression of LMP1 in MTLM-17 and MTLM-6 was tested and was found to be expressed at comparable levels in both clones (Figure 5.8). The levels of FOXO1 in these cells were subsequently tested. FOXO1 was found to be significantly downregulated in the LMP1 expressing MTLM-6 cells when compared to its corresponding vector control cell line,

GPT2. This observation, however, was not repeated in the other LMP1 expressing clone, MTLM-17, in comparison to its corresponding vector control line, GPT1. This discrepancy prompted us to investigate the signalling capacity of the LMP1 molecules expressed. The expression levels of STAT1 were therefore analysed. STAT1 was found to be constitutively expressed in MTLM-6 cells, but not in MTLM-17 cells, suggesting that the signalling potential of LMP1 in MTLM-17 cells is impaired. This explained the discrepancy observed in the effect of LMP1 on FOXO1 between these cells, and suggested that LMP1 may have a role in the downregulation of FOXO1.

To further evaluate the role of LMP1, and also to investigate whether LMP2A has a role in mediating the downregulation of FOXO1, DG75 cells, which express high levels of FOXO1, were transiently transfected with increasing amounts of either LMP1 or LMP2A expression vectors. The total amount of DNA transfected was kept constant (10μg) with the addition of the empty pSG5 vector. After 20 hours, cells were harvested, lysed and FOXO1 and bcl-6 protein levels were analyzed by immunoblotting. DG75 cells transfected with LMP1 demonstrated reduced levels of FOXO1 and also bcl-6 protein expression in a dose dependent manner (Figure 5.9a). Protein analysis of LMP2A transfected cells showed a similar decrease of FOXO1 expression (Figure 5.9b). A clear downregulation of bcl-6 was also detected with transfection of 10μg of LMP2A. However, at the lower doses of 1 and 5μg, LMP2A did not repress bcl-6 protein expression. Actin was used as a loading control. Luciferase reporter assays were carried out in parallel with the lysates generated from DG75 cells transfected with increasing amounts of either LMP1 or LMP2A expression vectors. Cells were also transfected with a fixed amount of a bcl-6 reporter construct containing a FOXO consensus binding sequence upstream of a luciferase gene. The b*cl-6* promoter sequence was mutated to lack the consensus bcl-6 binding site in the *bcl-6* gene that is thought to act as a negative autoregulatory loop (Tang *et al*, 2002). LMP1 repressed *bcl-6* transcription in a dose dependent manner (Figure 5.10a). LMP2A however, did not repress the transcriptional activity of the bcl-6 gene (Figure 5.10b). The expression levels of LMP1 and LMP2A in the transfected

cells were confirmed by immunoblotting with specific antibodies (Figure 5.9a, Figure 5.9b, Figure 5.10a, Figure 5.10b).

The effect of LMP1 and LMP2A on protein levels of FOXO1 and bcl-6, protein expression levels were also analyzed in stable DG75 transfectants containing inducible LMP1 and LMP2A genes. In these cell lines, the LMP1 and LMP2A genes were cloned downstream of a promoter containing binding sites for a hybrid tetracycline-regulated transacivator (tTA) that is constitutively expressed from a second co-transfected plasmid DNA. Tertracycline binds to the tTA and prevents binding of the promoter which remains silent. However, on removal of tetracycline, the tTA binds to the promoter sequences and activates the transcription of the gene of interest, in this case, LMP1 and LMP2A (Floettmann *et al*, 1996). The effects of another EBV encoded gene, EBV nuclear antigen 2A (EBNA2A) on FOXO1 and bcl-6 expression was also analysed in a stable DG75 transfectant containing inducible EBNA2A. EBNA2A is a viral transcriptional activator of several viral and cellular genes, and is a crucial mediator of the transforming effects of EBV (Kutok & Wang, 2006).

FOXO1 and bcl-6 protein levels were compared with those detected in two lymphoblastoid cell lines, IARC-171, and an early passage LCL generated from the B cells of a healthy donor with the B95.8 stain of EBV, CMc. In the presence of tetracycline, protein expression of FOXO1 and bcl-6 were detectable in EBNA2A, LMP1 and LMP2A inducible lines, although FOXO1 expression is higher in the EBNA2A line (Figure 5.11). On induction of LMP1 gene expression, by culturing the cells in the absence of tetracycline, a clear decrease of both FOXO1 and bcl-6 levels was detected at 24 hours, and to a greater degree at 48 hours. The induction of LMP2A expression also resulted in the repression of the bcl-6 protein at both time points. The induction of LMP2A expression did not significantly reduce FOXO1 expression, with a slight decrease being observed 48 hours post induction. The subtle effect of LMP2A may be due to the basal expression of LMP2A expression seen in the presence of tetracycline. FOXO1 levels in the LMP1 and LMP2A inducible lines were considerably reduced when compared to the EBNA2A line,

demonstrating the sensitivity of FOXO1 protein expression to the residual low levels of both LMP1 and LMP2A signals in the presence of tetracycline. Induction of EBNA2A did not affect the expression of FOXO1 or bcl-6. Calregulin was used as a loading control in this experiment. The data using stable cell lines expressing EBV genes was generated in collaboration with Martin Rowe and is included with permission.

In, summary, the results from the transient transfection and the stable lines demonstrate that both LMP1 and LMP2A can downregulate the expression of FOXO1 and bcl-6.

5.10 LMP1 mediated repression of FOXO1 and Bcl-6 is not mediated by the C-Terminal activating region -1 (CTAR-1) of LMP1

LMP1 gave a more dramatic reduction in FOXO1 down regulation than LMP2A. There has been significant analysis of LMP1 signalling that has identified at least two different signalling domains, known as C-Terminal activating region-1 (CTAR1) and CTAR2. CTAR1 has been shown to activate PI3K in epithelial cells (Dawson *et al*, 2003) and fibroblasts (Maniou *et al*, 2005). To investigate whether CTAR1 can regulate FOXO1, an LMP1 mutant was employed in which three critical amino acids within the CTAR1 domain, Proline 204, Glycine 206 and Threonine 208, were mutated to alanine. This mutant is referred to $LMP1^{AAA}$. This mutation blocks CTAR1 mediated NFκB activation by abrograting the binding of TRAF (TNF-receptor associated factor) molecules, which is important for the LMP1 induced phenotypic and cellular changes associated with EBV mediated transformation (Eliopoulos *et al*, 1997, Brodeur *et al*, 1997). DG75 cells were therefore transfected with increasing amounts of either the wild type LMP1 or $LMP1^{AAA}$ expression vectors. After 24 hours the cells were harvested and lysates were generated. The protein extract was resolved by SDS-PAGE and analysed by immunoblotting with antibodies specific for FOXO1, bcl-6 and LMP1 (Figure 5.12). The protein levels of STAT1 were also analysed with a specific

antibody as this transcription factor has been demonstrated to be increased in expression by LMP1 through an NFκB pathway (Richardson *et al*, 2003). Actin was used as a loading control. Both the wild type LMP1 and the $LMP1^{AAA}$ mutant were able to cause a decrease in FoxO1 and bcl-6 protein levels. However, only wild type LMP1 was able to induce STAT1. These data suggests that distinct domains of LMP1 are involved in the regulation of FOXO1 and bcl-6 transcription factor protein levels, and that this may not be due to the direct activation of PI3K by the CTAR1 region of LMP1.

This work has been accepted for publication; *'Epstein-Barr Virus represses FoxO1 transcription factor through Latent Membrane Protein 1 and Latent Membrane Protein 2A'*, manuscript in press, *Journal of Virology* (see appendix III).

5.11 Discussion

In this chapter the interplay between EBV and FOXO1 was investigated. FOXO1 has a well established role in the regulation of cell survival and so it was logical to suspect that it is a target of regulation by EBV. The data clearly demonstrated that EBV has a repressive effect on FOXO1 protein expression in B cells and that this effect can be mediated by both EBV encoded latent membrane proteins-1 and 2A. The repression of FOXO1 may be due to the ability of these membrane proteins to activate PI3K signalling. However, the CTAR1 component of LMP1, which has previously been shown to mediate PI3K activation in other cell types, was found not to be essential for the LMP1 mediated downregulation of FOXO1. Furthermore, EBV can also repress FOXO1 mRNA levels, suggesting alternative mechanisms for the regulation of FOXO1.

A correlation was observed between EBV status and activity of FOXO target genes, *bcl-6* and *cyclin D2,* which are essential for B cell proliferation and development. The levels of bcl-6, which is a direct target gene for activation by FOXO (Tang *et al*, 2002), correlated with FOXO1 expression. Bcl-6 is a transcriptional repressor of both lymphocyte differentiation and apoptosis and is the most frequently targeted proto-oncogene in non-Hodgkin's lymphomas (Ye, 2000). It was demonstrated that LMP1 is sufficient for this pathway as inducible expression of LMP1 and transfection of a plasmid encoding LMP1 was enough to repress both FOXO1 and its target gene, bcl-6. An inverse correlation between LMP1 and bcl-6 has previously been described at the transcriptional and protein levels in the B-cells of transgenic mice expressing LMP1 or a chimeric LMP1CD40 molecule (Panagopoulos *et al*, 2004). Other studies have also observed the repressive effect of CD40 activation or EBV genome expression on bcl-6 expression in B cells and dendritic cells (Allman *et al*, 1996, Carbone *et al*, 1998, Moschella *et al*, 2001). The function of bcl-6 repression by LMP1 is believed to be germinal centre suppression, allowing exit of EBV infected cells from the germinal centre. On the basis of this data, FOXO1 can be proposed as a molecular intermediate by which LMP1 can regulate bcl-6.

The role of LMP2A in the regulation of FOXO1 and bcl-6 is more complex. Activation of PI3K through LMP2A has previously been shown to induce phosphorylation of FOXO1 in epithelial cells (Morrison *et al*, 2003) which subsequently targets FOXO1 for degradation. However, the effects of LMP2A on the total protein levels of both FOXO1 and bcl-6 had not been previously defined. A clear downregulation of FOXO1 protein expression was observed in this study with LMP2A transient transfection but this did not translate to a corresponding dose responsive decrease in bcl-6 expression. However, induction of LMP2A in stable transfectants did result in a clear repression of bcl-6 protein expression. This may be due to differences in the time or levels of expression. In the system with stable expression, more LMP2A is expressed and for a longer period of time than the 20 hours studied in the transient transfections.

In contrast to bcl-6, an inverse correlation was observed between Cyclin D2 protein expression and FOXO1. It has previously been shown that PI3K regulates the cyclin D2 protein (Brennan *et al*, 2002) and the *cyclin D2* promoter (White *et al*, 2006) so this may in part be due to FOXO1. It is also possible that the repressive effect of FOXO transcription factors on Cyclin D2 transcription may be indirect (Schmidt *et al*, 2002) possibly through bcl-6, via an interaction with the STAT5 transcription factor (Fernandez de Mattos *et al*, 2004). The *cyclin D2* gene has a complex promoter and is subject to regulation by a diverse range of cellular stimuli (Martino *et al*, 2001, White *et al*, 2006).

A difference was observed in the kinetics of FOXO1 regulation by the PI3K pathway when comparing EBV negative BL cells and LCLs. In IARC-171 cells, 24 hour incubation with LY294002 was required to detect an increase in nuclear FOXO1 but one hour was sufficient for an increase of a similar level in the nuclei of the EBV negative BL41 cells. This suggests extra mechanisms for the repression of FOXO1 in EBV immortalized cells, an observation supported by the detection of lower levels of FOXO1 mRNA in these cells. A difference in the contribution of the PI3K pathway towards survival of these two cell types is evident when analyzing cell survival post treatment with the

PI3K inhibitor (Brennan *et al*, 2002). EBV negative BL lines rapidly undergo apoptosis but in contrast, LCL lines do not die, but are growth arrested. The difference in sensitivity to LY294002 is also seen for other agents and may be due in part to the delay in induction of FOXO1 as well as the other molecules such as NFκB that are increased in EBV immortalized cells.

2D-electrophoretic analysis of DNA bound FOXO1 shows that post-translational modification patterns are distinct in EBV positive B cells (BL41+B95.8) when compared with EBV negative B cells (BL41) suggesting a further level of control of FOXO1. It is possible that the multiple isoforms of FOXO1 detected bound to DNA in EBV positive cells are differentially phosphorylated forms but this may be unlikely as phosphorylation targets FOXO1 molecules towards nuclear exclusion, and so would not be easily detected by this assay. Acetylation of nuclear FOXO proteins has also been reported to affect the transcriptional programmes controlled by FOXO proteins due to interference with the balance of co-activator and co-repressor recruitment (Fukuoka *et al*, 2003, Van der Horst *et al*, 2004, Perrot & Rechler, 2005). Again, a role for the PI3K pathway is proposed as acetylation was reported to increase the sensitivity of FOXO1 to phosphorylation, contributing towards degradation (Matsuzaki *et al*, 2005). A recent study has demonstrated the role of ubiquitinylation by the Skp2/Cul-1/F-box protein ubiquitin complex in the targeting of FOXO1 molecules for degradation following PKB activation (Huang *et al*, 2005). However, the pattern of spots observed did not show a significant increase in the molecular weight of the protein (> 1000 Da) which would be suggested following ubiquitinylation (Jensen, 2006). Thus, while pathways activated by EBV lead to differential modifications of FOXO1, as detected in BL41+B95.8, that may repress transcriptional activity, their identity is currently unknown and requires further investigation.

Integrating the data in this chapter provides evidence that EBV regulates FOXO1 expression by three distinct mechanisms: the prototypic repression of FOXO1 by PI3K; alternative post-translational modifications observed in BL41 cells infected by EBV, BL41+B95.8, and a repression of FOXO1 expression at the mRNA level observed in LCLs. These diverse mechanisms

ensure that FOXO1 expression and activity is inhibited. The existence of these distinct mechanisms suggests that repressing FOXO1 activity is an important part of EBV infection of B-cells.

In summary, these data identify FOXO1 as an EBV regulated transcription factor and adds to a body of work demonstrating the suppression of pro-apoptotic pathways by EBV. Furthermore, FOXO1 has been identified as one of the few proteins regulated by both LMP1 and LMP2A. LMP1 and LMP2A have been shown to activate a diverse range of signalling pathways contributing towards cell survival. These include the activation of tyrosine kinases, mitogen-activated protein kinases (MAPKs), JNK, p38, the transcription factor, NFκB, as well as the PI3K pathway. This chapter describes another nuclear target by which EBV latent membrane proteins work to shift the balance of anti- and pro- apoptotic pathways towards survival. Interplay between EBV and FOXO1 is likely to contribute towards the characteristic apoptotic resistance of immortalized B cells in the context of EBV associated malignancies.

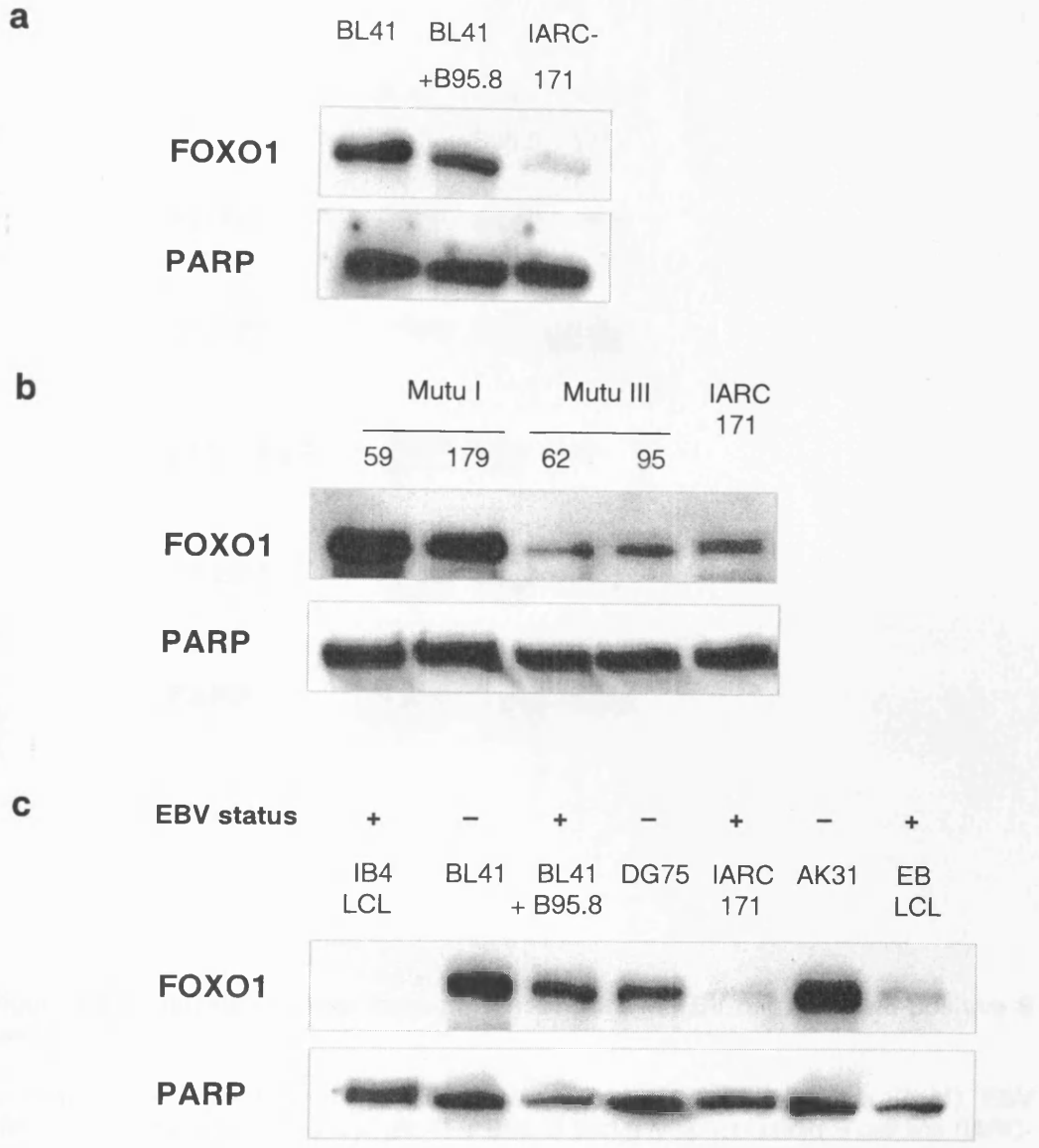


Figure 5.1 EBV infection downregulates FOXO1 expression

Nuclear protein extracts were generated from a) Burkitt's Lymphoma line (BL41), EBV infected Burkitt's Lymphoma line (BL41 + B95.8) and a lymphoblastoid B cell line (IARC-171) b) EBV positive Burkitt's line expressing Latency I genes (Mutu I, clones 59 and 179), EBV positive Burkitt's line expressing Latency III genes (Mutu III,clones 62 and 95), and two lymphablastoid cell lines (IARC-171, EB-LCL) c) EBV negative BL lines (BL41, DG75, AK31), EBV positive BL line (BL41 + B95.8) and lymphoblastoid cell lines (IB4-LCL, IARC-171, EB-LCL). Proteins were resolved by SDS-PAGE and FOXO1 protein levels were determined by immunoblotting with a specific FoxO1 antibody. Poly-ADP ribose polymerase (PARP) was used as a loading control.

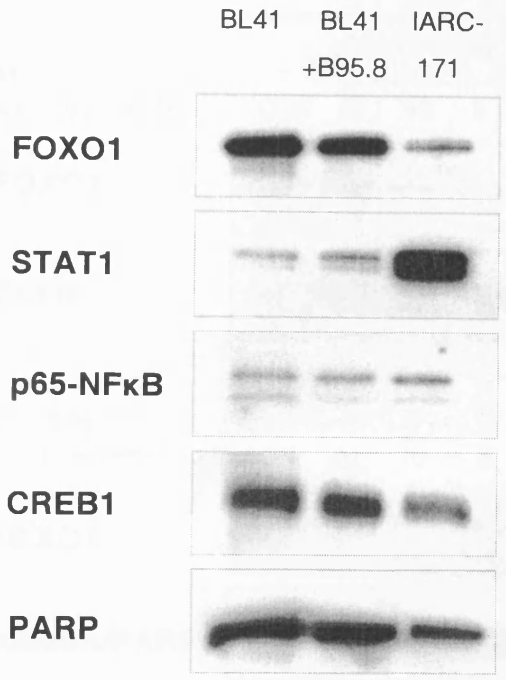


Figure 5.2 Analysis of other transcription factors in EBV negative and positive B cell lines

Nuclear protein extracts were generated from a Burkitt's Lymphoma line (BL41), EBV infected Burkitt's Lymphoma line (BL41 + B95.8) and a lymphoblastoid B cell line (IARC-171) Proteins were resolved by SDS-PAGE. Levels of expression of transcription factors were determined by immunoblotting with antibodies specific for FOXO1, STAT1, p65-NFκB and CREB1. Poly-ADP ribose polymerase (PARP) was used as a loading control.

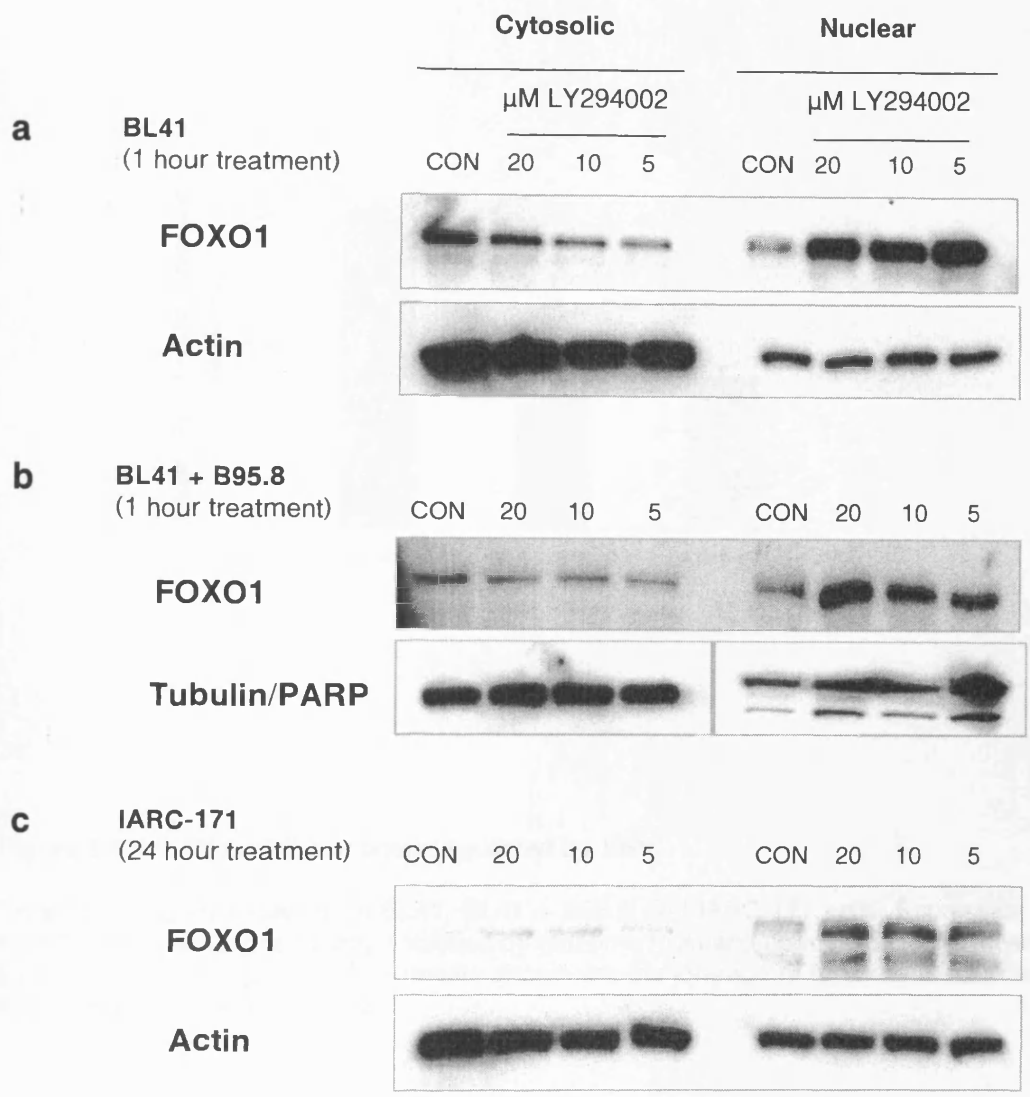


Figure 5.3 PI3K inhibition can increase nuclear FOXO1 in all cell types but with different kinetics.

Nuclear and cytosolic protein extracts were generated from a) BL41 and b) BL41 + B95.8 and c) IARC-171 B cell lines. Proteins were resolved by SDS-PAGE and immunoblotted with an antibody specific to FOXO1. Cells were either left untreated or were treated with varying concentrations of LY294002 (20μM, 10μM, 5μM) for either one hour (BL41, BL41 + B95.8) or 24 hours (IARC-171). Antibodies specific for actin, tubulin and PARP were used as a loading controls. FOXO1 is represented by the higher molecular weight band in part c.

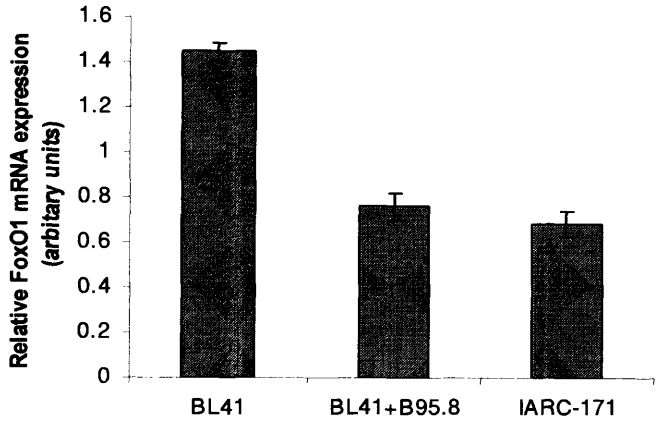


Figure 5.4 FOXO1 mRNA is downregulated by EBV

Total RNA was extracted from BL41, BL41 + B95.8 and IARC-171 cells. Expression of FOXO1 RNA was subsequently analyzed by real-time PCR and normalised to the level of L19, a house keeping gene. The results shown are the average of triplicate results from three independent experiments.

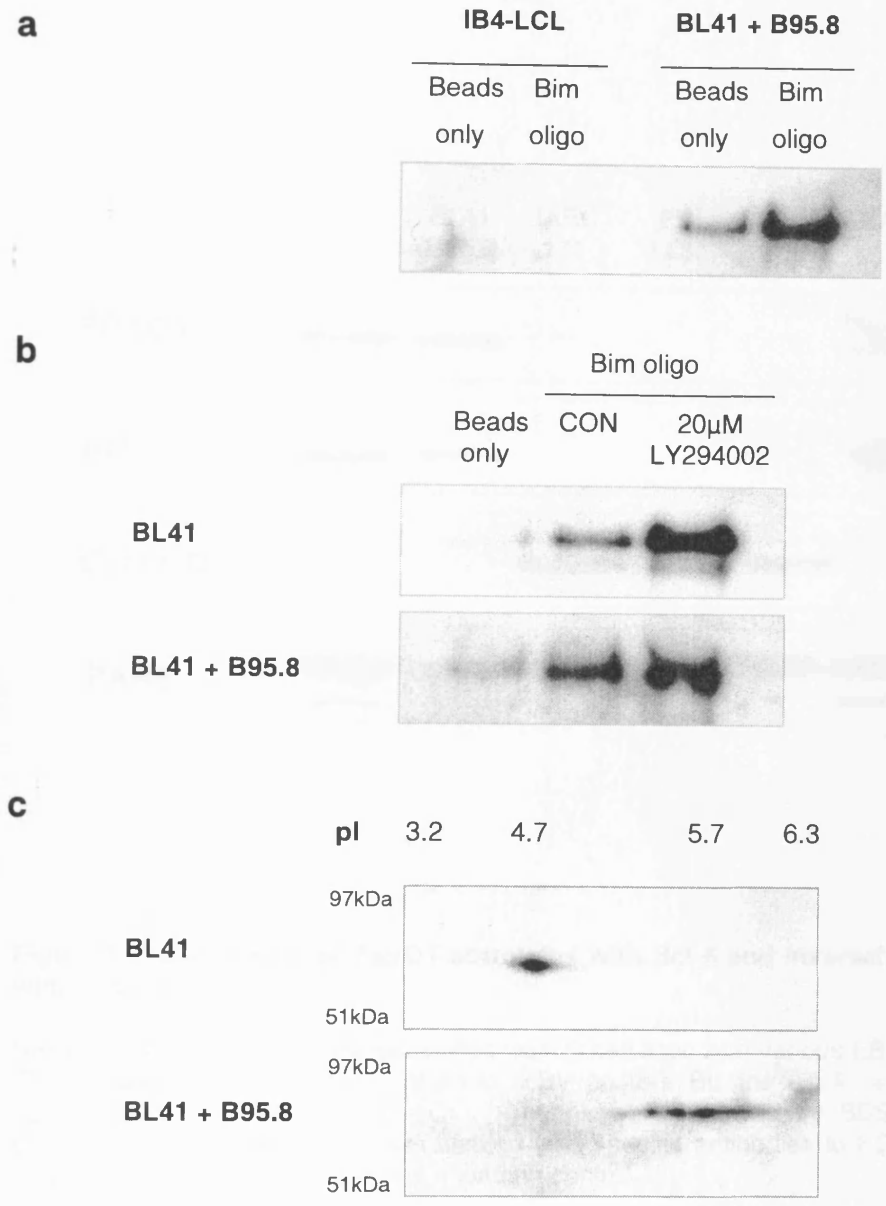


Figure 5.5 FOXO1 can bind DNA and is present in multiple forms

Nuclear protein extracts were generated from IB4-LCL, BL41 and BL41 + B95.8 cells. Nuclear proteins were subsequently DNA-Affinity precipitated using streptavidin coated agarose beads with or without biotinylated bim oligonucleotides. Bound proteins were eluted and resolved by SDS-PAGE and immunoblotted using the anti-FOXO1 antibody a) IB4-LCL and BL41 + B95.8 cells b) BL41 and BL41 + B95.8 cells untreated or treated with LY294002 (20μM, 1 hour) c) DNA-Affinity precipitated proteins using the bim oligonucleotide from BL41 and BL41 + B95.8 cells treated with LY294002 (20μM, one hour) were analysed by 2D-electrophoresis. Eluted proteins were isoelectrically focused using a pH 3-10 nonlinear immobilised pH gradient and separated in the second dimension by SDS-PAGE. FOXO1 isoforms were detected by immunoblotting with the anti-FOXO1 antibody.

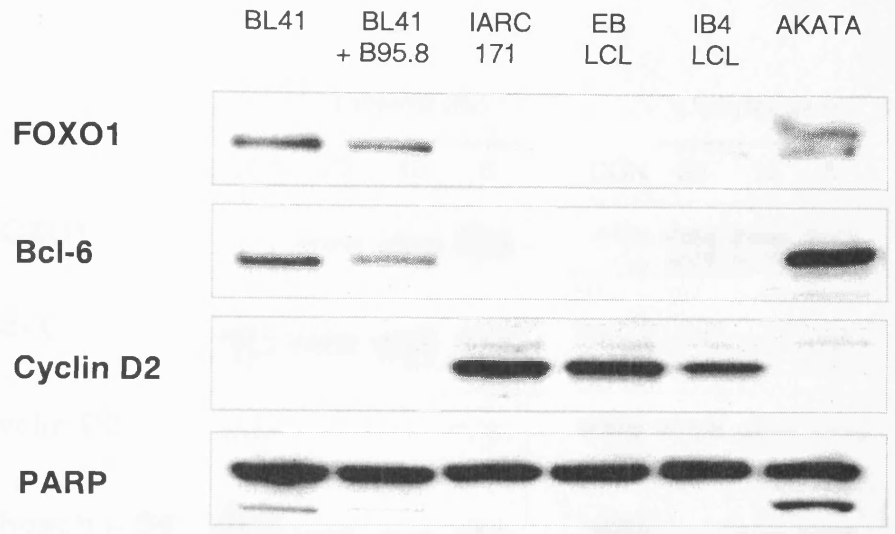


Figure 5.6 Expression of FoxO1 correlates with Bcl-6 and inversely correlates with Cyclin D2

Nuclear protein extracts were generated from B cell lines with various EBV statuses – EBV negative BL lines (BL41, AKATA), EBV positive BL line (BL41 + B95.8) and LCL's (IARC-171, EB-LCL, IB4-LCL). Proteins were resolved by SDS-PAGE and subsequently analysed by immunoblotting with specific antibodies to FOXO1, Bcl-6, and Cyclin D2. PARP was used as a loading control.

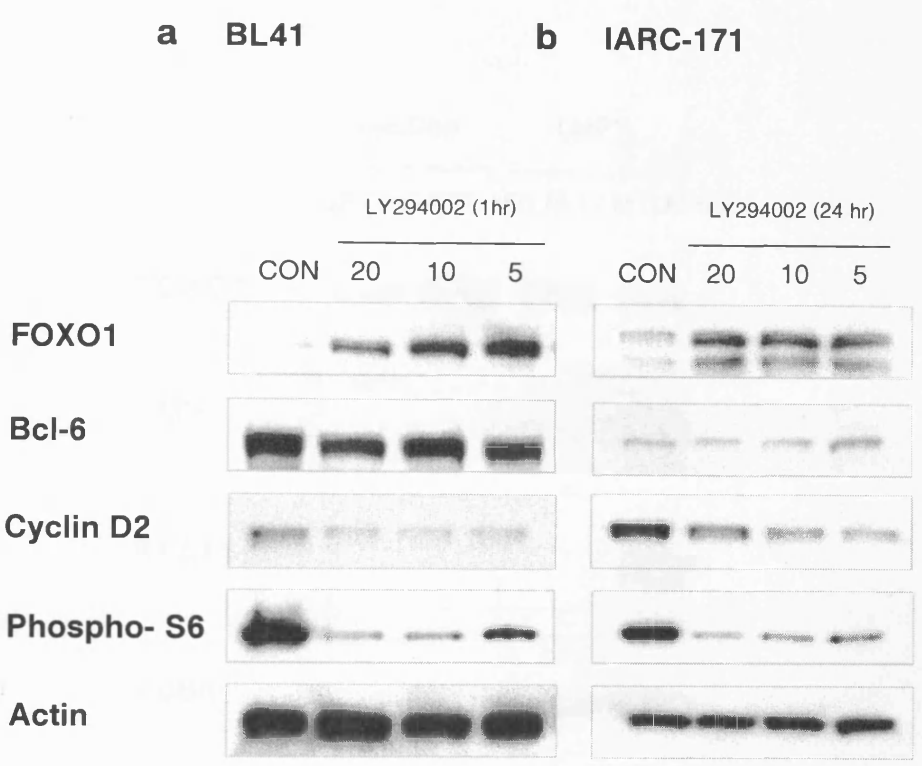


Figure 5.7 PI3K activity can regulate Cyclin D2 but not Bcl-6 levels in EBV negative and EBV positive B cells

Nuclear protein extracts were generated from (a) BL41 and (b) IARC-171 cell lines. Cells were either left untreated or were treated with varying concentrations of LY294002 (20μM, 10μM, 5μM) for either one hour (BL41) or 24 hours (IARC-171). Proteins were resolved by SDS-PAGE and immunoblotted with an antibodies specific to FOXO1, Bcl-6, Cyclin D2 and phospho-S6RP. Actin levels was used as a loading control.

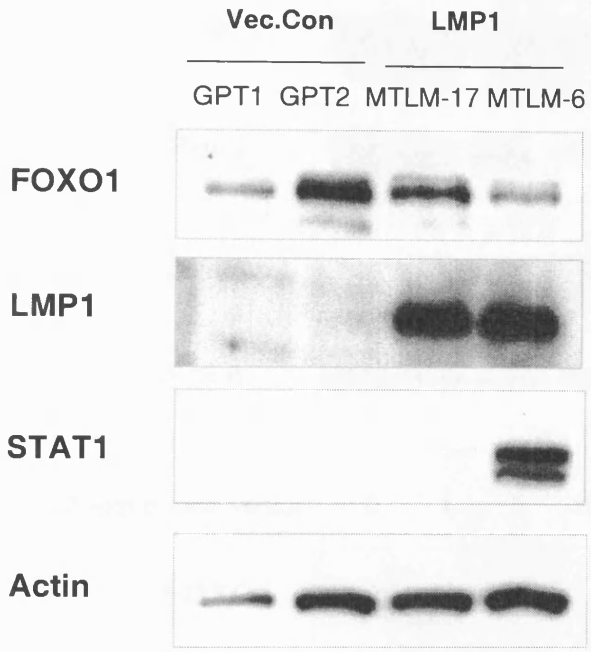


Figure 5.8 Role for LMP1 in the downregulation of FOXO1

Nuclear protein extracts were generated from EBV negative BJAB human B cell lymphoma cell lines stably transfected with LMP1 (MTLM-17 and MTLM-6) and vector control tranfectants (GPT1 and GPT2). Proteins were analysed by SDS-PAGE and immunoblotting with antibodies specific for FOXO1, LMP1 and STAT1. Actin levels were used as a loading control.

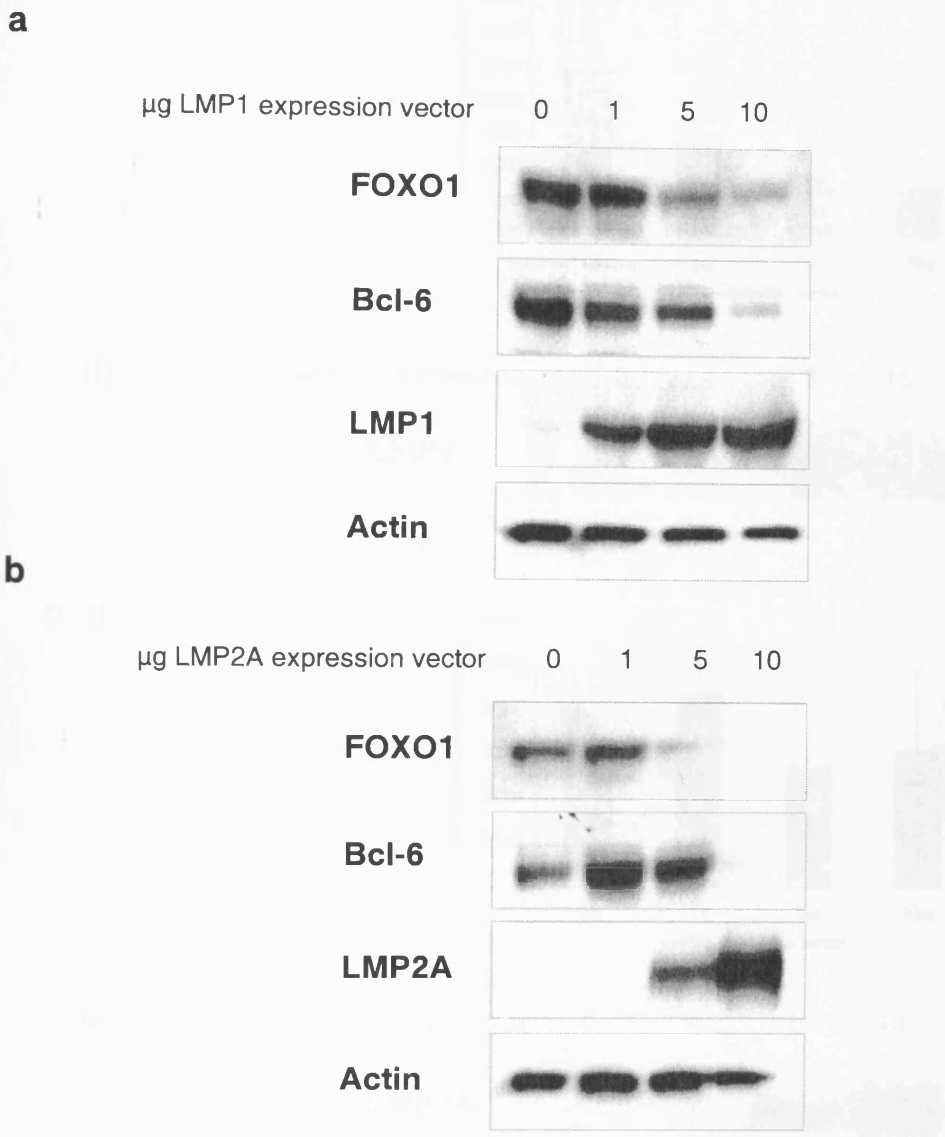


Figure 5.9 LMP1 and LMP2A downregulate FOXO1 and Bcl-6 protein expression

DG75 cells were transiently transfected with varying amounts of a) LMP1 and b) LMP2A expression vectors. Cells were harvested, and lysates generated were analysed by SDS-PAGE and western blotting. Protein levels of FOXO1 and Bcl-6 were analysed using specific antibodies. LMP1 and LMP2A protein levels were also checked using specific antibodies. Actin was used as a loading control.

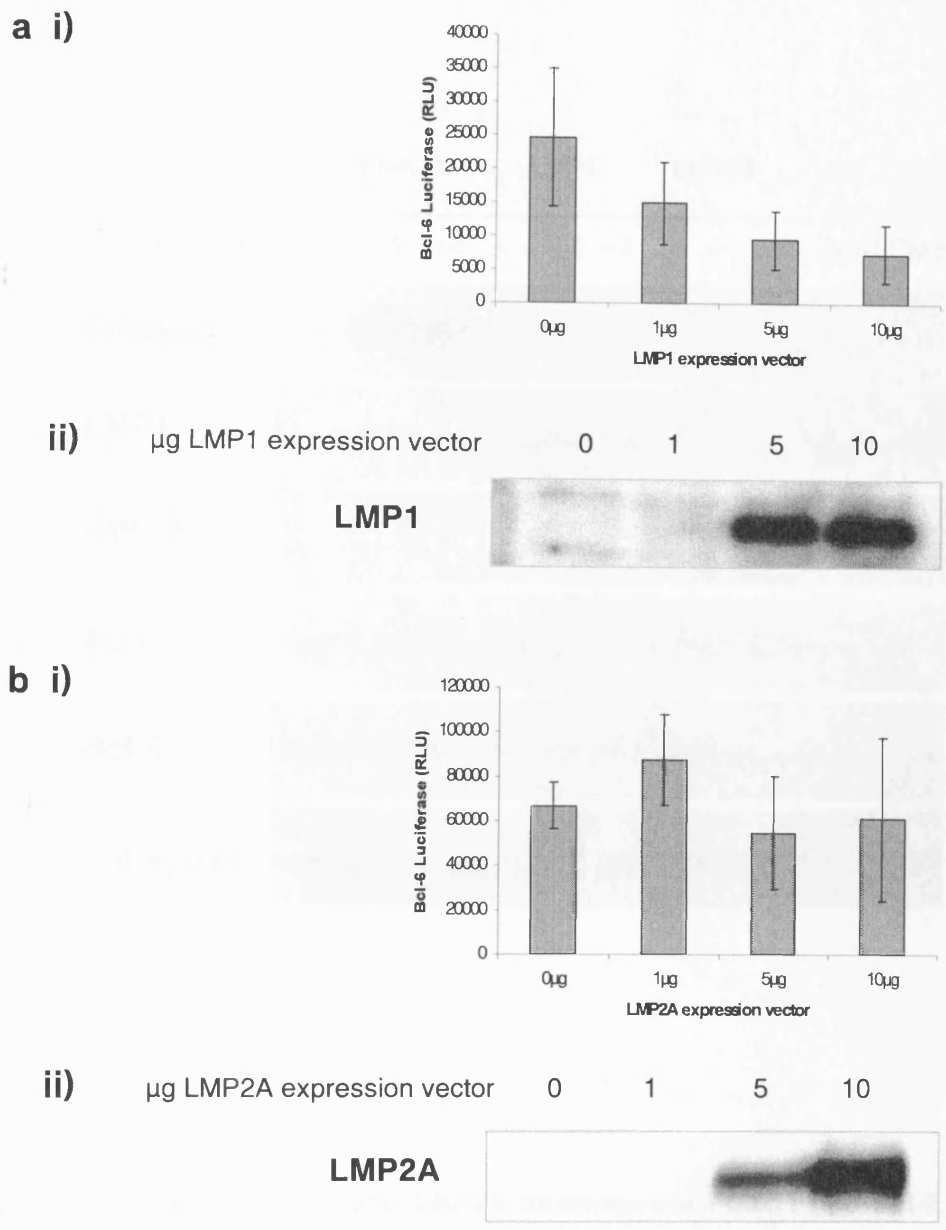


Figure 5.10 LMP1 downregulates Bcl-6 at the transcriptional level

EBV negative DG75 cells were transfected with varying amounts of a) LMP1 and b) LMP2A expression vectors, along with a fixed amount of a Bcl-6 reporter construct containing a FoxO consensus binding sequence upstream of a luciferase gene. The *Bcl-6* promoter sequence was mutated to lack the consensus Bcl-6 binding site in the *Bcl-6* gene promoter that is thought to act as a negative autoregulatory loop. i) Luciferase was measured after transfection ii) Protein expression levels of a) LMP1 and b) LMP2A in transfected cells were analysed by immunoblotting with specific antibodies.

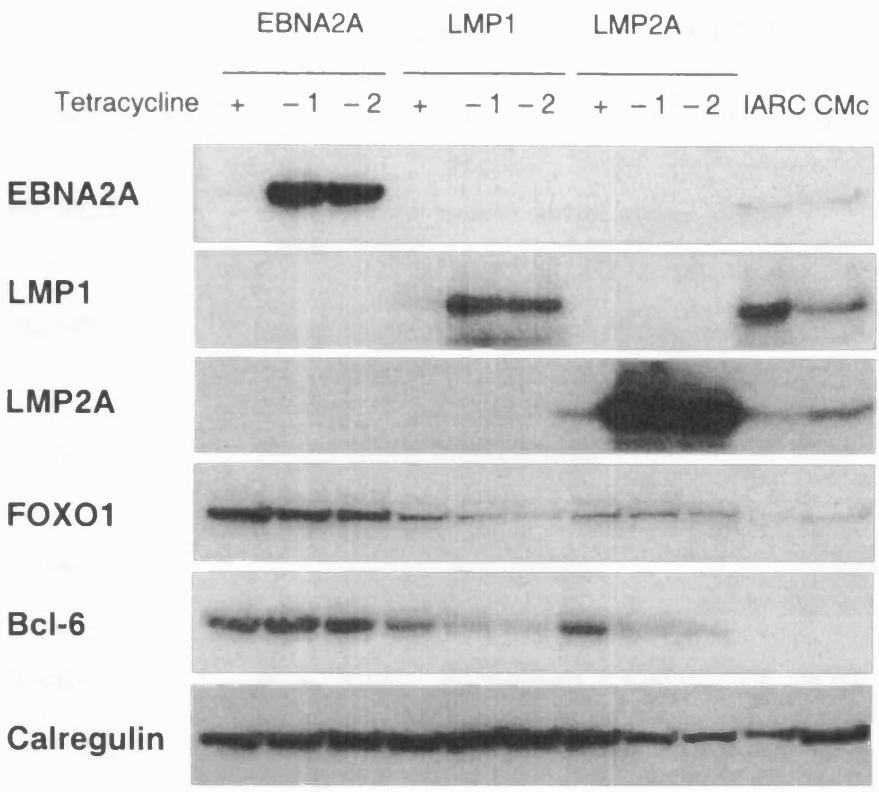


Figure 5.11 Inducible LMP1 and LMP2A downregulate FOXO1 and Bcl-6 protein expression.

Stable DG75 transfectants containing inducible EBNA2A, LMP1 or LMP2A genes were maintained under drug selection and in 1μg/ml tetracycline until required for an experiment. When required, cells were washed five times in RPMI 1640 medium and were re-cultured without drug selection and in the presence or absence of 1μg/ml tetracycline for a period of either 24 (–1) or 48 hours (–2). Protein levels of FOXO1 and Bcl-6 were subsequently analysed by immunoblotting using specific antibodies. Protein expression levels were also analysed in IARC-171 and CMc Lymphoblastoid cell lines. EBNA2A, LMP1 and LMP2A protein levels were also checked using specific antibodies. Calregulin was used as a loading control.

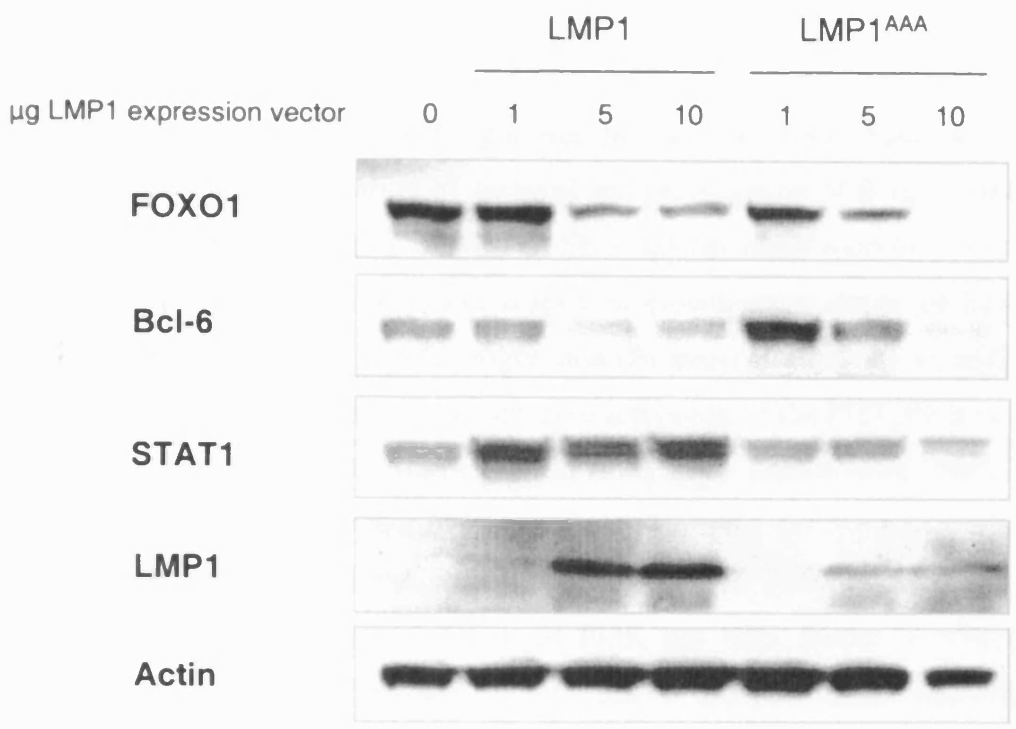


Figure 5.12 FOXO1 regulation by LMP1 is not mediated through the CTAR1

DG75 cells were transiently transfected with varying amounts of wild type or CTAR1 inactivated LMP1 mutants ($LMP1^{AAA}$). Cells were harvested, and lysates generated were analysed by SDS-PAGE and western blotting. Protein levels of FOXO1, Bcl-6, and STAT1 were analysed using specific antibodies. LMP1 protein levels were also checked using specific antibodies. Actin was used as a loading control.

CHAPTER 6

FINAL DISCUSSION

The activation of the PI3K/PKB signalling pathway has been shown to play an important role in the regulation of survival and proliferation of B-cells (Brennan, 2001). The inhibition of PI3K in BL cell lines induces rapid apoptosis. The same treatment of EBV immortalized B cells leads to growth arrest due to the inhibition of proteins required for cell cycle progression (Brennan *et al*, 2002). In addition, a number of studies have reported constitutive activation of the PI3K/PKB pathway in EBV associated malignancies (Morrison *et al*, 2004, Dutton *et al*, 2005, Nagel *et al*, 2005). Activation of the PI3K/PKB pathway may contribute significantly towards cell survival and the morphological changes observed during B cell transformation by EBV, as inhibition of PI3K has been shown to reverse the transformed phenotype (Dawson *et al*, 2003). Despite these observations, relatively little is known about the downstream targets of PI3K activation in lymphocytes that mediate these effects, particularly within EBV immortalized lymphocytes. The purpose of this study was to investigate downstream targets of the PI3K pathway in transformed lymphocytes in order to further our understanding of the contribution of PI3K activation towards lymphocyte proliferation and survival, particularly within the context of EBV-associated B-cell lymphomas.

The present thesis has identified two important targets of PI3K signalling in EBV immortalized B cells: S6, a component of the mammalian ribosome (Chapter 3), and the pro-apoptotic transcription factor, FOXO1 (Chapter 5). In addition, S6 was found to be a major target for PI3K signalling in other transformed lymphocyte cell lines, including EBV negative BL cells and IL-2 dependent leukaemic T cells. Both of these proteins have been shown to be PI3K regulated

targets in other systems (Biggs *et al*, 1999, Brunet *et al*, 1999, Kops & Burgering 1999, Rena *et al*, 1999, Tang *et al*, 1999, Takaishi *et al*, 1999, Kane *et al*, 2002, Zhang *et al*, 2002b, Ly *et al*, 2003, Wedel *et al*, 2004).

The constitutive phosphorylation of S6, and the dramatic inhibition of S6 phosphorylation in response to PI3K and mTOR inhibition in all lymphocyte cell lines tested suggests that S6 is an important target for activation of these pathways in lymphocytes. Since S6 activation is associated with cellular growth and translational control (Volaveric *et al*, 2000, Ruvinsky *et al*, 2005), the constitutive phosphorylation of this protein may contribute towards the oncogenic capacity of deregulated PI3K activation in lymphocytes, and may therefore be a contributory factor towards the development of lymphoid malignancies such as EBV associated lymphomas and leukaemia.

Inhibitors targeting pathways leading to S6 phosphorylation are being evaluated for the treatment of EBV-associated malignancies. A study by Majewski *et al* investigated the effects of a macrolide immunosuppressant, RAD001, a rapamycin derivative, on the growth of LCLs and PTLD-like EBV positive B cells xenotransplanted into SCID mice (Majewsky *et al*, 2000). This study demonstrated that RAD001 inhibited the growth of LCLs, which reflects the findings of other studies investigating the effects of mTOR inhibition on lymphocyte growth and proliferation (Breslin *et al*, 2005, Fumarola *et al*, 2005). In the *in vivo* model, RAD001 significantly delayed the growth or induced regression of the established tumours (Majewsky *et al*, 2000). The potent inhibition of S6 phosphorylation in response to mTOR inhibiton as demonstrated by this study and others (Ly *et al*, 2003, Kane *et al*, 2002, Wedel *et al*, 2004, Breslin *et al*, 2005), demonstrates that S6 is a major target for activation by mTOR, and suggests that this may be an important factor in the immunosupressive properties of rapamycin and its analogues. Indeed, the phosphorylation status of S6 is increasingly being evaluated as a potential biomarker for the activation status of the PI3K and mTOR pathways, and for the

monitoring of response to treatment with inhibitors of these pathways in several disease contexts, including acute myeloid leukaemia, prostate cancer, breast cancer, and antibody-mediated rejection in heart allografts (Thomas *et al*, 2004, Thomas, 2006, Lepin *et al*, 2006, Chow *et al*, 2006). These studies demonstrate the potential of analyzing the phosphorylation status of S6 in evaluating aberrant signal transduction contributing towards disease and malignancy.

The FOXO transcription factors have been shown to co-ordinate cell cycle progression and cell survival by the activation of antiproliferative and pro-apoptotic genes in many systems. The target genes of FOXO proteins that mediate these effects include components of the cell cycle machinery such as $p27^{Kip1}$ and cyclin G2, as well as pro-apoptotic genes such as Fas Ligand (FasL), bcl-6 and the bcl-2 family member bim (Brunet *et al*, 1999, Medema *et al*, 2000, Dijkers *et al*, 2002, Tang *et al*, 2002, Furukawa-Hibi *et al*, 2002, Essafi *et al*, 2005). Studies in mammalian cells have shown that the overexpression of FOXO1, FOXO3 and FOXO4 causes a strong inhibition of cell proliferation (Collado *et al*, 2000, Medema *et al*, 2000, Nakamura *et al*, 2000, Kops *et al*, 2002). In the context of the immune system, the overexpression of FOXO proteins promotes cell cycle arrest at the G_1/S boundary in B cells (Yusuf *et al*, 2004) and T cells (Fabre *et al*, 2005). Furthermore, the optimal proliferation of primary B cells requires the inactivation of FOXO transcription factors in a PI3K dependent manner (Yusuf *et al*, 2004). A recent study by Uddin *et al*, has implicated a role for FOXO inactivation in the survival of diffuse large B-cell lymphomas (DLBCL) (Uddin *et al*, 2006). The inhibition of PI3K in primary DLBCL cells and DLBCL cell lines and induced apoptosis, and correlated with the de-phosphorylation FOXO transcription factors. The FOXO proteins have therefore emerged as important targets of the PI3K pathway in lymphocytes that regulate cell survival and cell proliferation.

With regards to the present thesis, this was the first study to demonstrate a definitive link between EBV infection and FOXO expression, and formed the

basis of a manuscript accepted for publication (Shore *et al*, 2006, *manuscript in press*). This work demonstrated that a latency III pattern of EBV viral gene expression can regulate the expression of FOXO1. An investigation into the mechanism by which this EBV latency pattern downregulates FOXO1 expression identified a role for two EBV latent genes, LMP1 and LMP2A.

With respect to LMP1, it is clear that this protein utilizes several mechanisms to contribute towards the survival of EBV infected cells. The most well characterized role of LMP1, in this regard, is its ability to activate the NFκB transcription factor. The current view is that EBV utilizes the activation of the NFκB transcription factor as a primary mechanism to promote the survival and transformation of infected cells, which is reflected by the upregulation of several NFκB target genes in EBV immortalized B cells. For example, the upregulation of anti-apoptotic genes such as A20 and cIAP2 have been demonstrated to occur via LMP1 activation of NFκB (Laherty *et al*, 1992, Hong *et al*, 2000). The STAT transcription factors, which promote oncogeneis by inhibiting apoptosis and promoting cell cycle progression (Bowman *et al*, 2000) are also constitutively activated in LMP1 expressing cells, by a mechanism that involves NFκB (Richarson *et al*, 2003, Zhang *et al*, 2004, Najjar *et al*, 2005). This role for NFκB is highlighted by the fact that suppression of EBV-mediated NFkB activation leads to spontaneous apoptosis in EBV-transformed LCLs (Cahir-McFarland *et al*, 2000). Furthermore, inhibition of the NFκB pathway diminishes the ability of LMP1 to induce cellular transformation and tumourigenesis in rat fibroblasts (He *et al*, 2000). However, it is also clear that LMP1 can utilize NFκB independent pathways to promote cell survival, as demonstrated by a study showing that LMP1 can induce the upregulation of another anti-apoptotic protein, bcl-2, to confer protection from apoptosis (Henderson *et al*, 1991, Rowe *et al*, 1994). The downregulation of FOXO1 by LMP1 provides a further insight into NFκB independent mechanisms employed by this protein to promote the survival of virus infected cells.

The role of LMP2A in providing survival signals to EBV infected cells has mainly been attributed to its ability to mimic a constitutively activated BCR (Burkhardt *et al*, 1992, Miller *et al*, 1995). In transgenic mice, LMP2A provides developmental and survival signals to BCR-deficient B cells, allowing their survival in the periphery (Caldwell *et al*, 1998), and has a critical role in maintaining viral latency (Miller *et al*, 1994). The activation of Ras and the PI3K/PKB pathway have a central role in this process, as B cells from LMP2A transgenic mice are sensitive to apoptosis in the presence of specific inhibitors of Ras, PI3K and PKB (Portis & Longnecker, 2004a). This induction of apoptosis correlated with a decrease in the expression of an anti-apoptotic protein, Bcl-X_L, implicating a role for this protein in apoptosis inhibition mediated by LMP2A. DNA microarrays of primary B cells from LMP2A-transgenic mice, LMP2A-expressing human B-cell lines and LCLs have indicated that LMP2A has multiple effects on global gene expression. These effects include an increase in the expression of genes associated with cell-cycle induction and inhibition of apoptosis, a decrease in the expression of B-cell specific factors and genes associated with immunity, and also alterations in the expression of genes involved in DNA and RNA metabolism (Portis & Longnecker, 2003, 2004b, Portis *et al*, 2003). Despite these observations, little is known about the effects of LMP2A on BCR-induced apoptosis in B cells. The demonstration in this study that LMP2A expression can reduce protein levels of the FOXO1 pro-apoptotic transcription factor contributes towards our understanding of these effects, and suggests another potential mechanism by which PI3K activation by LMP2A contributes towards the survival of BCR-deficient B cells.

Together, the findings that LMP1 and LMP2A are capable of suppressing the expression of the pro-apoptotic FOXO1 transcription point towards a mechanism contributing towards the characteristic apoptotic resistance of EBV immortalized B cells, and improves our understanding of how EBV may influence malignancy. In this respect, the analysis of the effects of FOXO1 on LCL proliferation would provide a further insight into the role of FOXO1 in EBV-mediated cell

transformation, and would be the next step in the project. The key experiment may be to introduce a tetracycline-regulated FOXO1 transgene into an LCL and analyze the effects of FOXO1 on LCL proliferation. A finding of growth suppression would provide further evidence to support a critical role for LMP1 and LMP2A-mediated down-regulation of FOXO1 in cell transformation. Further analysis of the effects of EBV on FOXO1 target genes, such as bim and Fas-L would also enhance our understanding of the consequences of FOXO1 downregulation by EBV. In addition, it would be of interest to analyse the expression profiles of other members of the FOXO transcription factor family, such as FOXO3 and FOXO4, in EBV negative and positive cells to further evaluate the ability of EBV to suppress pro-apoptotic pathways. During the course of this project, attempts were made to address this question by analysing the expression levels of FOXO3 and FOXO4 in a range of cell lines by immunoblotting. These attempts were unsuccessful as the antibodies employed failed to detect protein bands that correlated with the known molecular weights of FOXO3 and FOXO4. The availability of better antibodies should resolve this issue.

The high levels of FOXO expression in BL cells and the repression of FOXO expression in LCLs raises another interesting question with regards to the levels of FOXO1 expression in other EBV-associated lymphoid malignancies such as Hodgkin's disease (HD). As mentioned in section 1.1.5.2, EBV positive HD cells display a latency II pattern of EBV gene expression in which EBERs, EBNA1, LMP1 and LMP2A are expressed. LMP1 is expressed at high levels in the large atypical tumour Hodgkin and Reed-Sternberg (HRS) cells that characterize HD (Pallesen *et al*, 1991, Murray *et al*, 1992). Furthermore, the PI3K/PKB pathway has been demonstrated to be constitutively activated in HRS cell lines and in most primary HRS cells, and contributes towards their survival (Morrison *et al*, 2004, Dutton *et al*, 2005, Nagel *et al*, 2005, Georgakis *et al*, 2006). With these factors in consideration, it would be reasonable to hypothesize that FOXO1 levels are repressed in Hodgkin's lymphoma cells. This hypothesis has not been tested by

this study or others, and would be an interesting question to address in order to further our understanding of the role of FOXO transcription factors, and the contribution of EBV induced activation of the PI3K pathway towards the pathogenesis of HD.

The prominent role of the PI3K/PKB pathway in regulating cellular survival and proliferation, particularly within the context of human malignancies, suggest that components of this pathway may be attractive targets for cancer therapy, and is an area of ongoing cancer research and investigation by pharmaceutical companies FOXO1 is among many of the PI3K targets that are under evaluation in pre-clinical models for their potential as targets for therapeutic benefit (Kau *et al*, 2003, Schroeder *et al*, 2005). Since the tumour-suppressor activities of FOXO molecules require FOXO nuclear localization, the development of chemical inhibitors of nuclear export could prove beneficial. A screen of small molecules for their ability to relocalize FOXO1 to the nucleus in PTEN deficient cells resulted in the identification of two general classes (Kau *et al*, 2003). These were general inhibitors of the nuclear export protein CRM1, and specific inhibitors of the PI3K/PKB-dependent export pathway. A subsequent screen of extracts derived from natural marine products resulted in the identification of a bromotyrosine derivative, psammaplysene A, as a compound that can induce the relocalization of FOXO1 to the nucleus in PTEN deficient cells (Schroder *et al*, 2005). The value of these compounds as therapeutic approaches requires further investigation. In the context of EBV-associated malignancies, such strategies are unlikely to prove beneficial due to the very low levels of FOXO1 detected in the cytosol of EBV infected cells (Figure 5.3). Moreover, the involvement multiple pathways in mediating the survival of virus-infected cells suggests that more-than one, or indeed several, of the pathways activated by the virus may need to be targeted to observe a repressive effect on EBV-induced proliferation.

In terms of targeting PI3K itself, the therapeutic use of PI3K inhibitors is controversial. This is due to the inability of many of the developed compounds to

discriminate between the different isoforms of PI3K. As many classes and isoforms of PI3K control distinct cellular processes, this is a concern. However, isoform selective PI3K inhibitors have been described (Sadhu *et al*, 2003a, 2003b, Finan & Thomas, 2004) and may make the therapeutic use of PI3K inhibitors feasible in the future. Isoform-specific inhibitors of PKB have been proposed to provide effective anti-tumour activity with fewer toxic side effects. Indeed, a number of such compounds are being developed (Defeo-Jones *et al*, 2005, Lindsley *et al*, 2005, Barnett *et al*, 2005). The identification of a small molecule inhibitor of PKB that inhibits the growth of PKB expressing tumours *in vivo*, whilst not affecting PDK-1 or PI3K activity (Yang *et al*, 2004) has further highlighted the benefit of targeting PKB as a therapeutic approach in cancer therapy.

In some respects, it was disappointing that during the course of this study only two targets of PI3K were identified in lymphocytes, and that no novel PI3K targets could be identified. This is especially the case when considering that two distinct approaches were used, namely the use of a phospho-specific antibody directed against the consensus phosphorylation motif of PKB (Chapter 3), and the application of 2DE for the study of PI3K signalling on a wider scale (Chapter 4). As discussed previously, phospho-PKB substrate antibodies have been successfully used by other studies for the identification of novel targets of PKB (Manning et al, 2002, Kane *et al*, 2002, Kovacina *et al*, 2003, Astoul *et al*, 2003, Jiang *et al*, 2005). In many instances, 2DE has also proved to be a useful approach for screening molecular changes occurring in response to the selective activation or inhibition of signalling pathways. Previously uncharacterised targets of the MAPK pathway (Lewis *et al*, 2000, Ueda *et al*, 2004), Fas-dependent apoptosis (Gerner *et al*, 2000), TGF-β1 mediated signalling (Kanamoto *et al*, 2002), Rho-GTPases (Kabuyama *et al*, 2006) and growth factor stimulation of RTKs (Saridaki & Penayotou, 2005) have been identified using this approach.

With this in mind, it becomes apparent that the molecular changes that occur in the nuclei of EBV immortalized B cells in response to PI3K/PKB activation are relatively subtle and is likely to involve low abundance proteins that could not be detected using a 2DE minigel system. The studies described above which successfully identified previously uncharacterized targets of signalling pathways all utilized larger 2D gel formats that allow the larger amount of protein to be analyzed at increased resolution. This suggests that the use of larger gel formats, as opposed to the 2D minigel system, may have been beneficial in this study. Another factor that is important to consider is that EBV can activate multiple signalling pathways upon infection. The specific detection of PI3K regulated proteins in EBV immortalized cells with the use of a PI3K inhibitor can therefore be considered as a relatively simplified approach in what is a complicated setting. As discussed previously in section 4.8, it is possible that the use of more sensitive mass spectrometric based approaches would be more useful for screening the molecular changes that occur in the lymphocyte nucleus in response to PI3K inhibition.

Despite the limitations of 2DE for the detection of PI3K targets, this approach was successfully used to elucidate protein expression differences in cells that differed more dramatically in phenotype, that is EBV negative BL cells and EBV immortalised LCLs. In this regard, three proteins were detected that were differentially expressed. The identification and characterization of these proteins would be the next step in the project, and may increase our understanding of the molecular changes that occur in the B cell nucleus in response to EBV infection. The detection of distinct post-translational modification patterns of the FOXO1 transcription factor between BL41 and BL41 + B95.8 cells further suggests that mechanisms of protein regulation are distinct in EBV negative and EBV infected B cells. Multiple forms of the CREB1 and STAT1 transcription factors were also detected in LCLs. This suggests that EBV employs many strategies for the regulation of its transcription factor targets. These observations bring to light the complexity of the virus-host interaction, and emphasise the fact that still much is

unknown about the mechanisms employed by EBV to mediate the regulation of target proteins. Previous studies employing proteomic strategies have detected greater than 100 methyl-accepting (Huang *et al*, 2002) and 400 tyrosine-phosphorylated proteins (Caron *et al*, 2002) in LCLs. A more recent study by Yan *et al* demonstrated that LMP1 can increase the total phosphoprotein component of epithelial cells by 18% (Yan *et al*, 2006). These studies further support the idea that the alteration of post-translational modification patterns, particularly via phosphorylation, may be an important mechanism by which EBV can regulate its target proteins.

One interesting point that was not addressed by this project is the question of which molecules regulate the activation of PI3K in EBV immortalized cells. As mentioned in section 1.1 and 1.2, LMP1 and LMP2A as well as cytokines that are induced during EBV infection have all been shown to activate PI3K. The multiple mechanisms EBV utilize to activate this pathway demonstrate its importance for the virus. LMP2A has been shown to activate PI3K signalling in B cells (Swart *et al*, 2000) and epithelial cells (Scholle *et al*, 2000, Morrison *et al*, 2003), whereas the regulation of PI3K by LMP1 has only been demonstrated in epithelial (Dawson *et al*, 2003) and fibroblasts cells (Maniou *et al*, 2005). The ability of LMP1 to activate PI3K in B cells therefore remains to be defined. Interestingly, the present study demonstrated that both LMP1 and LMP2A were capable of repressing FOXO1, a target for PI3K activation, in B cells. This supports the hypothesis both of these molecules can activate PI3K in B cells, although this was not investigated or demonstrated by this study. Further investigations into the mechanisms employed by EBV to activate PI3K will contribute towards our understanding of the role of this pathway in the pathogenesis of EBV-associated malignancies.

In conclusion, this work has explored the use of antibody detection and proteomic techniques for the identification and analysis of nuclear proteins and transcription factors regulated by PI3K and EBV. Together, these investigations have deepened

our understanding of the molecular changes that occur in lymphocytes in response to EBV infection, and how EBV may influence malignancy.

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Appendix I

List of suppliers

LIST OF SUPPLIERS

Amersham Pharmacia	Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK.
Beckman	Beckman Instruments Ltd., High Wycombe, UK.
Biorad	Biorad, Hemel Hempstead, Watford, Herts, UK.
Boehringer Mannheim	Roche Diagnostics Ltd., Bell Lane, Lewes, UK.
Cell Signaling Technology	Cell Signaling Technology Inc., Boston, US.
Calbiochem	Merck Biosciences Ltd., Beeston, Nottingham, UK
Chiron	Chiron Therapeutics, Emeryville, California, US.
Fisher	Fisher Scientific Ltd., Loughborough, Leics, UK.
Fluka	Sigma Aldrich Company Ltd., Poole, Dorset, UK.
Gibco BRL	Life Tchnologies Ltd., Inchinnan Bus. Park, Paisely, UK.
Greiner	Greiner, Stonehouse, Gloucestershire, UK.
Heatsystems	Heatsystems-Ultrasonics Inc., Farmingdale, New York, US.
Heraeus	Heraeus Holding GmBH, Hanau, Germany.
Invitrogen	Invitrogen Ltd., Groningen, The Netherlands.
Jencons	Jencons-PLS, Leighton Buzzard, UK.
Kodak	Kodak (IBI Ltd.), Cambridge, UK.
Merck	Merck Ltd., Poole, Dorset, UK.
Molecular Probes	Invitrogen, Groningen, The Netherlands.
MWG Biotech	Wolverton Mill South, Milton Keynes, UK.
New England Biolabs	New England Biolabs Ltd, Hitchin, Herts, UK
Nonlinear Dynamics	Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK.
Novagen	Merck Biosciences Ltd., Beeston, Nottingham, UK.
Oxoid	Oxoid Ltd., Basingtoke, Hampshire, UK.
PAA	PAA Laboratories, Linz, Austria.
Promega	Promega, Southampton, UK.
Qiagen	Qiagen, Surrey, UK.
Santa Cruz	Autogen Bioclear UK Ltd., Calne, Wiltshire, UK.
Sigma	Sigma, Poole, Dorset, UK.
Sorvall	Kendro Lab, Products Ltd., Bishops Stortford, Herts, UK.

Tropix	Cambridge Bioscience, Cambridge, UK.
Tyco Healthcare	Tyco Healthcare, Mansfield, Massachusetts, US.
USB	Cleveland, Ohio, US.
Whatman	Whatman International Ltd., Maidstone, Kent, UK.

Appendix II

List of publications

List of publications

1. Breslin, E. M., P.C. White, A. M. Shore, M. Clement, and P. Brennan. 2005. LY294002 and rapamycin co-operate to inhibit T-cell proliferation. *British Journal of Pharmacology* **144:** 791-800.

2. White, P. C., A.M. Shore, M. Clement, J. McLaren, I. Soeiro, E.W. Lam, and P. Brennan. 2006. Regulation of cyclin D2 and the cyclin D2 promoter by protein kinase A and CREB in lymphocytes. *Oncogene* **25:** 2170-80.

3. Shore, A.M., P.C. White, R.C. Hui, A. Essafi, E.W. Lam, M. Rowe, and P. Brennan. Epstein-Barr Virus Represses the FoxO1 Transcription Factor through Membrane protein 1 and Latent Membrane Latent Protein 2A. 2006. *Journal of Virology* **80:** 11191-11199.

Appendix III

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Epstein-Barr Virus Represses the FoxO1 Transcription Factor through Latent Membrane Protein 1 and Latent Membrane Protein 2A▽

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Epstein-Barr virus (EBV) infection is associated with the development of many B-cell lymphomas, including Burkitt's lymphoma, Hodgkin's lymphoma, and posttransplant lymphoproliferative disease. The virus alters a diverse range of cellular molecules, which leads to B-cell growth and immortalization. This study was initiated to investigate the interplay between EBV and a proapoptotic transcription factor target, FoxO1. In this report, we show that EBV infection of B cells leads to the downregulation of FoxO1 expression by phosphatidylinositol 3-kinase-mediated nuclear export, by inhibition of FoxO1 mRNA expression, and by alteration of posttranslational modifications. This repression directly correlates with the expression of the FoxO1 target gene *Bcl-6* and inversely correlates with the FoxO1-regulated gene *Cyclin D2*. Expression of the EBV genes for latent membrane protein 1 and latent membrane protein 2A decreases FoxO1 expression. Thus, our data elucidate distinct mechanisms for the regulation of the proapoptotic transcription factor FoxO1 by EBV.

Epstein-Barr virus (EBV) is a member of the human γ-herpesvirus family. Greater than 90% of the adult population worldwide is infected with the virus. In the majority of cases, EBV infection is asymptomatic for the lifetime of the host due to cytotoxic T-lymphocyte-mediated targeting of infected cells (42). EBV primarily infects B cells but has also been reported to infect T cells and epithelial cells. Primary infection with EBV during adolescence or adulthood can be accompanied by the development of a self-limiting T-cell lymphocytosis known as infectious mononucleosis. However, EBV is also potentially oncogenic. The virus has been detected in malignancies of lymphoid as well as epithelial origin (41). The EBV genome is detected in most cases of posttransplant lymphoproliferative disease, where patients are immunosuppressed and thus cannot control the proliferation of virus-infected cells (36). Essentially every case of endemic Burkitt's lymphoma (BL) is EBV positive, in contrast with between 10 and 90% of sporadic cases. The virus has also been implicated in cases of Hodgkin's lymphoma, nasopharyngeal carcinoma, and some T-cell lymphomas.

The in vitro infection of primary B cells with EBV leads to the establishment of immortalized lymphoblastoid cell lines (LCLs). The cooperative actions of several EBV genes contribute to this effect by generating survival and proliferation signals (56). Activation of the transcription factor NF-κB and the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) signaling pathway has been shown to play an important role in the regulation of survival and proliferation of B cells (6). While many of the targets of NF-κB have been characterized, the nuclear targets of PI3K are relatively poorly characterized for EBV-immortalized B cells.

Proapoptotic forkhead box class O (FoxO) transcription factors are direct targets of PI3K-mediated signal transduction in a variety of cell systems. Phosphorylation of members of this transcription factor family by PKB, the main downstream effector of PI3K activity, results in the nuclear exclusion and inhibition of transcriptional activity (3, 9, 49). FoxO transcription factors coordinate cell cycle progression and cell survival by the activation of antiproliferative genes, such as those encoding $p27^{Kip1}$ and cyclin G2, as well as proapoptotic genes, such as those encoding Fas ligand (FasL), Bcl-6, and the Bcl-2 family member Bim (9, 13, 15, 20, 33, 50). Initial identification of this transcription factor family in humans occurred when three members were identified at chromosomal translocations in human tumors, namely, FoxO1 (FKHR) in alveolar rhabdomyosarcomas, FoxO3a (FKHR-L1) in acute myeloblastic leukemia, and FoxO4 (AFX) in acute lymphocytic leukemia (5, 11, 21, 22, 38). These discoveries were the first indications that FoxO transcription factors have a role in tumor development. Recent studies have demonstrated that a loss of FoxO activities due to protein degradation contributes to cellular transformation of primary breast cancer tumors (23) and mouse primary lymphomas (24).

This study was initiated to investigate the interplay between FoxO1 and EBV. We focused on one member of the FoxO family, FoxO1, and have shown that it is downregulated in EBV-infected B cells. This repression was found to correlate with the expression of two FoxO1 target genes, *bcl-6* and *cyclin D2*. PI3K regulation of FoxO1 protein levels and subcellular localization were also found to differ between EBV-negative and -positive B-cell lines. In addition, two EBV proteins, latent

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membrane protein 1 (LMP1) and LMP2A, have been identified as sufficient for the downregulation of FoxO1 expression.

MATERIALS AND METHODS

Cell culture. All cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics (200 U/ml penicillin and 200 μg/ml streptomycin) and were maintained at 37°C in a 5% CO_2 humidified incubator.

Nuclear and cytosolic protein extraction. Nuclear and cytosolic extracts were generated using a modification of a previously published protocol (8). Following the application of inhibitors as indicated, 1×10^7 cells were harvested and placed on ice. They were washed in 1 ml of hypotonic buffer (10 mM HEPES [pH 7.9], 1.5 mM magnesium chloride, 10 mM potassium chloride, 10 mM phenylmethylsulfonyl fluoride [PMSF]) and centrifuged at 10,000 × *g* for 1 min. Cells were lysed in 100 μl of hypotonic buffer with 0.1% Nonidet P-40 and placed on ice for 5 min. The mixture was centrifuged for 5 min at 10,000 × *g*. The supernatant was removed and retained as the cytosolic fraction. The remaining pellet was incubated with 60 μl of high-salt buffer (20 mM HEPES [pH 7.9]), 420 mM NaCl, 1.5 mM $MgCl_2$, 25% glycerol, 0.5 mM PMSF) to release the transcription factors from the DNA. This mixture was incubated on ice for 15 min and centrifuged for 5 min at 10,000 × *g*. The supernatant was retained as the nuclear fraction and stored at −20°C until further use.

Transfection, Western blotting, and antibody detection. For transient transfections, 1×10^7 DG75 cells from a suspension culture were transfected by electroporation using a Bio-Rad Genepulser II electroporator at 300 V and 950 μF at room temperature in 500 μl growth medium. Following electroporation, the cells were transferred to 3.5 ml of fresh medium per sample and incubated at 37°C in a 5% CO_2 humidified incubator for 20 h. Total protein lysates were generated using the passive lysis buffer provided with the Promega Dual Luciferase reporter assay system (E-1910). The lysates were clarified by centrifugation at 13,000 × *g* for 5 min, and the soluble fraction was added to an equal volume of 2× gel sample buffer (0.1 M Tris buffer, pH 6.8, 0.2 M dithiothreitol, 4% sodium dodecyl sulfate [SDS], 20% glycerol, 0.1% bromophenol blue) and boiled for 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham) for immunoblotting. Specific antibody-protein complexes were detected using alkaline phosphatase-conjugated secondary antibodies and CDP-Star (Tropix) chemiluminescence reagent.

Antibodies to poly(ADP) ribose polymerase (PARP; sc-7150), cyclin D2 (sc-593), Bcl-6 (sc-858), and calregulin (sc-11398) were obtained from Santa Cruz Biotechnology and used at a concentration of 200 ng/ml. An antibody to FoxO1 (9462) was purchased from Cell Signaling Technologies and used at a 1/1,000 dilution of the stock supplied. An antibody to actin (A-2066), obtained from Sigma, was used at a 1/1,000 dilution of the stock supplied. Anti-LMP1 (CS.1-4) (45), anti-EBNA2A (PE2) (55), and anti-LMP2A (14B7) (18) monoclonal antibodies have been described previously.

DNA affinity precipitation. Nuclear extracts were diluted with 20 volumes of salt-free buffer (50 mM Tris-HCl, pH 8, 0.25 mM EDTA, 10 mM NaF, 25% glycerol, 0.5 mM PMSF, 10 μl/ml phosphatase inhibitor cocktail I [P-2850; Sigma] and phosphatase inhibitor cocktail II [P-5726], 1 mM $NaVO_4$). Streptavidin-conjugated agarose beads (30 μl of a 50% slurry) and a biotinylated double-stranded oligonucleotide (1 μg) were added to the lysate, which was rotated for 1 h at 4°C. The mixture was centrifuged at 12,000 × *g*, and the supernatant was removed. The beads were washed in buffer three times, and the proteins were eluted from the beads by the addition of 2× gel sample buffer (0.1 M Tris buffer, pH 6.8, 0.2 M dithiothreitol, 4% sodium dodecyl sulfate, 20% glycerol, 0.1% bromophenol blue) for one-dimensional analysis by SDS-PAGE. Proteins were eluted from the beads by the addition of sample buffer {7 M urea, 2 M thiourea, 0.4% 3-[(3-cholamidopropyl)-dimethylamino]-1-propanesulfonate (CHAPS)} for analysis by two-dimensional (2D) electrophoresis. Separated proteins were transferred to PVDF membranes and analyzed using specific antibodies. The sequence of the oligonucleotide corresponding to the *bim* promoter was CAG AGTTACTCCGGTAAACACGCCAGGGAC (15).

2D electrophoresis. DNA affinity-precipitated proteins were eluted from streptavidin-coated agarose beads using 100 μl sample buffer (7 M urea, 2 M thiourea, 0.4% CHAPS). A 7-cm pH 3-10 NL Immobiline Drystrip gel (IPG; Amersham) was rehydrated for 12 h at 20°C with 80 μl of the eluted sample in a total volume of 125 μl of sample buffer supplemented with 50 mM dithiothreitol, 1% bromophenol blue, and 0.5% IPG pH 3-10 NL buffer (Amersham). Isoelectric focusing (IEF) of the samples was performed on an Ettan IPGphor II IEF system using the following program: 1 h at 500 V; 2 h at 1,000 V (gradient); 1 h at 1,000 V and 2 h at 8,000 V (gradient); and 8 h at 8,000 V. IPG strips were then equilibrated for 15 min in equilibration buffer (1× NuPAGE LDS sample buffer; Invitrogen) containing 10× NuPAGE sample reducing agent (0.5 ml) (Invitrogen). The IPG strips were subsequently equilibrated for 15 min in equilibration buffer containing 125 mM iodoacetamide. Equilibrated IPG strips were transferred to the IPG wells of NuPAGE 4 to 12% bis-Tris Zoom gels (Invitrogen) and separated in the second dimension for 1 h at 200 V. Separated proteins were subsequently transferred to PVDF membranes and analyzed by Western blotting as described previously.

Real-time quantitative reverse transcription-PCR. Total cellular RNA was isolated using an RNeasy kit (QIAGEN) according to the manufacturer's instructions. RNAs were treated with RNase-free DNase I (QIAGEN) for subsequent real-time quantitative PCR. Total RNA was reverse transcribed with Superscript III reverse transcriptase (Invitrogen). The resulting cDNAs were amplified using an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) with the following primer pairs: *FOXO1*-sense (TGG ACA TGC TCA GCA GAC ATC), *FOXO1*-antisense (TTG GGT CAG GCG GTT CA), *L19*-sense (GCG GAA GGG TAC AGC CAA T), and *L19*-antisense (GCA GCC GGC GCA AA). *L19*, a nonregulated ribosomal housekeeping gene, was used as an internal control to normalize input RNA. All measurements were performed in triplicate (28).

Plasmids. The pSG5 empty vector, pSG5-LMP1 (25), pSG5-LMP1^{AAA} (14), and pSG5-LMP2A (29) have been described previously. The Bcl-6ΔBcl-6-luciferase reporter was a kind gift from Tracy Tang and Laurence Lasky (Genentech Inc., South San Francisco, Calif.) and has been described previously (16, 50). The phRL-SV40 luciferase reporter vector was purchased from Promega (E-6261).

Inducible expression of LMP1, LMP2A, and EBNA2A in stable transfectants. Stable DG75 transfectants containing an inducible LMP1, LMP2A, or EBV nuclear antigen 2A (EBNA2A) gene (17) were maintained under drug selection and in 1 μg/ml tetracycline until required. Prior to each experiment, cells were washed five times in RPMI 1640 medium and were recultured without drug selection and in the presence or absence of 1 μg/ml tetracycline for a period of either 24 or 48 h. For the generation of total cell lysates, cells were counted on a hemocytometer and resuspended in 50 μl phosphate-buffered saline per 10^6 cells. An equal volume of 2× gel sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 0.4 M sodium 2-mercaptoethane sulfonate, 4% SDS, 0.02% bromophenol blue) was added, and the cells were sonicated using a W385 sonicator (Heat Systems Ultrasonics). Following sonication, samples were heated at 100°C for 5 min. The solubilized proteins were separated by SDS-PAGE and transferred to a PVDF membrane (Amersham) for immunoblotting as described above.

RESULTS

FoxO1 expression is downregulated by EBV infection. To investigate the relationship between EBV infection and FoxO1 expression, nuclear FoxO1 protein levels were analyzed by Western blotting, using cell lines with different EBV status and a specific antibody. PARP protein levels were also analyzed to verify equal protein loading, as PARP is found in the nucleus and PARP expression levels are not affected by EBV status. BL41, BL41+B95.8, and IARC-171 are from the same individual and thus have the same genetic background. They differ in their patterns of EBV gene expression and the presence of the *myc* translocation in BL41 that is typical of Burkitt's lymphoma cells (44). BL41 is an EBV-negative BL line. BL41+B95.8 is the same line after infection with the B95.8 strain of EBV. IARC-171 is an EBV B95.8-immortalized LCL derived from the same patient as BL41 (Fig. 1a). Our results show high levels of FoxO1 protein expression in the EBV-negative BL line (BL41). In contrast, FoxO1 expression was markedly downregulated in EBV-immortalized B cells (IARC-171). To investigate whether the presence of EBV was the cause of downregulation, FoxO1 expression was also analyzed in BL41+B95.8 cells. Less FoxO1 was detected in BL41+B95.9 cells than in BL41 cells, suggesting that EBV gene products have a role in downregulating FoxO1 protein expression.

EBV infection of B cells can lead to the establishment of distinct latency programs as a result of the different expression

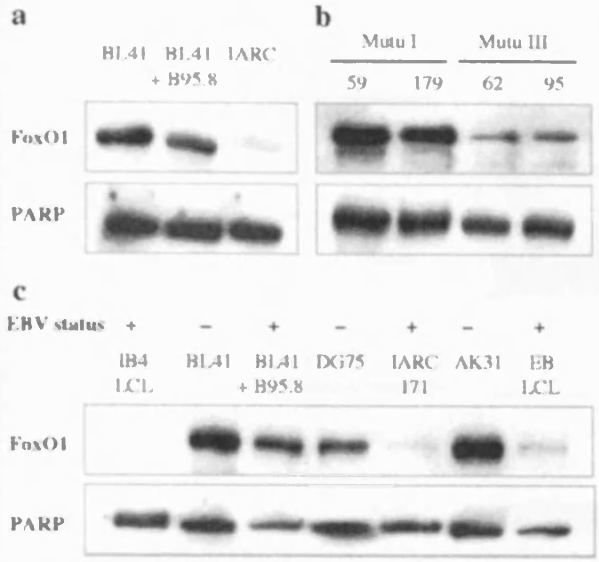

FIG. 1. EBV infection downregulates FoxO1 expression. Nuclear protein extracts were generated from the following cell lines: (a) a Burkitt's lymphoma cell line (BL41), an EBV-infected Burkitt's lymphoma line (BL41+B95.8), and a lymphoblastoid B-cell line (IARC-171); (b) an EBV-positive Burkitt's line expressing latency I genes (Mutu I, clones 59 and 179) and an EBV-positive Burkitt's line expressing latency III genes (Mutu III, clones 62 and 95); and (c) EBV-negative BL lines (BL41, DG75, and AK31), an EBV-positive BL line (BL41+B95.8), and lymphoblastoid cell lines (IB4-LCL, IARC-171, and EB-LCL). Proteins were resolved by SDS-PAGE, and FoxO1 protein levels were determined by immunoblotting with a specific FoxO1 antibody. PARP was used as a loading control.

patterns of viral genes (27). To establish whether the latency program affects FoxO1 expression, protein levels were compared using four EBV-positive sublines of a BL tumor. Mutu I cells express only EBNA1 and two small polyadenylated RNA molecules (EBER1 and EBER2), a restricted pattern of viral gene expression termed latency I. Mutu III cells express the full complement of EBV latency genes (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA-LP, LMP1, LMP2, EBER1, EBER2, and BamHI A rightward transcripts), termed the latency III pattern (43). Our results clearly demonstrate that a latency III pattern of viral gene expression is required for the repression of FoxO1 protein levels (Fig. 1b).

Additional EBV-negative and -positive lines were also tested for FoxO1 protein expression (Fig. 1c). Three EBV-negative Burkitt's lymphoma lines were analyzed. BL41 is described above. DG75 is another EBV-negative BL line (2). AK31 is an EBV-negative subclone of Akata (26), an EBV-positive BL line displaying a latency I pattern of gene expression. Again, FoxO1 protein expression was high in EBV-negative tumor lines. Three LCLs were also tested. IARC-171 is described above. IB4-LCL is an LCL generated from cord blood B cells immortalized with EBV B95.8 (46). EB-LCL is an LCL generated in-house by infection of primary B cells from the blood of a healthy donor with the B95.8 strain of EBV. In accordance with previous results, FoxO1 expression was very low in EBV-immortalized B cells. The level of FoxO1 expression in the BL41+B95.8 cells was again between those observed in BL41 and IARC-171 cells.

PI3K inhibition increases nuclear FoxO1. One mechanism whereby FoxO activity is regulated is by nuclear exclusion and subsequent degradation once it is in the cytoplasm. Stimulation of the PI3K pathway has a central role in this process by activating the kinase activity of PKB. FoxO transcription factors contain three consensus PKB phosphorylation sites that, when phosphorylated, target the proteins for nuclear exclusion, thereby increasing the survival potential of the cell (4). In order to investigate the role of PI3K in regulating the subcellular localization and protein levels of FoxO1, a specific inhibitor of PI3K, LY294002, was employed (52). BL41 and IARC-171 cells were either left untreated or treated with increasing doses of LY294002. Nuclear and cytosolic FoxO1 protein levels were subsequently analyzed by Western blotting.

Although basal levels of FoxO1 were relatively high in the nuclei of BL41 cells, this could be increased further following PI3K inhibition by LY294002 for 1 h (Fig. 2a). In parallel with this increase, a decrease in cytosolic FoxO1 was observed following LY294002 treatment. In contrast, no nuclear increase or cytosolic decrease in FoxO1 level could be detected in the LCL (IARC-171) after 1 h of treatment with the PI3K inhibitor (data not shown). Only after treatment with LY294002 for 24 h could an increase in FoxO1 nuclear protein be detected (Fig. 2b). This increase was not accompanied by a decrease in cytosolic FoxO1. These results show that PI3K inhibition can increase the FoxO1 level in both cell types, but with distinct mechanisms, suggesting a difference in the ways in which PI3K regulates FoxO1 activity in cells with different EBV statuses.

The slow kinetics of upregulation of FoxO1 by treatment with LY294002 in IARC-171 cells suggests a different mechanism of regulation in these cells. For this reason, we investigated whether FoxO1 repression by EBV could occur at the transcriptional level. Total RNAs were purified from BL41, BL41+B95.8, and IARC-171 cells, and FoxO1 mRNA activity was measured relative to that of a housekeeping gene (L19) by real-time PCR. The results showed that steady-state levels of FoxO1 mRNA were reduced by half in EBV-infected cells compared to those in EBV-negative cells (Fig. 2c), indicating that FoxO1 is downregulated at the transcriptional level by EBV.

FoxO1 can bind DNA and is posttranslationally modified in EBV-infected cells. DNA binding is required for transcription factors to regulate target genes and downstream effects. It was therefore important to check whether the FoxO1 protein detected was capable of binding DNA for mediation of its effects on cell proliferation and survival. DNA affinity precipitation experiments were therefore carried out using an oligonucleotide containing a forkhead response element (5′-TAAACAC-3′) from the *bim* promoter (15). FoxO1 molecules from nuclear extracts of BL41 and BL41+B95.8 cells, either left untreated or treated with LY294002 (20 μM) for 1 h, were DNA affinity precipitated using the *bim* promoter oligonucleotide. FoxO1 DNA binding was subsequently analyzed by Western blotting (Fig. 3a). DNA binding of FoxO1 to the *bim* promoter oligonucleotide was detected in untreated BL41 and BL41+B95.8 cells, confirming that the FoxO1 protein detected is capable of binding to target promoter sequences. The effect of PI3K inhibition on DNA binding was also tested. Following LY294002 treatment, the amount of DNA-bound FoxO1 was significantly increased, to similar degrees, in both BL41 and

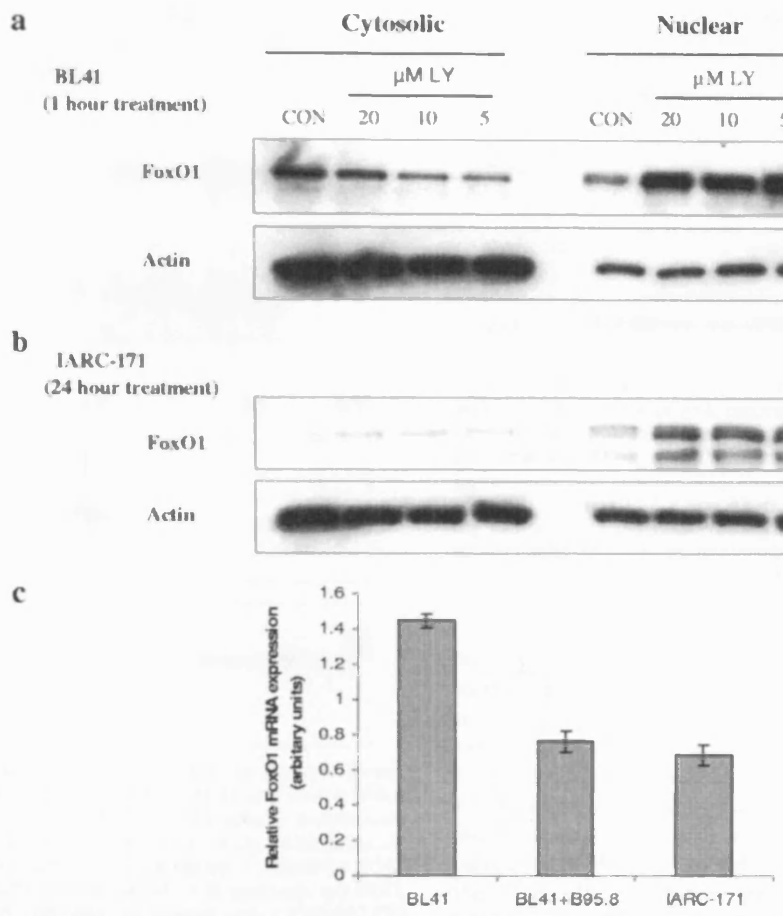

FIG. 2. PI3K inhibition can increase nuclear FoxO1 in all cell types, but with different kinetics. Nuclear and cytosolic protein extracts were generated from (a) BL41 and (b) IARC-171 B-cell lines. Proteins were resolved by SDS-PAGE and immunoblotted with the anti-FoxO1 antibody. Cells were either left untreated or treated with various concentrations of LY294002 (20 μM, 10 μM, and 5 μM) for either 1 h (BL41) or 24 h (IARC-171). Human anti-actin was used as a loading control. (c) The expression of FoxO1 RNA was analyzed by real-time PCR and normalized to the level of L19. The results shown are the averages of triplicate results.

BL41+B95.8 cells. This suggests that a direct correlation exists between the amount of FoxO1 localized within the nucleus and DNA binding.

The role of posttranslational modifications in the regulation of transcription factor activity is well established. Phosphorylation events are well known to have important effects on the activities of many transcription factors. FoxO members contain multiple PKB phosphorylation sites that mediate subcellular localization and DNA binding activity. Both ubiqitinylation and acetylation have also been reported to modulate members of the FoxO family by directing degradation and attenuation of DNA binding, respectively (24, 32, 39). To establish how many isoforms of FoxO1 exist in the nuclei of EBV-negative and -positive BL41 cells, FoxO1 was analyzed by 2D electrophoresis (Fig. 3b). DNA affinity-precipitated FoxO1 eluted from *bim* promoter oligonucleotides was isoelectrically focused, using a pH 3-to-10 nonlinear immobilized pH gradient, and subsequently separated in the second dimension by SDS-PAGE. Western blot detection of FoxO1 with a specific antibody revealed that multiple isoforms of FoxO1 bind to DNA in BL41+B95.8 cells. The isoelectric points (pI) of the different FoxO1 isoforms range between 5.4 and 6.1, and the isoforms are present in various amounts. The most abundant isoform has a pI of approximately 5.7. In contrast, only one isoform of FoxO1 could be detected in BL41 cells, with a lower pI value of approximately 4.7. We can therefore conclude that FoxO1 is posttranslationally modified and that multiple isoforms of FoxO1 are capable of binding DNA in EBV-positive cells (BL41+B95.8) but not in an EBV-negative B-cell line (BL41).

FoxO1 expression correlates with Bcl-6 expression and inversely correlates with cyclin D2 expression. Having established that the FoxO1 protein detected was capable of binding DNA, it was then important to determine whether DNA binding initiated transcriptional activation and to measure the effect of EBV on target gene activity. To address these issues, expression levels of FoxO1 target genes were analyzed by using various cell lines (Fig. 4). Bcl-6 is a sequence-specific transcriptional repressor of proteins mediating lymphocyte apoptosis and differentiation, and *bcl-6* transcription is activated by FoxO1 (50). Nuclear Bcl-6 from various cell lines was analyzed by Western blotting. A direct correlation between FoxO1 and Bcl-6 protein levels was observed, with a high level of Bcl-6 expression in cells with high FoxO1 expression and less Bcl-6 detected in EBV-transformed LCL cells. The Bcl-6 levels did

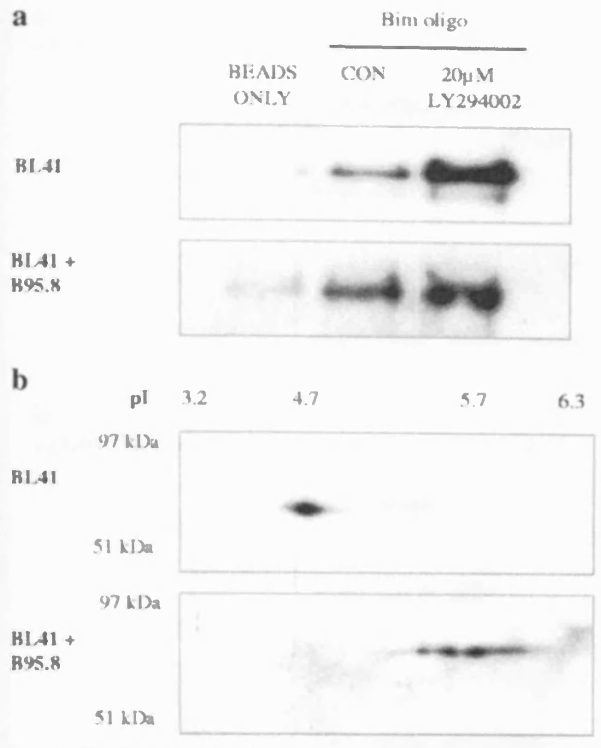

FIG. 3. FoxO1 can bind DNA and is present in multiple forms. Nuclear protein extracts were generated from BL41 and BL41+B95.8 cells. Nuclear proteins were subsequently DNA affinity precipitated using streptavidin-coated agarose beads with or without biotinylated *bim* oligonucleotides. Bound proteins were eluted, resolved by SDS-PAGE, and immunoblotted using the anti-FoxO1 antibody. (a) BL41 and BL41+B95.8 cells left untreated or treated with LY294002 (20 μM, 1 h). (b) DNA affinity-precipitated (using the *bim* oligonucleotide) proteins from BL41 and BL41+B95.8 cells treated with LY294002 (20 μM, 1 h) were analyzed by 2D electrophoresis. Eluted proteins were isoelectrically focused using a pH 3-to-10 nonlinear immobilized pH gradient and separated in the second dimension by SDS-PAGE. FoxO1 isoforms were detected by immunoblotting with the anti-FoxO1 antibody.

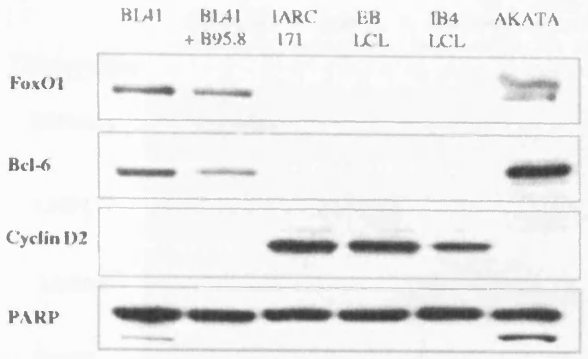

FIG. 4. Expression of FoxO1 correlates with expression of Bcl-6 and inversely correlates with expression of cyclin D2. Nuclear protein extracts were generated from the following B-cell lines with various EBV statuses: BL41, an EBV-negative BL line; BL41+B95.8, an EBV-positive BL line (latency III); three EBV-positive LCLs (IARC-171, EB-LCL, and IB4-LCL); and Akata, an EBV-positive BL line expressing the latency I pattern. Proteins were resolved by SDS-PAGE and subsequently analyzed by immunoblotting with specific antibodies to FoxO1, Bcl-6, and cyclin D2. PARP was used as a loading control.

not correspond with EBV status per se, as Akata cells, which are EBV positive but have a latency I pattern of gene expression, expressed high levels of FoxO1 and Bcl-6. This agrees with the data for Mutu I cells and suggests that one or more of the EBV genes expressed in latency III are likely to regulate FoxO1. Cyclin D2 protein levels were also analyzed. Cyclin D2 is a cell cycle protein required for progression through the G_1 phase of the cell cycle and has been shown to be repressed transcriptionally by FoxO (16, 47). Western blot analysis showed an inverse correlation between cyclin D2 expression and the expression of both FoxO1 and Bcl-6. These data suggest that FoxO1 is transcriptionally active and that the repressive effect that EBV has upon FoxO1 also extends to its target genes.

Both LMP1 and LMP2A can downregulate FoxO1 expression. The establishment of EBV latency requires the expression of a repertoire of EBV-carried latent genes. LMP1 is the major transforming protein of EBV and is required for the transforming effects of EBV on primary B cells. LMP1 mimics constitutively activated CD40, a member of the tumor necrosis factor receptor superfamily, mediating ligand-independent signaling through a range of key signaling pathways essential for survival, including the NF-κB, mitogen-activated protein kinase, Jun N-terminal protein kinase (JNK), p38, and JAK/STAT pathways as well as the PI3K pathway (56). LMP2A, which is also encoded by EBV, is another potent activator of the PI3K pathway, acting as a constitutively activated B-cell receptor (BCR), thereby inhibiting normal signaling through the BCR (34, 48). Since the activities of both LMP1 and LMP2A have been shown to activate PI3K signaling, it was reasonable to suspect that they may have a role in the repression of FoxO1.

To test this hypothesis, DG75 cells, which express high levels of FoxO1, were transiently transfected with increasing amounts of either an LMP1 or LMP2A expression vector. After 20 h, cells were harvested and lysed, and FoxO1 and Bcl-6 protein levels were analyzed by Western blotting. DG75 cells transfected with LMP1 demonstrated reduced levels of FoxO1 and Bcl-6 protein expression in a dose-dependent manner (Fig. 5a). A reporter plasmid carrying a *Bcl-6* promoter containing a FoxO consensus binding sequence (50) was also repressed by LMP1 in a dose-dependent manner (data not shown). Protein analysis of LMP2A-transfected cells showed a similar decrease of FoxO1 expression. A clear downregulation of Bcl-6 was also detected with transfection of 10 μg of LMP2A. However, at the lower doses of 1 and 5 μg, LMP2A did not repress Bcl-6 protein expression (Fig. 5b). Transfection of DG75 cells with an EBNA2A expression vector did not repress either the protein level of FoxO1 or Bcl-6 or the transcriptional activity of the Bcl-6 gene (not shown).

To further analyze the effects of LMP1, LMP2A, and EBNA2A on protein levels of FoxO1 and Bcl-6, protein expression levels were analyzed in stable DG75 transfectants containing an inducible LMP1, LMP2A, or EBNA2A gene. Protein levels were compared with those detected in two lymphoblastoid cell lines, namely, IARC-171 and an early-passage LCL (CMc) generated from the B cells of a healthy donor with the B95.8 strain of EBV (Fig. 6). In the presence of tetracy-

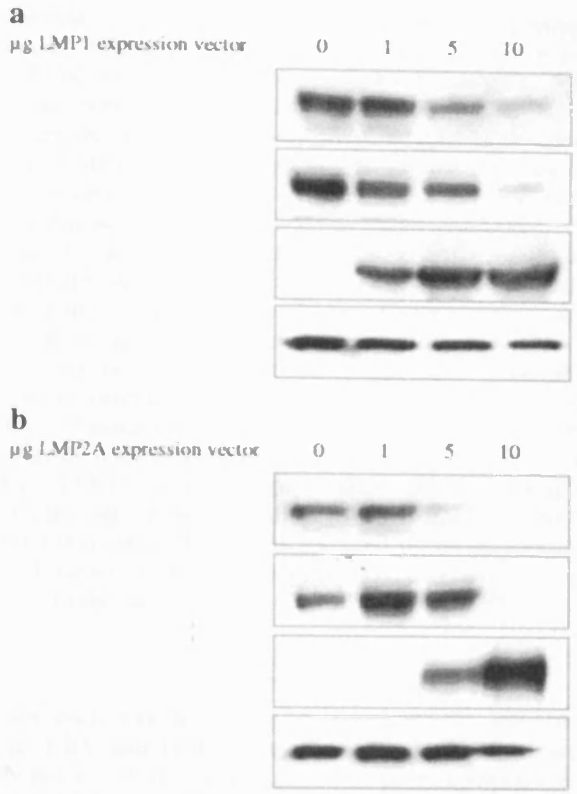

FIG. 5. Roles for LMP1 and LMP2A in FoxO1 downregulation. DG75 cells were transfected with various amounts of (a) LMP1 and (b) LMP2A expression vectors. Cells were harvested, and lysates generated were analyzed by SDS-PAGE and Western blotting. Protein levels of FoxO1 and Bcl-6 were analyzed using specific antibodies. LMP1 and LMP2A protein levels were also checked using specific antibodies. Actin was used as a loading control.

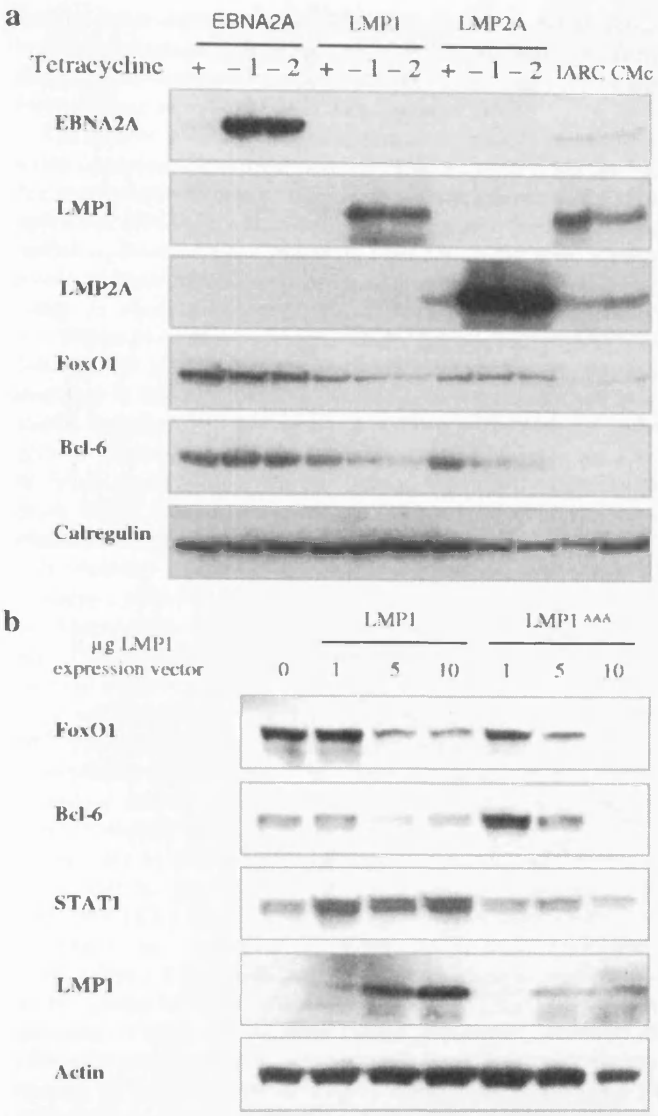

FIG. 6. Inducible LMP1 and LMP2A downregulate FoxO1 and Bcl-6. (a) Stable DG75 transfectants containing an inducible EBNA2A, LMP1, or LMP2A gene were maintained under drug selection and with 1 μg/ml tetracycline until required for experiments. When required, cells were washed five times with RPMI 1640 medium and were recultured without drug selection and in the presence or absence of 1 μg/ml tetracycline for a period of either 24 (−1) or 48 h (−2). Protein levels of FoxO1 and Bcl-6 were subsequently analyzed using specific antibodies. Protein expression levels were also analyzed in the IARC-171 and CMc lymphoblastoid cell lines. EBNA2A, LMP1, and LMP2A protein levels were also checked using specific antibodies. Calregulin was used as a loading control. (b) In a similar experiment to that described in the legend to Fig. 5, three different amounts of an expression vector for wild-type and a mutant LMP1 ($LMP1^{AAA}$) were transiently transfected into DG75 cells. Cells were harvested, and the lysates generated were analyzed by SDS-PAGE and Western blotting. Protein levels of FoxO1, Bcl-6, LMP1, and STAT1 were analyzed using specific antibodies. Actin was used as a loading control.

cline, protein expression of FoxO1 and Bcl-6 was clearly detectable in EBNA2A-, LMP1-, and LMP2A-inducible lines, although FoxO1 expression was higher in the EBNA2A line and the parent DG75 cells (not shown). Upon induction of LMP1 gene expression by reculturing the cells in the absence of tetracycline, clear decreases in both FoxO1 and Bcl-6 levels were detected at 24 h and, to a greater degree, at 48 h. The induction of LMP2A expression did not significantly reduce FoxO1 expression. However, the FoxO1 level was lower in the DG75 cells that contained LMP2A, even when they were cultured in the presence of tetracycline, than in cells with the tetracycline transactivator alone (not shown) or cells expressing EBNA2A. The cells clearly also expressed LMP2A. However, the induction of higher levels of LMP2A caused a decrease in Bcl-6 protein levels, suggesting that LMP2A may contribute to the regulation of this protein in a FoxO1-independent fashion. Similar to the LMP2A level, the FoxO1 level in the LMP1-inducible line was reduced compared to that in the EBNA2A line. Together, these observations demonstrate the sensitivity of FoxO1 protein expression to the residual low levels of both LMP1 and LMP2A signals in the presence of tetracycline. Induction of EBNA2A did not affect the expression of FoxO1 or Bcl-6. The results from the transiently transfected and stable lines demonstrate that both LMP1 and LMP2A can downregulate the expression of FoxO1.

LMP1 gave a more dramatic downregulation of FoxO1 than did LMP2A. There has been significant analysis of LMP1 sig-

naling that has identified at least two different signaling domains, CTAR1 and CTAR2. CTAR1 has been shown to activate PI3K in epithelial cells and fibroblasts (12, 30). To determine whether CTAR1 can regulate FoxO1, we expressed a mutant that had three amino acids changed in this domain, entitled LMP1^{AAA}, where proline 204, glycine 206, and threonine 208 were all mutated to alanine. Three different amounts of an expression vector for wild-type LMP1 or LMP1^{AAA} were transiently transfected into DG75 cells, and the cells were left for 24 hours. After this time, the cells were harvested and lysed by the addition of gel loading buffer. The protein extract was resolved by SDS-PAGE and transferred to a PVDF membrane, and the levels of four different proteins were detected by specific antibodies (Fig. 6b). Both wild-type LMP1 and the LMP1^{AAA} mutant were able to cause a decrease in FoxO1 and Bcl-6 protein levels. However, only wild-type LMP1 was able to induce STAT1, a transcription factor that is increased by LMP1 through an NF-κB pathway (40). These data show that distinct domains of LMP1 are involved in the distinct regulation of transcription factor protein levels and that this may not be due to the direct activation of PI3K by this molecule.

DISCUSSION

This study was performed to investigate the interplay between EBV and FoxO1. Our data clearly demonstrate that EBV has a repressive effect on FoxO1 protein expression in B cells and that this effect can be mediated by both latent membrane proteins 1 and 2A. The repression of FoxO1 may be due to the ability of these membrane proteins to activate PI3K signaling, although our data show that EBV can also repress FoxO1 mRNA levels, suggesting alternative mechanisms for the regulation of this molecule.

FoxO1 has a well-established role in the regulation of cell survival, and thus it is logical to suspect that it is a target of regulation by EBV. It is important, however, that all of the cell lines used in this study proliferate, suggesting that FoxO1 regulation can be circumvented, perhaps by the c-*myc* oncogene, in Burkitt's lymphoma cells. In this study, a correlation was observed between EBV status and the activities of the FoxO target genes *Bcl-6* and *cyclin D2*, which are essential for B-cell proliferation and development. The level of Bcl-6, a direct target gene for activation by FoxO (50), correlated with FoxO1 expression. However, this does not prove that Bcl-6 is a direct target in these cells. The introduction of a FoxO1 transgene into an LCL, probably in some system that allows regulated gene expression, could be used to directly identify FoxO1 target genes in EBV-immortalized cells. Interestingly, Bcl-6 is a transcriptional repressor of both lymphocyte differentiation and apoptosis and is the most frequently targeted proto-oncogene in non-Hodgkin's lymphomas (54). LMP1 is sufficient for this pathway, as inducible expression of LMP1 and transfection of a plasmid encoding LMP1 were enough to repress both FoxO1 and the target gene encoding Bcl-6. An inverse correlation between LMP1 and Bcl-6 has previously been described at the transcriptional and protein levels for the B cells of transgenic mice expressing LMP1 or a chimeric LMP1-CD40 molecule (37). Other studies have also observed the repressive effect of CD40 activation or EBV genome expression on Bcl-6 expression in B cells and dendritic cells (1, 10, 35). The function of Bcl-6 repression by LMP1 is believed to be germinal center suppression, allowing exit of EBV-infected cells from the germinal center. This study identifies FoxO1 as a molecular intermediate by which LMP1 can regulate Bcl-6.

The role of LMP2A in the regulation of FoxO1 and Bcl-6 is more complex. The activation of PI3K through LMP2A has previously been shown to induce phosphorylation of FoxO1 in epithelial cells (34), which subsequently targets FoxO1 for degradation. However, the effects of LMP2A on the total protein levels of both FoxO1 and Bcl-6 had not been defined previously. A clear downregulation of FoxO1 protein expression was observed in this study upon LMP2A transient transfection, but this did not translate to a corresponding dose-responsive decrease in Bcl-6 expression. However, induction of LMP2A in stable transfectants did result in a clear repression of Bcl-6 protein expression. This may be due to differences in the time or level of expression. In the system with stable expression, more LMP2A is expressed for a longer time than the 20 h studied for the transient transfections.

In contrast to Bcl-6, an inverse correlation was observed between cyclin D2 protein expression and that of FoxO1. We have previously shown that PI3K regulates the cyclin D2 protein (7) and the *cyclin D2* promoter (53), so this may be due, in part, to FoxO1. However, the repressive effect of FoxO transcription factors on cyclin D2 transcription may be indirect (47), perhaps through Bcl-6, via an interaction with the STAT5 transcription factor (16). The *cyclin D2* gene has a complex promoter and is subject to regulation by a diverse range of cellular stimuli (31, 53).

Our data have revealed a difference in the kinetics of FoxO1 regulation by the PI3K pathway between EBV-negative BL cells and LCLs. In IARC-171 cells, 24 h of incubation with LY294002 was required to detect an increase in nuclear FoxO1, but 1 h was sufficient for an increase of a similar level in the nuclei of EBV-negative BL41 cells. This suggests the presence of extra mechanisms for the repression of FoxO1 in EBV-immortalized cells, an observation supported by the detection of lower levels of FoxO1 mRNA in these cells. A difference in the contributions of the PI3K pathway towards the survival of these two cell types is evident when analyzing cell survival after treatment with the PI3K inhibitor (7). EBV-negative BL lines rapidly undergo apoptosis; in contrast, LCL lines do not die but are growth arrested. This difference in sensitivity to LY294002 is also seen for other agents and may be due, in part, to the delay in induction of FoxO1 as well as other molecules, such as NF-κB, that are increased in EBV-immortalized cells.

Our 2D-electrophoretic analysis of DNA-bound FoxO1 showed that posttranslational modification patterns are distinct for EBV-positive B cells (BL41+B95.8) compared with those for EBV-negative B cells (BL41), suggesting a further level of control of FoxO1. Interestingly, there was also more than one band present in IARC-171 nuclear extracts, particularly when they were treated with LY294002 (Fig. 2a). Changes in molecular weight are often indicative of posttranslational modification. However, the small amount of FoxO1 in these cells precluded the analysis of FoxO1 by 2D gel electrophoresis. It is possible that the multiple isoforms of FoxO1 detected bound to DNA in EBV-positive cells are differentially phosphorylated forms, but this is unlikely, as phosphorylation tar-

gets FoxO1 molecules for nuclear exclusion and thus would not be easily detected by this assay. Acetylation of nuclear FoxO proteins has also been reported to affect the transcriptional programs controlled by FoxO proteins due to interference with the balance of coactivator and corepressor recruitment (19, 39, 51). Again, a role for the PI3K pathway is evident, as acetylation was reported to increase the sensitivity of FoxO1 to phosphorylation, contributing to degradation (32). A recent study demonstrated the role of ubiquitinylation by the Skp2/Cul-1/F-box-protein–ubiquitin complex in the targeting of FoxO1 molecules for degradation following PKB activation (24). However, the pattern of spots observed did not show a significant increase in the molecular weight of the protein, which would be suggested following ubiquitinylation. Thus, while pathways activated by EBV lead to differential modifications of FoxO1, as detected in BL41+B95.8 cells, that may repress transcriptional activity, their identity is unknown and currently under investigation.

Integrating the data generated in this study provides evidence that EBV regulates FoxO1 expression by three distinct mechanisms, i.e., the prototypic repression of FoxO1 by PI3K, alternative posttranslational modifications observed in BL41 cells infected by EBV (BL41+B95.8 cells), and a repression of FoxO1 expression at the mRNA level. These diverse mechanisms ensure that FoxO1 expression and activity are inhibited. The existence of these distinct mechanisms suggests that repressing FoxO1 activity is an important part of EBV infection of B cells.

In summary, our data identify FoxO1 as an EBV-regulated transcription factor and, as such, add to a body of work demonstrating the suppression of proapoptotic pathways by EBV. Furthermore, we have identified FoxO1 as one of the few proteins regulated by both LMP1 and LMP2A. Putting this in context, LMP1 and LMP2A have been shown to activate a diverse range of signaling pathways contributing to cell survival. These include the activation of tyrosine kinases, mitogen-activated protein kinases, JNK, p38, the transcription factor NF-κB, and the PI3K pathway. Distinctly, this study describes another nuclear target by which EBV latent membrane proteins work to shift the balance of anti- and proapoptotic pathways towards survival. The interplay between EBV and FoxO1 is likely to contribute to the characteristic apoptotic resistance of immortalized B cells in the context of EBV-associated malignancies.

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