

Regulation of the STAT1 by the Epstein-Barr virus

James Edward McLaren

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LIST OF ABBREVIATIONS

Ad5	Adenoviral type 5
AIDS	Acquired immune deficiency syndrome
AML	Acute myelogenous leukaemia
AP	Alkaline phosphatase
AP-1	Activating protein-1
APS	Ammonium persulphate
ATF	Activator transcription factor
BART	<i>Bam</i> HI A rightward transcript
Bcl-2	B-cell lymphoma protein-2
BCR	B-cell receptor
BL	Burkitt's lymphoma
BSA	Bovine serum albumin
CaM-KII	Ca ²⁺ /calmodulin-dependent protein kinase II
CAR	Coxsackie-Adenovirus receptor
CAS	Cellular apoptosis susceptibility protein
CBP	CREB-binding protein
CDK	Cyclin dependent kinase
CIS	Cytokine inducible SH2 domain-containing
CLL	Chronic lymphocytic leukaemia
CNS	Central nervous system
Cp	<i>Bam</i> HI C promoter
CSF	Colony stimulating factor
CTAR	C-terminal activating region
CTL	Cytotoxic T-lymphocyte
DLCL	Diffuse large cell lymphoma
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-AP	DNA-affinity precipitation

DTT	Dithiothreitol
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	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal-regulated protein kinase
FasL	Fas-ligand
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GAS	γ -activated sequence
GC	Germinal centre
gp	Glycoprotein
GSB	Gel sample buffer
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
HA	Haemagglutinin
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HL	Hodgkin's lymphoma
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HRS	Hodgkin and Reed-Sternberg
HTLV	Human T-lymphocyte leukaemia virus
ICAM	Intercellular adhesion molecule
IEF	Isoelectric focusing
IFN	Interferon

Ig	Immunoglobulin
IL	Interleukin
IM	Infectious mononucleosis
IPG	Immobilised pH gradient
IR	Internal direct repeats
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
ISGF3	Interferon-stimulated gene factor 3
ISRE	Interferon sequence response element
ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
JNK	Jun N-terminal kinase
Kb	Kilobasepairs
LAR	Luciferase assay reagent
LB	Luria-Bertani
LCL	Lymphoblatoid cell line
LCV	Lymphocryptovirus
LD	Lymphocyte depleted
LMP	Latent membrane protein
LPS	Lipopolysaccharide
LTR	Long Terminal Repeat region
MAPK	Mitogen-activated protein kinase
MEK	MAPK-ERK kinase
MESNA	2-mercaptoethansulphonate
MHC	Major histocompatibility complex
miRNA	Micro RNA
MOI	Multiplicity of Infection
MoMLV	Moloney Murine Leukaemia Virus
mRNA	Messenger RNA
MC	Mixed cellularity
Mw	Molecular weight

NFκB	Nuclear factor-κB
NGS	Normal goat serum
NHS	Normal human serum
NLS	Nuclear localization signal
Nmi-1	<i>N-myc</i> interacting protein-1
NLS	Nuclear localization signal
NK	Natural killer
NPC	Nasopharyngeal carcinoma
NS	Nodular sclerosing
OHL	Oral hairy leukoplakia
oriP	Origin of replication
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDK1	Phosphoinositide dependent kinase 1
PEL	Primary effusion lymphoma
PKC	Protein kinase C
PKR	Protein kinase R
PI	Propidium iodide
PIAS	Protein inhibitor of activated STAT
PI3K	Phosphatidylinositol-3-kinase
PML	Promyelocytic leukaemia
PMSF	Phenylmethylsulfonyl fluoride
PTLD	Post-transplant lymphoproliferative disease
PTM	Post-translational modification
PTP	Protein tyrosine phosphatase
PVDF	Polyvinylidene difluoride
Qp	<i>Bam</i> HI Q promoter
qRT-PCR	Quantitative reverse-transcriptase PCR
Rb	Retinoblastoma
RBP-Jκ	J-kappa recombination-binding protein

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CHAPTER 1

Review of the Literature

1.1 Epstein-Barr Virus (EBV)

1.1.1 Discovery of EBV

Epstein-Barr Virus (EBV) was initially discovered through the clinical observations of Denis Burkitt, a British missionary surgeon working in Uganda in the late 1950s. Burkitt discovered the prevalence of a childhood lymphoma among various tribes in areas of sub-Saharan Africa which were supportive of holoendemic malaria (Burkitt & O'Connor, 1961). Given the geographical distribution and unusual clinical features of this childhood tumour, now referred to as Burkitt's lymphoma (BL), Burkitt suspected that an infectious agent, possibly arthropod-borne, was the causative agent (Burkitt, 1962). In 1964, Anthony Epstein and his colleagues Yvonne Barr and Burt Achong identified herpesvirus-like particles in the electron micrographs of lymphoma cells that were successfully cultured from BL tumour biopsies (Epstein et al., 1964). This herpesvirus differed from other identified human herpesviruses since it was unable to replicate in other cultured cells was non-reactive with antibodies directed against other known human herpesviruses. The B-lymphocyte transforming ability of EBV was subsequently demonstrated (Pope et al., 1968, Pattengale et al., 1973) and lead to the emergence of EBV as the first candidate human tumour virus.

1.1.2 EBV classification and structure

Gamma-herpesviruses (γ -herpesviruses) are a sub-family of human herpesviruses which replicate in epithelial cells and are characterised by their tropism for cells of

lymphoid origin. EBV belongs to the γ 1- or lymphocryptovirus (LCV) genus of the γ -herpesvirus family and is classified by taxonomists as human herpesvirus-4 (HHV-4). EBV is the only known LCV to infect humans and shares similar homology to other LCV genomes in structure and genomic organisation. LCV genomes encode various viral genes and homologues that are essential for the establishment of latent infection, regulation of viral replication and cell survival (Kieff & Rickinson, 2007). Two EBV types have been identified (Types 1 and 2) and differ largely in their allelic expression of EBV-encoded nuclear antigens (EBNAs). They also differ in geographic localisation with Type 1 pre-dominantly found in developed countries whereas Type 2 is prevalent in equatorial regions of Africa and New Guinea (Kieff & Rickinson, 2007).

EBV has a toroid shaped protein core that is wrapped with a linear, double stranded 184 kb DNA genome. This DNA-protein core is surrounded by an icosahedral shaped nucleocapsid which is composed of 162 capsomeres and is approximately 100-110nm in diameter. Outside the nucleocapsid lies a protein tegument which is composed of a number of EBV-encoded proteins such as BPLF1 and BNRF1 which are γ -herpesvirus specific (Johannsen et al., 2004). Surrounding all these components is an outer envelope that contains surface viral glycoprotein spikes. The major glycoprotein components of this envelope include gp350 (BLLF1), gH (BXLF2), gB (BALF4), gp42 (BZLF-2), gM (BBRF3), gN (BLRF2), gp78 (BILF2), gL (BKRF2) and gp150 (BDLF3) (Johannsen et al., 2004). The structure of the EBV genome is characterised by a number of genomic features. These include short and long unique sequence domains (U_S and U_L) that contain almost all of the genome coding capacity, tandem direct repeats (TR) of 0.5 kDa at both termini of the genome and six to twelve tandem repetitions of internal direct repeats (IR-1) (Given et al., 1979, Cheung & Kieff, 1982).

1.1.3 EBV latent infection *in vitro*

Epstein-Barr Virus (EBV) is a ubiquitous human γ -herpesvirus which has a potent B-lymphocyte transforming function. Although the main targets of EBV are primary B-lymphocytes, it also infects other cell types such as T-lymphocytes, natural killer (NK) cells and epithelial cells (Kieff & Rickinson, 2007). The route of entry for EBV differs

among cell types and in B-lymphocytes involves endocytosis (Miller & Hutt-Fletcher, 1992). EBV first gains entry into the B lymphocyte through binding of the viral envelope glycoprotein gp350/220, encoded by BLLF1, to two amino terminal domains in the CD21 receptor located on the B-lymphocyte cell surface (Thorley-Lawson et al., 1979, Fingerroth et al, 1984, Hummel et al., 1984, Beisel et al., 1985, Nemerow et al., 1987, Tanner et al, 1987, Martin et al., 1991, Molina et al., 1991, Moore et al., 1991). A second interaction also occurs between gp42 (BZLF-2) and MHC class II molecules, which act as a co-receptor for EBV on the B-lymphocyte surface (Li et al., 1995, Li et al., 1997, Borza & Hutt-Fletcher, 2002, Mullen et al., 2002). This second interaction is critical since EBV deficient in gp42 cannot successfully infect B-lymphocytes (Wang et al., 1998). Following these interactions, CD21 becomes aggregated at the cell surface and enables EBV to be internalised by endocytosis where it then fuses with an endocytic vesicle membrane and releases the viral capsid into the cytoplasm of the target cell (Nemerow & Cooper, 1984, Carel et al., 1990). Alternative mechanisms of EBV internalization are also likely, however, since EBV deficient in gp350/220 is able to infect B-lymphocytes and epithelial cells (Janz et al., 2000). Following internalisation and release of the viral capsid, the linear EBV genome circularises into a viral episome through the joining of the tandem direct repeats located at each end of the genome.

The B-lymphocyte transforming function of EBV enables it to successfully transform and immortalise primary B-lymphocytes, derived from peripheral, tonsillar or foetal cord blood, *in vitro* into proliferating lymphoblastoid cell lines (LCLs). These LCLs provide a cellular model of post-transplant lymphoproliferative disease (PTLD), an EBV-associated B-lymphocyte malignancy that is manifested in immunocompromised individuals (Kieff & Rickinson, 2007). They have also proved indispensable for studying EBV latent infection and the transforming effects of the virus. These EBV-transformed LCLs contain extrachromosomal copies of the viral episome and express a distinct pattern of viral latent gene expression. This viral latency program, termed Latency III, encodes proteins that induce cell cycle entry, continual proliferation and the maintenance of viral latency. These include the EBV nuclear antigens EBNA1, 2, 3A, 3B, 3C and LP; the latent membrane proteins LMP1, 2A and 2B; two EBV-encoding nuclear RNAs (EBERs); the highly spliced *Bam*HI A rightward transcripts (BARTs) and EBV

microRNAs (Young et al., 1989, Kieff & Rickinson, 2007). The expression of all six EBNAs is driven from either the *Bam*HI W promoter (Wp) or C promoter (Cp) as a long Wp/Cp-initiated transcript which is differentially spliced into each individual EBNA (Sample et al., 1986, Woisetschlaeger et al., 1989, Kieff & Rickinson, 2007), and all three LMPs are transcribed from EBNA2-responsive promoters (Abbot et al., 1990, Fahraeus et al., 1990, Young et al., 1991, Zimmer-Strobl et al., 1991). Out of all these latent gene products, only EBNA1, EBNA2, EBNA3A, EBNA3C and LMP1 are considered essential for EBV-induced B-cell transformation (Yates et al., 1985, Cohen et al., 1989, Hammerschmidt & Sugden, 1989, Mannick et al., 1991, Swaminathan et al., 1981, Tomkinson & Kieff, 1992, Kaye et al., 1993, Longnecker et al., 1993, Tomkinson et al., 1993, Robertson et al., 1994, Kilger et al., 1998, Lee et al., 1999a, Speck et al., 1999, Humme et al., 2003, Chen et al., 2005). LCLs grow similarly to B-lymphocytes proliferating in response to antigenic and mitogenic stimulation and display high levels of adhesion molecules such as CD54 (ICAM-1, intercellular adhesion molecule 1) on their cell surface which makes them form characteristic cell clumps (Wang et al., 1988, Kieff & Rickinson, 2001).

The *in vitro* transformation of EBV-negative BL cells can also be achieved but at a reduced efficiency compared to normal primary B-lymphocytes (Kieff & Rickinson, 2007). EBV-negative BL cells display continual proliferation due to the constitutive expression of *c-myc*, a transcription factor that promotes cell proliferation (Dang, 1999). This enhanced expression of *c-myc* is caused by a reciprocal chromosomal translocation, characteristic of BL, that places *c-myc* at chromosome 8q24 adjacent to one of three immunoglobulin heavy or light chain genes on chromosomes 2, 14 or 22 (Hecht & Aster, 2000). When EBV infects BL cells *in vitro*, they initially display a Latency III viral program as seen with LCLs (Calender et al., 1990, Wang et al., 1991). They also grow in tight clumps, similar to seen in LCLs, whereas uninfected BL cells grow as single cells. However, continuous culture of these cells *in vitro* can lead to a loss of the EBV genome which converts the cells to an EBV-negative BL phenotype. Other cells may also still maintain the EBV episome and revert to a viral program termed Latency I where EBNA1, EBERs and BARTs are only expressed (Kieff & Rickinson, 2007). This Latency I viral program is also seen in BL cells infected with EBV *in vivo*.

EBV also establishes another type of viral latency, termed Latency II, which is seen in other malignancies of lymphoid and epithelial origin such as classic Hodgkin's lymphoma and nasopharyngeal carcinoma. Latency II is characterised by the expression of EBNA1, the latent membrane proteins LMP-1, -2A and -2B, EBERs and BARTs (Herbst et al., 1991, Deacon et al., 1993, Grasser et al., 1994, Niedobitek et al., 1997b). All the latency programmes expressed by EBV are summarised in Table 1.1 below.

Latency	EBV latent genes expressed	Malignancies
I	EBNA1, EBERs, BARTs	Burkitt's Lymphoma Primary Effusion Lymphoma
II	EBNA1, LMP1, LMP2A, LMP2B, EBERs, BARTs	Hodgkin's Lymphoma Nasopharyngeal Carcinoma T-lymphocyte and NK-cell Lymphoma Gastric Carcinoma
III	EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA-LP, LMP1, LMP2A, LMP2B, EBERs, BARTs, miRNAs	PTLD X-linked lymphoproliferative syndrome AIDS-associated B-lymphocyte lymphomas

Table 1.1 EBV latency gene expression patterns

1.1.3.1 EBNA1

The main functions of EBNA1 in latently infected cells are to maintain the persistence and replication of the EBV episome during cell division and to enhance transcription from the genome (Yates et al., 1984, Sugden et al., 1985, Yates et al., 1985, Reisman & Sugden, 1996). These actions are achieved by sequence specific binding of EBNA1 to the plasmid origin of replication (oriP) in the viral episome and aiding association of the episome with mitotic chromosomes (Ohno et al., 1977, Rawlins et al.,

1985, Lee et al., 1999a, Marechal et al., 1999). In Latency I, EBNA1 is transcribed from the *Bam*HI Q promoter (Qp) as a *Bam*HI Q-U-K spliced 2.4kb transcript whereas in Latency III, EBNA1 is transcribed from the Wp/Cp promoters as a highly spliced 3.5kb transcript (Sample et al., 1986, Schaefer et al., 1991, Schaefer et al., 1995, Nonkwelo et al., 1996, Kelly et al., 2002). The Qp promoter is suppressed in Latency III by interferon-regulatory factors, such as IRF-2 (Zhang & Pagano, 1999) whereas it is positively regulated by STATs such as STAT1 in Latency I and II (Chen et al., 1999). EBNA1 is also the only EBNA that is transcribed when EBV enters its lytic cycle. During lytic cycle, the activity of the Wp and Cp promoters ceases and lytic EBNA1 transcripts initiate from a downstream Fp promoter (Heller et al., 1982, Weigel et al., 1985, Nonkwelo et al., 1996).

The mature EBNA1 protein contains a long irregular glycine-glycine-alanine repeat sequence between amino acids 90 and 327 in the prototype B95.8 strain of EBV (Kieff & Rickinson, 2007). This sequence protects EBNA1 from the degradative effects of the immunoproteasome and prevents the presentation of EBNA1-specific peptides by MHC class I molecules on the surface of latently infected cells (Levitskaya et al., 1995). Thus EBV-positive B-lymphocytes that only express EBNA1 (Latency I) are poorly recognised by the host EBV-specific immunosurveillance.

1.1.3.2 EBNA2

EBNA2 is one of the first EBV latent genes expressed following *in vitro* transformation of B-lymphocytes (Allday et al., 1989). EBNA2 is spliced from a primary transcript that is initiated from the Wp promoter 12-16 hours post-infection and achieves a level of expression that is maintained in LCLs 24-32 hours post-infection (Alfieri et al., 1991, Kieff & Rickinson, 2007). EBNA2 is essential for the transformation of B-lymphocytes and this dependence has been demonstrated using EBNA2-defective EBV viruses (Miller et al., 1974, Cohen et al., 1989, Hammerchmidt et al., 1989). EBNA2 acts as a transcriptional co-activator of host cellular genes, such as CD21, CD23 and *c-myc*, and a subset of EBV latent genes. All three LMPs (LMP1, LMP2A and LMP2B) are transcribed from EBNA2-responsive promoters (Abbot et al., 1990, Fahraeus et al., 1990,

Wang et al., 1990, Young et al., 1991, Zimmer-Strobl et al., 1991) and EBNA2, along with EBNA-LP, also transactivates the Cp promoter. The establishment of complete levels of EBNA1, EBNA3A, EBNA3B and EBNA3C expression in LCLs is dependent on the transactivation of Cp by EBNA2 and EBNA-LP (Kieff & Rickinson, 2007).

The action of EBNA2 on these host and viral promoters is not direct, however, as it stably interacts with a sequence-specific transcription factor complex, RBP-J κ /CBF-1, in order to exert its action (Grossman et al., 1984, Henkel et al., 1994, Kieff & Rickinson, 2007). RBP-J κ is a downstream component of the Notch signalling pathway and EBNA2 mimics a constitutive Notch receptor in this pathway (Zimmer-Strobl & Strobl, 2001). In the absence of EBNA2, RBP-J κ /CBF-1 acts as a transcriptional repressor by recruiting histone deacetylase complexes, such as HDAC2, to the host and viral promoters. The interaction between EBNA2 and RBP-J κ /CBF-1 removes this repressive effect and enables transactivation at RBP-J κ sites in these promoters (Kieff & Rickinson, 2007). Other sequences that are important in EBNA2 responsiveness include the PU.1 and CRE binding sites in the LMP1 promoter and the AUF1 site in the Cp promoter (Laux et al., 1994, Yalamanchilli et al., 1994, Johannsen et al., 1995, Sjoblom et al., 1998).

EBNA2 function is also negatively regulated by phosphorylation in latently infected cells (Grasser et al., 1992, Kwiatkowski et al., 2004, Yue et al., 2004). EBNA2 is phosphorylated by many host protein kinases, such as casein kinase II and cdc2, (Kwiatkowski et al., 2004, Yue et al., 2004) but also by a virally encoded kinase. The EBV lytic cycle gene BGLF4 encodes a protein kinase which serine phosphorylates EBNA2 and thus suppresses its ability to transactivate the LMP1 promoter (Yue et al., 2005). Since other promoters like Cp are EBNA2 responsive, it is reasonable to suggest that they may also be affected similarly.

1.1.3.3 EBNA3A, EBNA3B and EBNA3C

EBNA3A, EBNA3B and EBNA3C are nuclear proteins that are transcribed from tandemly placed genes downstream of the Wp/Cp promoter in the EBV genome. All three genes are expressed in Latency III, and EBNA3A and EBNA3C have been shown to be essential for the *in vitro* transformation of primary B-lymphocytes (Tomkinson et

al., 1993). Although the EBNA3 proteins are disparate in amino acid sequence between Type 1 and Type 2 EBV, their effects on B-lymphocyte transformation are indistinguishable (Tomkinson et al., 1992, Kieff & Rickinson, 2007). EBNA3B is not critical for the *in vitro* transformation of primary B-lymphocytes, the out-growth of LCLs or lytic cycle (Tomkinson et al., 1992, Chen et al., 2005). However, it would seem that EBNA3B is important in *in vivo* infection since EBNA3B epitopes are well recognized by host EBV-specific cytotoxic T-lymphocytes (Rickinson & Kieff, 2007).

Studies using conditional EBNA3A mutant LCLs have demonstrated that EBNA3A is critical for LCL growth and survival (Maruo et al., 2003). This effect requires transcriptional regulation through an association with RBP-J κ /CBF-1 (Maruo et al., 2005). EBNA3B and EBNA3C can also stably associate with RBP-J κ /CBF-1 and thus can compete with EBNA2 (Robertson et al., 1995, Robertson et al., 1996). All three EBNA3s bind more tightly to RBP-J κ /CBF-1 than EBNA2 and thus can negatively regulate EBNA2 responsiveness (Johannsen et al., 1996). EBNA3A and EBNA3C can also repress Cp promoter activity and this action is dependent on RBP-J κ and also partially on histone deacetylase 1 (Radkov et al., 1997, Cludts & Farrell, 1998, Radkov et al., 1999). However, EBNA3C can also positively regulate promoter activity since it can co-activate the LMP1 in co-operation with EBNA2 (Zhao & Sample, 2000, Lin et al., 2002).

Other functions of all three EBNA3's include regulating host cellular genes involved in cell proliferation and survival. Over-expression of EBNA3A in an LCL resulted in a down-regulation in *c-myc* expression which accompanied G₀/G₁ growth arrest (Cooper et al., 2003). The introduction of EBNA3B into an EBV-negative BL cell line up-regulated bcl-2 expression and rendered cells resistant to programmed cell death (Silins & Sculley, 1995). EBNA3C has been shown control cell cycle progression by regulating cyclin A/p27 complex formation, and manipulating p16^{INK4A} accumulation and retinoblastoma protein (Rb) phosphorylation (Knight & Robertson, 2004, Maruo et al., 2006).

1.1.3.4 EBNA-LP

EBNA-Leader Protein (EBNA-LP), like EBNA2, is one of the first EBV latent genes expressed following *in vitro* transformation of B-lymphocytes (Allday et al., 1989). EBNA-LP is spliced from the same Wp-initiated primary transcript as EBNA2 and only differs in its second exon (Kieff & Rickinson, 2007). Many of the functions of EBNA-LP are in co-operation with EBNA2. For example, EBNA-LP has been shown to co-activate EBNA2 responsive promoters (Alfieri et al., 1991, Harada & Kieff, 1997, Nitsche et al., 1997, McCann et al., 2001, Peng et al., 2004), and although it is not essential for the *in vitro* transformation of B-lymphocytes, it is required for LCL growth (Mannick et al., 1991). Alone EBNA-LP exhibits minimal promoter transactivation, but can dimerise with EBNA2, through the EBNA2 acidic activating domain, and elicit promoter co-activation (Harada & Kieff, 1997, Peng et al., 2004). Among the EBNA2 responsive promoters, EBNA-LP has significantly less co-activating properties on the viral LMP2A and host CD21 promoters (Peng et al., 2005). EBNA-LP, in conjunction with EBNA2, has also been shown to induce cyclin D2 expression in primary B-lymphocytes and enable the transition of quiescent infected cells into the G₁ phase of the cell cycle (Sinclair et al., 1994). These all highlight a critical role for EBNA-LP in regulating host and viral gene expression that is required for LCL out-growth.

1.1.3.5 LMP1

LMP1 is an integral latent membrane protein that is essential for the *in vitro* transformation of primary B-lymphocytes (Kaye et al., 1993, Kilger et al., 1998). LMP1 has the ability to transform rodent fibroblast cell lines (Wang et al., 1985) and this oncogenic capacity has lead to it becoming the most intensely studied gene in EBV biology. LMP1 is transcribed from one of two promoters, ED-L1 and L1-TR. LMP1 expression is driven from the EBNA2 responsive ED-L1 promoter in transformed B-lymphocytes whereas in Nasopharyngeal carcinoma and Hodgkin's lymphoma, both Latency II tumours, it is driven from the alternative L1-TR promoter located within the viral terminal repeats (Chen et al., 2001). The L1-TR promoter lies 600bp upstream of

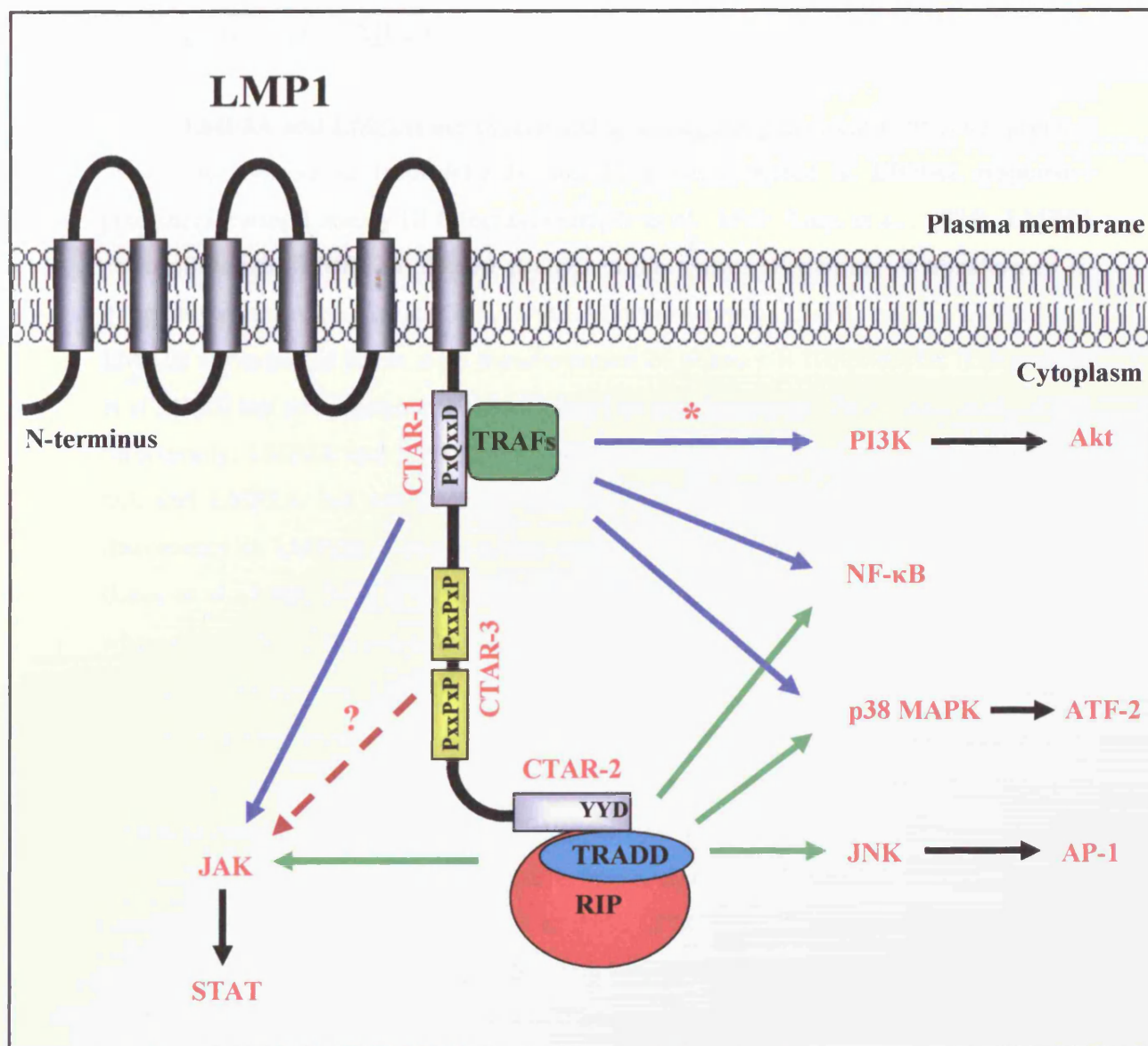
ED-L1 and although they transcribe LMP1 transcripts of different lengths (Sadler & Raab-Traub, 1997), the encoded LMP1 proteins are indistinguishable.

LMP1 mimics a constitutively active CD40 receptor (Mosialos et al., 1995, Gires et al., 1997, Izumi & Kieff, 1997) and, in LCLs, forms discrete patches in the plasma membrane (Hennessy et al., 1984, Liebowitz et al., 1986). Structurally, LMP1 has six hydrophobic transmembrane (TM)-spanning regions, a short, 24 amino acid cytoplasmic N-terminus and a long, 200 amino acid cytoplasmic C-terminal tail (Kieff & Rickinson, 2007). The short N-terminus tethers LMP1 to the plasma membrane (Izumi et al., 1994) and the six TM-spanning regions promote aggregation and oligomerisation of LMP1 molecules (Liebowitz et al., 1992). The first TM-spanning region and the short N-terminus have been shown to be important for LCL out-growth and B-lymphocyte transformation (Wang et al., 1988, Kaye et al., 1993). However, the long C-terminal tail is primarily responsible for the transduction of cellular signals that lead to primary B-lymphocyte transformation and LMP1 specific phenotypic changes (Eliopoulos & Young, 2001). This is highlighted by the fact that removal of this C-terminal tail renders EBV unable to transform primary B-lymphocytes (Kaye et al., 1995).

It has been confirmed by mutational analysis that the C-terminal tail contains two functional C-terminal activating regions (CTAR-1 and -2) (Huen et al., 1995) which function to activate cellular signalling (Brennan et al., 2001). CTAR-1 (amino acids 194-232), which is essential for B-lymphocyte transformation (Kaye et al., 1995, Izumi et al., 1999), is located proximally to the plasma membrane and binds heterotrimers of the tumour necrosis factor (TNF) receptor-associated factor (TRAF) family (TRAF1, 2, 3 and 5) (Mosialos et al., 1995, Devergne et al., 1996, Kaye et al., 1996, Brodeur et al., 1997) through a P₂₀₄XQ₂₀₆xxD₂₀₉ motif (Devergne et al., 1998). CTAR-2 (amino acids 351-386), which is required for the long-term growth of EBV-transformed LCLs (Eliopoulos & Young, 2001), is located at the end of the C-terminal tail and recruits TNF receptor-associated death domain (TRADD) proteins through a YYD₃₈₆ motif (Izumi et al., 1997, Izumi et al., 1999). A number of cellular signaling pathways are activated by these two domains. These include the nuclear factor-kappaB (NF-κB), p38 mitogen activated protein kinase (MAPK), janus kinase/ signal transducer and activator of transcription (JAK/STAT), phosphatidylinositol-3-kinase (PI3K) (in epithelial cells) and

c-Jun N-terminal kinase (JNK) pathways (Laherty et al., 1992, Huen et al., 1995, Kieser et al., 1997, Eliopoulos et al., 1998, Eliopoulos et al., 1999, Brennan et al., 2001b, Dawson et al., 2003, Richardson et al., 2003, Zhang et al., 2004) and are schematically described in Figure 1.1. The activation of the NF- κ B signalling pathway, in particular, is critical for the promotion of cell survival and for cellular transformation (Cahir-McFarland et al., 1999, Cahir-McFarland et al., 2000, He et al., 2000). Receptor interacting protein (RIP), which is a TNF receptor1-associated protein that induces cell survival, is also able to interact with the CTAR-2 domain of LMP1 (Izumi et al., 1999). However, the contribution of RIP to LMP1 signalling and B-lymphocyte transformation is not well characterised. A third CTAR domain, termed CTAR-3, which comprises of two repeat 33bp regions that contain a proline-rich PxxPxP sequence (amino acids 257-280 and 302-307) has also been described (Gires et al., 1999). This domain, although non-essential for B-lymphocyte transformation (Izumi et al., 1999), has been proposed to activate the JAK/STAT1 signalling pathway through JAK3 (Gires et al., 1999). However, this projected mechanism is controversial since an LMP1 mutant lacking functional CTAR-1 and CTAR-2, but with an intact CTAR-3 domain was unable to induce STAT transcriptional activity (Brennan et al., 2001). Also, there is evidence which shows that this CTAR-3 domain does not interact with JAK3 (Higuchi et al., 2002).

These signals induced by LMP1 lead to the up-regulation of a number of cellular genes to encode phenotype functions. These include up-regulation of the anti-apoptotic genes bcl-2, mcl-1, A20 and cIAP2 (Henderson et al., 1991, Laherty et al., 1992, Wang et al., 1996, Hong et al., 2000) which act to promote cell survival. Also, LMP1 up-regulates the expression of MHC class I and class II molecules, the TAP1 and TAP2 peptide transporter proteins as well as adhesion molecules, such as ICAM-1, which are all involved in immune recognition by EBV-specific cytotoxic T-lymphocytes (Wang et al., 1988, Rowe et al., 1995, Rickinson & Moss, 1997). Other functions, related to oncogenesis, stimulated by LMP1 include the induction of DNA synthesis (Peng & Lundgren, 1992), interference with cellular senescence (Yang et al., 2000) and promotion of angiogenesis and metastasis in EBV-associated tumours (Eliopoulos et al., 1999, Eliopoulos & Young, 2001). These evidence all highlight an essential role for LMP1 in EBV-associated malignancy.



Adapted from Eliopoulos & Young, 2001

Figure 1.1 Schematic representation of signalling pathways activated by LMP1

LMP1 activates a number of cellular signalling pathways through the CTAR-1 and CTAR-2 domains located in its C-terminal cytoplasmic tail. CTAR-1 activates the PI3K, NF-κB and p38 MAPK pathways whereas CTAR-2 activates the JNK pathway along with NF-κB and p38 MAPK, like CTAR-1. Both domains also activate the JAK/STAT pathway although a third domain, CTAR-3, has been implicated but as yet remains controversial in its involvement.

* PI3K activation by LMP1 is only observed in epithelial cells

1.1.3.6 LMP2A and LMP2B

LMP2A and LMP2B are twelve TM-spanning integral latent membrane proteins which are transcribed from RBP-J κ and PU.1 sites located in EBNA2 responsive promoters during Latency III infection (Sample et al., 1989, Laux et al., 1994). LMP2A co-localises with LMP1 in discrete patches in the plasma membrane of transformed B-lymphocytes (Longnecker & Kieff, 1990). However, unlike LMP1, neither LMP2A nor LMP2B are essential for *in vitro* transformation of primary B-lymphocytes (Longnecker et al., 1993) but do contribute to the efficiency of transformation (Briemeier et al., 1996). Structurally, LMP2A and LMP2B both contain a 27 amino acid C-terminal cytoplasmic tail, and LMP2A, but not LMP2B, also has a 119 amino acid N-terminal tail. This discrepancy in LMP2B is due to a non-coding exon 1 located in the LMP2B transcript (Laux et al., 1989, Sample et al., 1989). The function of LMP2A is well characterised whereas for LMP2B it remains relatively unclear. This is likely due to the fact that the phenotypic effects of LMP2A are accredited to the N-terminal cytoplasmic tail it possesses. However, LMP2B has been shown to negatively modulate LMP2A activity (Rovedo & Longnecker, 2007) and may be involved in the regulation of EBV lytic cycle gene expression in lytically-induced EBV-positive BL cells (Rechsteiner et al., 2007).

The N-terminal tail of LMP2A contains eight tyrosine residues which become constitutively phosphorylated, and two of these (Y74 and Y85) form an immunoreceptor tyrosine-based activation motif (ITAM) which stimulates the recruitment, activation and stable association of src family tyrosine kinases, such as Lyn and Fyn, and syk family tyrosine kinases. These events, which mirror normal B-cell receptor (BCR) signalling in non-LMP2A expressing cells, function to block BCR signaling in transformed B-lymphocytes (Burkhardt et al., 1992, Miller et al., 1995). In this respect, LMP2A is thought to mimic an activated BCR. The N-terminal ITAM motif and the association of Lyn at tyrosine 112, are essential for the blockade of BCR signalling (Fruehling & Longnecker, 1997, Fruehling et al., 1998). As a result, LMP2A functions to maintain viral latency by preventing the induction of EBV lytic cycle following cross-linking of the BCR or surface immunoglobulins (Miller et al., 1993, Miller et al., 1994, Miller et al., 1995). This is consistent with established evidence that shows that cross-linking of

surface immunoglobulins on EBV-positive BL cells, which are LMP2A negative, triggers EBV lytic cycle (Takada, 1984). However, it has been shown that LMP2A can induce entry into lytic cycle in EBV-infected B-lymphocytes in the absence of lytically-inducing stimuli such as anti-IgG (Schaadt et al., 2005). Therefore, it seems that LMP2A may exert a dual role on EBV reactivation.

LMP2A has also been shown to provide survival signals to B-lymphocytes *in vivo* in the absence of the BCR (Caldwell et al., 1998). Cell survival is promoted by inhibition of BCR signalling (Fukuda & Longnecker, 2005) or through activation of the PI3K/Akt signalling pathway (Fukuda & Longnecker, 2004). This signalling pathway is activated by LMP2A in both B-lymphocytes and epithelial cells (Scholle et al., 2000, Swart et al., 2000, Morrison et al., 2003). Interestingly, LMP2A, which is also expressed in latency II infections like nasopharyngeal carcinoma, can transform epithelial cells. This effect is associated with PI3K/Akt activation (Scholle et al., 2000). The role of the PI3K/Akt pathway in cell survival has also been demonstrated *in vivo*, as B-lymphocytes from LMP2A transgenic mice are sensitive to apoptosis in the presence of specific PI3K and Akt inhibitors (Portis & Longnecker, 2004).

1.1.3.7 EBERs

EBER1 and EBER2 are small non-polyadenylated RNA molecules that are expressed in all forms of EBV latent infection. Both EBER1 and EBER2 are transcribed by RNA polymerase III although EBER1 is ten times more abundant than EBER2 (Arrand & Rymo, 1982). The EBERs co-localise in the nucleus of infected cells and associate with chaperone proteins such as La (Fok et al., 2006). Both EBERs are not essential for the *in vitro* transformation of primary B-lymphocytes or the out-growth of transformed LCLs but contribute to the efficiency of transformation (Swaminathan et al., 1991, Yajima et al., 2005). However, the EBERs have been shown to increase the survival potential of EBV-positive BL cells by resisting IFN- α induced apoptosis (Nanbo et al., 2002). This effect is believed to be due to direct inhibition of the interferon-alpha (IFN- α) regulated gene double-stranded RNA activated protein kinase R (PKR) (Nanbo et al., 2002) although this has been debated (Ruf et al., 2005). To add to this, the EBERs

have also been reported to induce the secretion of interleukin-10 (IL-10), which may promote the growth of EBV-infected B-lymphocytes (Kitagawa et al., 2000). Therefore, the EBERs are postulated to have a role in the growth and survival of infected cells.

1.1.3.8 BARTs and miRNAs

The *Bam*HI A rightward transcripts (BARTs) are mRNAs expressed at low levels in all types of EBV latency and during EBV lytic cycle. The BARTs are likely transcribed from two TATA-less promoters, P1 and P2, and translate the BARF0, RK-BARF0, A73 and RPMS1 proteins (Fries et al., 1997, Kienzle et al., 1998, Smith et al., 2000, Kusano & Raab-Traub, 2001, Kieff & Rickinson, 2007). Although this is known, their biological function still remains unclear and also the translated proteins have yet to be detected in EBV-infected cells (Kieff & Rickinson, 2007).

The identification of EBV encoded microRNAs (miRNAs) was made recently (Pfeffer et al., 2004) and was speculated that they might have a role in regulating host and viral gene expression. At least seventeen miRNAs have now been identified and their relative expression is different between cell type and each viral latency program (Cai et al., 2006). Their function is still unknown but they may regulate viral gene expression and immune recognition as seen by SV40-encoded miRNAs in SV40-infected cells (Sullivan et al., 2005).

1.1.4 EBV latent infection *in vivo*

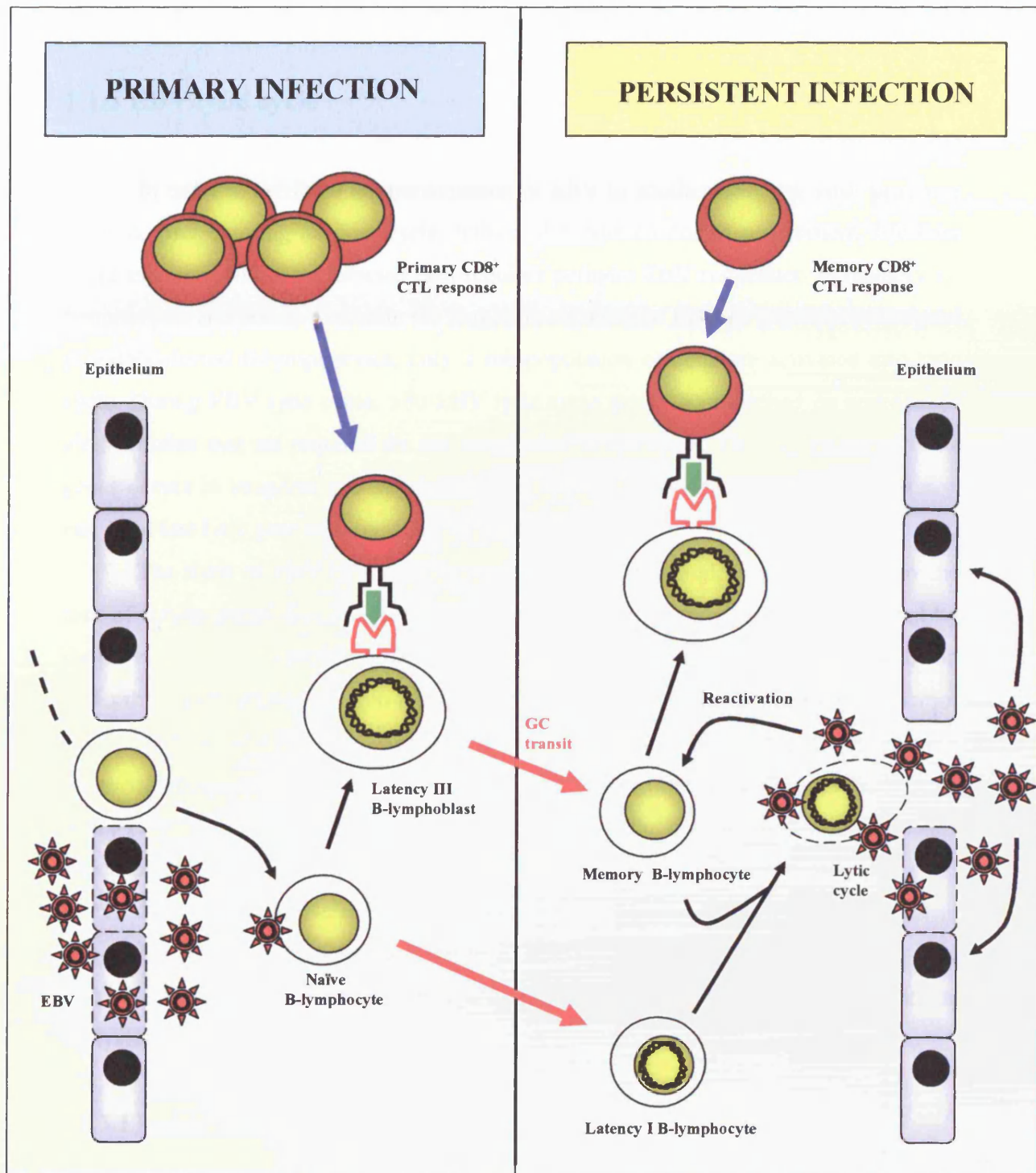
The site of primary EBV infection *in vivo* is widely accepted as being the mucosal lymphoepithelium in the oropharynx. EBV is transmitted orally through infected saliva and can be detected in throat washings from acute infectious mononucleosis (IM) patients and healthy, EBV-seropositive carriers (Niederman et al, 1976, Yao et al, 1989). In acute infection, EBV crosses the lymphoepithelium barrier through crypts located in areas of the oropharynx such as the tonsils, and targets a population of lymphocytes located in the mantle zone below. In persistent infection, virus is continuously released from cells, located in the tonsils that have entered lytic cycle (Joseph et al., 2000, Thorley-Lawson,

2001). However, it remains unclear whether EBV directly targets the epithelial cells in the mucosal lymphoepithelium of the oropharynx or naïve B-lymphocytes located in the mantle zone beneath (Thorley-Lawson et al., 1996). The following model of EBV latent infection and persistence *in vivo* summarized below was proposed by the group lead by David Thorley-Lawson (Thorley-Lawson, 2001, Thorley-Lawson, 2005) and is widely supported as a key explanation of how EBV persists in a healthy host.

Viral particles of EBV which pass through the mucosal lymphoepithelium during acute infection, or are released from cells in the tonsils during persistent infection, infect resting, naïve B-lymphocytes located in the follicular mantle and establish a latency III pattern of gene expression, also known as the growth programme (Babcock et al., 2000, Joseph et al., 2000). This stimulates the proliferation and clonal expansion of infected cells and establishes a pool of latently infected B-lymphocytes within the host. However, these proliferating B-lymphocyte blasts, which are continuously reproduced in the tonsils, are susceptible to attack by host EBV-specific cytotoxic T-cells (CTLs) (Rickinson & Moss, 1997). Some of these infected cells migrate into the follicles and undergo germinal centre (GC) differentiation into memory B-lymphocytes and revert to a latency II phenotype, also known as the default programme, where EBNA1, LMP1 and LMP2A are the only viral proteins expressed (Babcock et al., 2000). GC differentiation occurs to increase the pool of virally-infected cells and requires EBNA2 expression to be turned off since it is capable of blocking differentiation (Polack et al., 1996). EBNA1 is required for the replication of viral DNA (Marechal et al, 1999, Lee et al, 1999a) and LMP1 and LMP2A help drive the latently infected B-lymphocytes through GC differentiation by triggering immunoglobulin gene mutation (Casola et al, 2004) and isotype switching (He et al, 2003). LMP1 also down-regulates the expression of bcl-6 which signals the transition of memory B-lymphocytes out of the GC (Panagopoulos et al, 2004). These memory B-lymphocytes, which are thought to constitute the long term EBV reservoir in healthy hosts, exit the tonsils and enter the peripheral circulation (Babcock et al., 1999). EBV can persist in these cells *in vivo* for the life of the host by restricting latent gene expression to EBNA1 (Hochberg et al., 2004) or by switching off latent gene expression all together (Babcock et al., 1999). This persistence is also aided by the immune evasive properties of these memory B-lymphocytes as they do not express main CTL recognizing

antigens. These memory cells are also non-pathogenic and thus persist in a benign state in a vast majority of healthy hosts (Thorley-Lawson, 2005).

In order to persist long term within a host, EBV must replicate in order to produce and shed viral progeny. Memory B-lymphocytes are recruited from the peripheral blood reservoir into the tonsils and are stimulated to enter lytic cycle. This recruitment may be triggered by the same signals which facilitate terminal differentiation of long-live memory B-lymphocytes into antibody secreting plasma cells (Laichalk & Thorley-Lawson, 2005). The production of viral particles following lytic cycle enables the infection of new naïve B-lymphocytes and helps replenish the pool of infected cells. Therefore it seems that EBV persistence *in vivo* is a dynamic balance between various stages of infection and the host immunosurveillance. This proposed model of EBV infection, persistence and transmission *in vivo* is described in Figure 1.2. Although this model is widely accepted, it is worth acknowledging that certain caveats within the model have been opposed by other groups. However, further clarification will enable the validation of a model of for EBV persistence *in vivo*.



Adapted from Rickinson & Kieff 2007

Figure 1.2 A proposed model of EBV infection, transmission and persistence in an immunocompetent host

Viral particles, which enter through crypts in the lymphoepithelium or are released from infected tonsillar epithelial cells, infect naïve B-lymphocytes in the follicular mantle and establish latency III phenotype. These infected cells, which are susceptible to attack by host EBV-specific cytotoxic T-lymphocytes, can undergo germinal centre differentiation into memory B-lymphocytes. The long term persistence of EBV *in vivo* requires periodic reactivation in memory B-lymphocytes which produces new viral particles that are able to infect new naïve B-lymphocyte and replenish the pool of infected cells

1.1.5 EBV lytic cycle

In order to facilitate the transmission of EBV to another host, the virus produces progeny after entering its lytic cycle. Indeed, the maintenance of a persistent, life-long latent infection within an infected host requires periodic EBV replication in memory B-lymphocytes and occurs to sustain the long term reservoir of EBV *in vivo*. Out of the pool of EBV-infected B-lymphocytes, only a sub-population of cells are activated into lytic cycle. During EBV lytic cycle, >80 EBV lytic cycle genes are switched on and encode viral proteins that are required for the completion of the cycle. The expression of these genes occurs in temporal and sequential order and can be divided into immediate-early, early and late lytic gene subsets (Kieff & Rickinson, 2007).

The study of EBV lytic cycle *in vitro* has been hampered, to some degree, by the lack of a fully permissive *in vitro* culture system. However, this has been combated by the development of inducible systems such as EBV-positive BL cell lines, like Akata, and LCLs that can be stimulated into lytic cycle by treatment with chemicals, such as phorbol esters and *n*-butyrate, by calcium ionophore or by cross-linking of surface immunoglobulin (Saemundsen et al., 1980, Takada, 1984, Takada & Ono, 1989, Daibata et al., 1990, Kieff & Rickinson, 2007). In addition, a cellular model has been developed which enables immunosorting of cells induced into lytic cycle by anti-IgG (Ressing et al., 2005). Also, some EBV-transformed LCLs consistently contain a small population of cells (<5%) that have spontaneously entered lytic cycle (Kieff & Rickinson, 2007) and have, therefore, been utilised for lytic cycle research. These approaches have enabled the analysis of a number of EBV lytic cycle genes and some of these are described below.

1.1.5.1 Immediate-early lytic cycle genes

The expression of the immediate-early lytic cycle genes is induced directly from the BCR. Two critical immediate-early genes, BZLF-1 and BRLF1, were initially identified in experiments using P3HR-1 super-infected Raji BL cells (Miller et al., 1985, Biggin et al., 1987). BZLF-1 and BRLF1 both encode transcription factors which have been shown to control the transition from a latent to a lytic infection. BZLF-1 is

considered to be the major immediate-early EBV lytic protein and its expression alone is sufficient to trigger the lytic cycle cascade (Countryman et al., 1985, Rooney et al., 1989, Takada & Ono, 1989, Flemington & Speck, 1990). Indeed, the introduction of BZLF-1, or BRLF1, by transfection or viral-mediated transduction into EBV-associated tumour cell lines is sufficient to induce lytic cycle (Zalani et al., 1996, Ragoczy et al., 1998, Westphal et al., 1999, Feng et al., 2002).

BZLF-1 is transcribed as mRNA from the *Bam*HI Z promoter (Zp) and contains three exons which encode three separate functional domains within the BZLF-1 protein (Farrell et al., 1989, Lieberman & Berk, 1990). The first exon encodes a transactivating domain (amino acids 1-167), and the second exon encodes a DNA-binding domain (amino acids 168-202), homologous to *c-fos* and *c-jun*, which enables BZLF-1 to interact with AP-1 related DNA-binding sites and to be nuclear translocated (Mikaelian et al., 1993). The third exon encodes a coiled-coil domain (amino acids 203-245) which enables dimerisation of BZLF-1 at AP-1 related sites (Kieff & Rickinson, 2007). BZLF-1, along with BRLF1, transactivate the promoters of several early lytic cycle genes such as BSMLF1 and BMRF1 (Kenney et al., 1989, Holley-Guthrie et al., 1990, Quinlivan et al., 1993) which enable the continuation of the lytic cycle cascade. BZLF-1 also transactivates its own promoter as well as the adjacent BRLF1 promoter (Flemington & Speck, 1990).

Along with regulating the initiation of EBV lytic cycle, BZLF-1 can also modulate host cellular responses. BZLF-1 has been shown to interfere with p38 MAPK/ATF-2 (Adamson et al., 2000), NF- κ B (Gutsch et al., 1994, Morrison and Kenney, 2004), p53 (Zhang et al., 1994) and IFN- γ signalling (Morrison et al., 2001). It can also disperse nuclear promyelocytic leukaemia (PML) bodies (Adamson & Kenny, 2001), inhibit the anti-viral actions of interferon regulatory factor-7 (IRF7) (Hahn et al., 2005) and impede the ability of LMP1 to up-regulate cell surface MHC class I molecules (Keating et al., 2002). Indeed the last function contributes to the immune evasive properties of EBV infected cells in lytic cycle.

1.1.5.2 Early and late lytic cycle genes

Out of all the lytic cycle genes encoded by EBV, 38 are recognised as early lytic antigens (Lu et al., 2006, Yuan et al., 2006). BSMLF1 is the first early lytic cycle gene switched on during EBV lytic cycle (Yuan et al., 2006) and encodes a post-transcriptional regulatory protein, SM, which is essential for EBV replication (Gruffat et al., 2002, Ruvolo et al., 2004). Other encoded early lytic cycle proteins include BMRF1, the major DNA polymerase BALF5 and the alkaline exonuclease BGLF5 (Kieff & Rickinson, 2007). The function of DNA polymerase BALF5 is to produce new viral DNA during lytic cycle. BALF5 also recruits other EBV components, such as BMRF1, for this function and is dependent on transactivation by BSMLF1 and the immediate-early genes BZLF-1 and BRLF1 (Kieff & Rickinson, 2007). A number of early lytic cycle genes are also known to have immune evasive functions. BGLF5 has been shown to possess a host shutoff function in lytic cycle and blocks the synthesis of MHC class I and II molecules (Rowe et al., 2007). BNLF2a has also recently been implicated in reducing cell surface MHC class I and II expression during lytic cycle (Hislop et al., 2007), and BARF1 encodes a soluble human colony stimulating factor (CSF)-1 receptor that contributes to immune evasion (Strockbine et al., 1998).

Forty EBV lytic cycle genes are recognised as late antigens (Lu et al., 2006, Yuan et al., 2006) and are encoded to enable cleavage, packaging and envelopment of the viral DNA episome and formation of infectious virions. A large proportion of these late lytic genes encode virion proteins such as the viral glycoproteins gp350/220 (BLLF1), gH (BXLF2), gB (BALF4), gL (BKRF2), gp42 (BZLF-2) and gp150 (BDLF3) which form part of the outer membrane of EBV (Johannsen et al., 2004, Kieff & Rickinson, 2007). Other late lytic cycle genes include the viral capsid antigen (VCA), the protease BVRF2 and BCRF1. BCRF1, which shares 90% homology with human interleukin-10 (IL-10) (Hsu et al., 1990, Vieira et al., 1991), also has an immune evasive function since it can inhibit IFN- γ responses (Hsu et al., 1990, Kurilla et al., 1993). BZLF-2 also contributes to EBV immune evasion by obstructing interactions between with T-cell receptor on EBV-specific CD4⁺ T-cells and MHC class II molecules on EBV-infected cells (Ressing et al., 2003). It seems clear, therefore, that many early and late lytic cycle genes are expressed

not only to aid EBV replication within the host cell but also to prevent recognition by the host immunosurveillance.

1.1.6 EBV-associated disease

EBV is associated with the onset of diseases of B-lymphocyte origin, such as Burkitt's lymphoma, as well as those originating from epithelial cells, T-lymphocytes and natural killer (NK) cells. The majority of these diseases are EBV-associated malignancies (summarised in Table 1.1) which display different EBV latency programs. The association of EBV with a number of B-lymphocyte malignancies is not surprising considering that primary B-lymphocytes are the main targets of EBV. To add to this, the presence of EBV in epithelial malignancies, such as nasopharyngeal carcinoma (NPC) and gastric carcinoma (zur Hausen et al., 1970, Wolf et al., 1973, Shibata & Weiss, 1992, Tokunaga et al., 1993, Rickinson & Kieff, 2007), and in T-lymphocyte and NK cell lymphomas (Jones et al., 1988, Kikuta et al., 1988, Harabuchi et al., 1990, Rickinson & Kieff, 2007) indicates further that EBV is not B-lymphocyte specific. In this section, the B-lymphocyte infections and malignancies associated with EBV are discussed further.

1.1.6.1 Infectious mononucleosis

When primary EBV infection occurs during infancy or childhood, it presents as an asymptomatic infection. In contrast, if this primary EBV infection occurs later on during adolescence or early adulthood, it manifests as a self-limiting lymphoproliferative disease termed infectious mononucleosis (IM) in at least 25% of cases (Rickinson & Kieff, 2007). The symptoms of IM include pharyngitis, lymphadenopathy, headache and malaise and their severity is variable in IM patients (Rickinson & Kieff, 2007). A key feature of IM is a huge CD8⁺ cytotoxic T-lymphocytosis that is induced to control the extensive proliferation of EBV-infected B-lymphocytes in the tonsils. These reactive CD8⁺ T-cells recognise both latent and lytic viral antigens and become subsequently maintained in the memory T-lymphocyte reservoir (Hislop et al., 2002). It is believed that

many of the symptoms of IM are caused by excessive secretion of pro-inflammatory cytokines, such as IFN- γ and TNF- α , by these CD8⁺ T-lymphocytes following lysis of targeted EBV-infected cells (Anagnostopoulos et al., 1995, Niedobitek et al., 1997a, Wright-Browne et al., 1998).

1.1.6.2 X-linked lympholiferative syndrome

X-linked lymphoproliferative syndrome (XLP) is a rare familial disease that was first identified in the 1970s and is characterised by an extreme sensitivity to primary EBV infection in young boys (Bar et al., 1974, Purtilo et al., 1975). XLP results from a mutation in the XLP gene and, in 75% of cases, patients die from acute, fatal IM within one month of the primary EBV infection (Thorley-Lawson, 2001). This fatal form of IM produces a massive infiltration of CD8⁺ T-lymphocytes, macrophages and EBV-infected B-lymphocytes into the liver and bone marrow and results in severe hepatitis and bone marrow failure (Thorley-Lawson, 2001). The XLP gene encodes a src-homology 2 (SH2) domain-containing protein called signalling lymphocyte activation molecule-associated protein (SAP) which is involved in regulating both T- and B-lymphocyte signalling. A mutation in the SH2 domain leads to a failure in T-lymphocyte immunosurveillance (Coffey et al., 1998, Sayos et al., 1998) although the reason why it results in an extreme sensitivity to EBV is not known. A third of cases do survive the initial primary EBV infection but subsequently develop either hypogammaglobulinaemia or some form of malignant B-lymphocyte lymphoma (Rickinson & Kieff, 2007).

1.1.6.3 Burkitt's lymphoma

EBV was first discovered in cultured lymphoma cells from patients with the endemic form of Burkitt's lymphoma (BL) (Epstein et al., 1964). Endemic BL, which is one of three types of BL, is the most common malignancy in children throughout equatorial Africa with an incidence of 50-100 cases per million individuals each year (Rickinson & Kieff, 2007). This form of BL correlates geographically with areas supportive of holoendemic malaria, and shows the most frequent association with EBV

(95-100%). This tumour is also clinically distinct both in its unusual presentation and its histology. BL also manifests as a rare, sporadic tumour which, although histologically and clinically similar to endemic BL, has a much lower incidence rate (Rickinson & Kieff, 2007). Sporadic BL is seen at roughly 50-100 times lower incidence in Europe and the United States and its association with EBV is also much lower at 15-30%. The third form of BL manifests in individuals with acquired immunodeficiency syndrome (AIDS) and is described further in section 1.1.6.6.

All three forms of BL originate from proliferating GC B-lymphocytes and display a centroblast cell differentiation phenotype (Gregory et al., 1987, Ling et al., 1989, Rickinson & Kieff, 2007). Like centroblasts, BL tumour cells express bcl-6, CD10 and CD77 and lack surface markers associated with a lymphoblastoid phenotype like CD23, CD30, CD39, CD80 and the adhesion molecules CD54 (ICAM-1) and CD58 (LFA-3) (Rickinson & Kieff, 2007). BL tumour cells are monoclonal with respect to EBV infection (Raab-Traub & Flynn, 1986) and surface immunoglobulin rearrangement (Magrath, 1990), and also exhibit evidence of active somatic hypermutation (SHM) – a further characteristic of GC B-lymphocytes (Chapman et al., 1995, Chapman et al., 1996, Sale & Neuberger, 1998, Harris et al., 2001). The defining feature of BL tumours, irrespective of EBV status, is a reciprocal chromosomal translocation that places *c-myc* at chromosome 8q24 adjacent to one of three immunoglobulin heavy (IgH) or light chain (IgL) genes on chromosomes 2, 14 or 22 (Hecht & Aster, 2000). The most common translocation (in up to 80% of tumours) out of all these sees *c-myc* placed adjacent to the IgH gene on chromosome 14 (Rickinson & Kieff, 2007). This translocation results in the inappropriate expression and constitutive activation of *c-myc*, which promotes uncontrolled cell proliferation and is likely to have occurred as an error of SHM or Ig class switching (Goossens et al., 1998, Kuppers & Dalla-Favera, 2001). Mutations in the tumour suppressor genes p53 (Gaidano et al., 1991, Bhatia et al., 1992) and retinoblastoma-like 2 (RB2) (Cinti et al., 2000) have also been observed in cases of BL. The p53 mutations were found to be independent of geography and EBV status (Bhatia et al., 1992) whereas the RB2 mutations were more frequent in endemic BL (Cinti et al., 2000). It is possible that these genetic mutations may facilitate tumour progression in some cases.

Most EBV-positive BL tumours *in vivo* display a Latency I viral program where only EBNA1, the EBERs and BARTs are expressed (Rowe et al., 1987b). EBNA1, which maintains the viral DNA as a monoclonal episome (Neri et al., 1991), is transcribed from the Qp promoter in Latency I whereas Wp, Cp and LMP promoters are all silenced (Sample et al., 1986, Schaefer et al., 1991, Schaefer et al., 1995, Nonkwelo et al., 1996). Although considering this, it is not fully established whether EBV naturally adopts a Latency I infection in BL or whether BL arises from a clone selected from a pool of Latency III, EBV-transformed B-lymphocytes. However, there is evidence which implies that BL is selected from a Latency III progenitor pool through down-regulation of EBNA2 (Kelly et al., 2002). Indeed, this study identified a restricted form of latency in a subset of African endemic BL tumour biopsies where Wp, but not Qp promoter, was the active in the absence of EBNA2 (Kelly et al., 2002). This suggests that EBNA2 down-regulation may be a factor of BL tumour evolution.

The role of EBV in BL pathogenesis is still controversial. However, there is evidence which implicates EBV for this role. Firstly, it has been proposed that EBV may have an initiating role in establishing a pool of EBV-transformed B-lymphocytes that are prone to chromosomal *c-myc*/Ig translocations (Polack et al., 1996). Also, it has been suggested that EBNA1 and the EBERs contribute to malignancy since EBNA1 induces B-lymphocyte lymphomas in transgenic mice (Wilson et al., 1996), and the EBERs induce IL-10 secretion and mediate apoptotic resistance to IFN- α (Kitagawa et al., 2000, Nanbo et al., 2002) which may promote tumour growth and survival. Although the role of EBV in BL malignancy needs further clarification, it is known that EBV enables BL cells to evade the host immunosurveillance. Firstly, EBV restricts viral gene expression to EBNA1 in most *in vivo* BL tumours since it is poorly recognised by EBV-specific CD8⁺ CTLs (Levitskaya et al., 1995). Also, EBV-positive BL tumours exhibit low levels of MHC class I molecules and proteins of the endogenous antigen-processing pathway, such as TAP, which enables evasion from MHC class I restricted CD8⁺ CTLs (Rooney et al., 1985, Rowe et al., 1995).

1.1.6.4 Hodgkin's lymphoma

Hodgkin's lymphoma (HL) is characterised by a population of atypical tumour cells referred to as Hodgkin and Reed-Sternberg (HRS) cells within a granuloma composed of non-malignant infiltrating lymphocytes, macrophages, eosinophils and plasma cells (Harris et al., 1994, Kuppers, 2002). HRS cells represent less than 2% of cells within the tumour and, based on their morphology and the composition of the tumour infiltrate, HL is divided into three subtypes: nodular sclerosing (NS), mixed cellularity (MC) and the rarer lymphocyte depleted (LD) (Harris et al., 1994). HL has a worldwide distribution and incidence rates do not vary greatly between countries (Rickinson & Kieff, 2007). However, there are differences in age, sex and subtype distribution. These three subtypes, collectively known as classical HL, display a varied association with EBV within each subtype. HL tumours of the MC and LD subtypes show higher rates of association (60-90% EBV-positive) compared to tumours of the NS subtype (20-40% EBV-positive). Also, childhood HL is almost entirely EBV-associated in the developing world whereas, by contrast, half of childhood HL in the Western world is EBV-associated (Rickinson & Kieff, 2007).

The cellular origin of malignant HRS cells in classic HL remains elusive. However, there is evidence which implies that they may derive from pre-apoptotic GC B-lymphocytes that have acquired 'crippling' mutations in the variable regions of their Ig genes (Kanzler et al., 1996, Foss et al., 1999, Kuppers et al., 2002). Such B-lymphocytes are usually eliminated by apoptosis but are rescued by a transforming event (Kanzler et al., 1996). In EBV-associated HRS cells, the viral DNA is maintained as a monoclonal episome (Anagnostopoulos et al., 1989) and the cells display a Latency II viral program where EBNA1, LMP1, LMP2A, LMP2B, the EBERs and BARTs are expressed (Herbst et al., 1991, Deacon et al., 1993, Grasser et al., 1994, Niedobitek et al., 1997b). The transforming gene LMP1 and LMP2A may have a role in the rescue of the pre-apoptotic GC B-lymphocytes. LMP1 and, in most cases, LMP2A are expressed at unusually high levels in HRS cells (Pallesen et al., 1991, Niedobitek et al., 1997). LMP1 is transcribed from an alternative promoter, L1-TR, in classical HL which does not require EBNA2 and is responsive to the transcription factor STAT3 (Chen et al., 2001). LMP1 has been

shown to constitutively activate NF- κ B in HRS cells which aids apoptotic resistance (Bargou et al., 1997), and LMP2A has also been shown to provide survival signals to B-lymphocytes *in vivo* in the absence of the BCR (Caldwell et al., 1998). Also, the PI3K/Akt pathway, a signalling target of LMP1 is constitutively activated in HRS (Dutton et al., 2005) and contributes to their survival.

Other functions of EBV in the pathogenesis of classic HL include immune evasion in the HL tissue. Although EBV-associated HRS cells express LMP1 and LMP2A, which are both well recognised by EBV-specific CD8⁺ CTLs (Meij et al., 2002), LMP1 stimulates the induction of the CCL17 and CCL22 chemokines which attract CCR4-expressing T_H2 and regulatory T-lymphocytes (Van den Berg et al., 1999, Nakayama et al., 2004). This enables the HRS cells to receive survival signals from the T_H2 lymphocytes and the regulatory T-lymphocytes can inhibit the host EBV-specific CTL response. In addition, immune evasion can be achieved through LMP1-mediated production of the immunosuppressive cytokines IL-10 and transforming growth factor (TGF)- β (Marshall et al., 2004, Ishida et al., 2006). Also, the development of HL requires the impairment of B-lymphocyte differentiation which occurs due to a lack of EBNA2 expression in Latency II (Kuppers, 2002).

1.1.6.5 Post-transplant lymphoproliferative disease

The T-lymphocyte immunosuppression that is pharmacologically induced in patients after transplantation is associated with an enhanced risk of developing post-transplant lymphoproliferative disease (PTLD). The link between EBV and many cases of PTLD is well documented and most PTLDs arise within the first year after transplantation (Rickinson & Kieff, 2007). The incidence rates of PTLD differ between the types of transplant and the intensity of immunosuppressive therapy associated with them. Incidence rates in renal and liver transplants are lower (1-5%) than that seen with heart, lung or small bowel transplants (5-15%) (Rickinson & Kieff, 2007). To add to this, EBV serostatus is also a determining factor since solid organ transplant recipients showed a 20 times higher incidence of PTLD if they were EBV-seronegative (Ho et al., 1985). This highlights why young children are at particular risk.

Most PTLDs present as monoclonal or polyclonal lesions and are nearly always of B-lymphocyte origin (Knowles, 1998). EBV is associated with 100% of early onset PTLDs and only 80% of late onset PTLDs (Capello et al., 2003, Rickinson & Kieff, 2007). The presence of SHM activity (Brauninger et al., 2003, Timms et al., 2003, Capello et al., 2003) and the cell surface expression of the centroblast marker CD77 (Randhawa et al., 1994, Arbus et al., 2000) in PTLD lesions provides strong evidence that these B-lymphocytes are GC-derived B-lymphocytes. The majority of EBV-transformed B-lymphocytes in PTLD lesions display a Latency III viral program where all six EBNA (EBNA-1, -2, -3A, -3B, -3C and -LP), LMP1, LMP2A, LMP2B, the EBERs, the BARTs and microRNAs are expressed (Young et al., 1989, Kieff & Rickinson, 2007). This Latency III program in PTLD is captured in EBV-transformed LCLs which are generated by *in vitro* transformation of primary B-lymphocytes (Kieff & Rickinson, 2007). However, more restricted patterns of EBV latency have also been observed in PTLD (Oudejans et al., 1995). PTLD is thought to arise as the T-lymphocyte immunosuppressive regime impairs the function of EBV-specific CD8⁺ CTLs, which usually control the proliferation of EBV-infected B-lymphocytes (Rickinson & Moss, 1997). Thus, it is clear that, in the immunocompromised host, the normal T-lymphocyte control over EBV-infected B-lymphocytes is lost and leads to the manifestation of PTLD (Haque et al., 2002). This model is supported by the observation that relaxation of T-lymphocyte immunosuppression can lead to tumour regression (Starzl et al., 1984, Tsai et al., 2001).

1.1.6.6 AIDS-associated B-lymphocyte lymphoma

Individuals with AIDS are at a high risk of developing B-lymphocyte lymphomas, similar to that seen in transplant patients (Duval et al., 2004). Many types of AIDS-associated B-lymphocyte lymphoma exist and display a variable association with EBV (Carbone, 2003). These include immunoblastic or centroblastic diffuse large-cell lymphomas (DLCL), tumours of the primary central nervous system (CNS) and primary effusion lymphomas (PEL). EBV is associated with 90% of immunoblastic tumours and with 100% of CNS-associated tumours although 70% of centroblastic DLCL tumours are

EBV-negative (Rickinson & Kieff, 2007). Many of these tumours originate from post-GC B-lymphocytes (Gaidano et al., 2003) and display a Latency III viral program although in centroblastic DLCL and PEL this viral program can be restricted to Latency I (Gaidano et al., 1998, Hamilton-Dutoit et al., 1993).

Unlike immunosuppressed transplant patients, AIDS-affected individuals can also develop AIDS-associated BL tumours which contain the characteristic chromosomal *c-myc* translocation (Hamilton-Dutoit et al., 1991). Between 30-40% of these BL tumours are EBV-associated and appear to manifest in patients who are relatively immunocompetent (Carbone, 2003). They also display a restricted Latency I viral program and seem to have a similar pathogenesis to sporadic BL (Hamilton-Dutoit, 1993).

In summary, it is clear that EBV infection in an immunocompetent host can be harmless for the life of the host. However, it is evident that EBV can become pathological as observed by the consistent detection of EBV in many human lymphoid and epithelial malignancies. The host EBV immunosurveillance is vital for controlling unwanted growth of EBV-infected cells and any impairment lead to the infected cells overpowering the immune response. The recent identification of new tumours associated with EBV, such as the smooth muscle leiomyosarcomas, highlights that are knowledge of EBV is still incomplete. Therefore, a better understanding of EBV biology *in vitro* and *in vivo* is clearly vital.

1.2 Signal Transducer and Activator of Transcription 1

1.2.1 Discovery of STATs

The Signal Transducer and Activators of Transcription (STAT) proteins are a family of latent cytoplasmic transcription factors that contain seven mammalian members. They were named after their dual role as 'signal transducers' and 'activators of transcription' and were initially identified in the early 1990s as targets of IFN activation by purifying proteins bound to the promoters of IFN-responsive genes (Fu et al., 1992, Schindler et al., 1992a, Schindler et al., 1992b Shuai et al., 1992, Darnell et al., 1994). STAT1 and STAT2 were the first STATs identified as components of either an IFN- α induced DNA-bound complex termed interferon-stimulated gene factor -3 (ISGF3) or an IFN- γ induced DNA-bound complex termed γ -activated factor (Fu et al., 1992, Shuai et al., 1992). The identification and cloning of the other five members of the STAT family: STAT3, STAT4, STAT5a, STAT5b and STAT6 soon followed (Akira et al., 1994, Hou et al., 1994, Yamamoto et al., 1994, Zhong et al., 1994, Hou et al., 1995, Liu et al., 1995, Quelle et al., 1995, Wakao et al., 1995, Lin et al., 1996).

1.2.2 STAT structure

The genes of seven mammalian members of the STAT family of transcription factors are localised to three human chromosomal regions: chromosome 2 (STAT1 and STAT4, chromosome 12 (STAT2 and STAT6) and chromosome 17 (STAT3, STAT5a and STAT5b) (Ihle, 2001). All seven STATs range in size between 750 and 850 amino acids in length. STAT1 is composed of 750 amino acids and has a molecular weight of 91kDa. STAT1, also known as STAT1 α , has a naturally occurring spliced variant, STAT1 β , which is reported to act as a dominant-negative inhibitor of STAT1 α (Bromberg et al., 1996, O'Shea et al., 2002). Studies using X-ray crystallography revealed several domains are conserved in all STAT proteins (Becker et al., 1998, Chen et al., 1998, Vinkemeier et al., 1998), each of which has a key function for STAT

activity. It is now known that each STAT contains 6 six functional domains. The domain structure of STAT1 α and its naturally occurring spliced form STAT1 β are illustrated in Figure 1.3.

The N-terminal domain (amino acids 1-135) is functionally necessary for protein-protein interactions. This enables the formation of STAT dimers and also mediates the oligomerisation of STAT dimers to form tetramers (Vinkemeier et al., 1996). The tetramer formation of STATs contributes to the stabilisation of STAT-DNA binding complexes (John et al., 1999). The coiled coil domain (amino acids 136-317) has been found to interact and associate with other regulatory proteins such as the *N-myc* interacting protein-1 (Nmi-1) and IRF9 (Xu et al., 1996, Zhu et al., 1999, Horvath, 2000). The DNA-binding domain (amino acids 318-488) contains several β -pleated sheets, folded similarly to the DNA-binding domains of NF- κ B and p53 (Levy & Darnell, Jr., 2002), which interact directly with STAT binding sites in gene promoters at the core consensus sequence TT(N₄₋₆)AA (Horvath et al., 1995). The DNA-binding specificity of each STAT is determined by this domain (Horvath, 2000). The linker domain (amino acids 488-576) bridges the DNA-binding domain to the SH2 domain (Chen et al., 1998), and it has been shown that mutations within this domain affect DNA-binding stability (Yang et al., 2002).

The SH2 domain (amino acids 577-683), which is a phosphotyrosine binding domain found commonly in signalling molecules, is essential for the reciprocal interactions between STAT monomers during dimer formation (Horvath, 2000). This domain also mediates receptor-STAT interactions and confers selectivity for different receptors by each STAT protein (Shuai et al., 1994, Becker et al., 1998). The C-terminal transactivation domain (TAD) (amino acids 684-750) promotes transcriptional activity for all STATs and the interactions with co-activators, like MCM5, BRCA1 and the histone acetyltransferase, CREB-binding protein (CBP)/p300 (Bhattacharya et al., 1996, Zhang et al., 1996, Horvai et al., 1997, Morrigl et al., 1997, Korzus et al., 1998, Zhang et al., 1998, Ouchi et al., 2000). Within this domain all STATs contain a critical tyrosine residue (positioned at ~700 amino acids) whose phosphorylation is thought to be vital for STAT activation (Shuai et al., 1993, Becker et al., 1998) as described in section 1.2.3. In addition, all STATs, except STAT2 and STAT6, contain a serine residue, located around

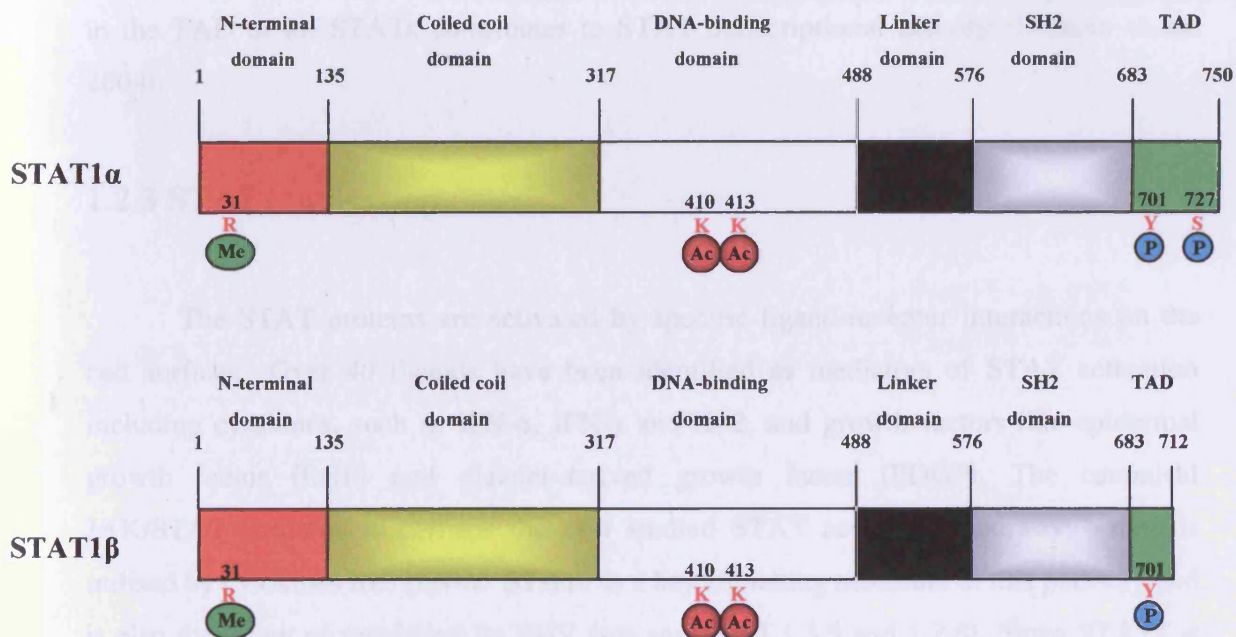


Figure 1.3 Domain structure of STAT1α and STAT1β

STAT1α and its naturally truncated form STAT1β contain six functional domains: The N-terminal domain, the coiled coil domain, the DNA-binding domain, the linker domain, the SH2 domain and the transactivation domain (TAD). STAT1β has a shorter TAD (29 amino acids long) which does not contain the serine 727 amino acid that is phosphorylated in order for STAT1 to achieve its maximal transcriptional potential. The sites of STAT1 tyrosine and serine phosphorylation, arginine methylation and lysine acetylation are shown.

amino acid 730, within the TAD whose phosphorylation is crucial for maximal STAT transcriptional activation (Wen et al., 1995, Cho et al., 1996, Decker & Kovarik, 2000). The naturally occurring spliced forms of both STAT1 and STAT3, formed by alternative mRNA splicing, lack part of the TAD domain including the serine residue and mediate inhibitory effects against full length STAT1 α and STAT3 α (Bromberg et al., 1996, Caldenhoven et al., 1996, O'Shea et al., 2002). Also, a recently identified proline residue in the TAD of all STATs contributes to STAT transcriptional activity (Gamero et al., 2004).

1.2.3 STAT1 activation

The STAT proteins are activated by specific ligand-receptor interactions on the cell surface. Over 40 ligands have been identified as mediators of STAT activation including cytokines, such as IFN- α , IFN- γ and IL-2, and growth factors like epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). The canonical JAK/STAT pathway is perhaps the best studied STAT activation pathway, which is utilised by cytokines like IFN- α . STAT1 is a key signalling molecule in this pathway and is also the target of regulation by EBV (see sections 1.1.3.5 and 1.2.8). Since STAT1 is the focus of investigation in this thesis, this introduction will concentrate mainly on the activation and function of STAT1 but will also compare its role to other STAT proteins.

The activation of STAT1 by IFN, involving the canonical JAK/STAT pathway is shown schematically in Figure 1.4. The interaction between IFN and its cell surface receptor results in the tyrosine phosphorylation of two recruited tyrosine kinases called Janus kinases (JAKs) which associate with the receptor. The JAKs are a family of four receptor-associated tyrosine kinases, JAK1, JAK2, JAK3 and TYK2, which are activated by receptor dimerisation or oligomerisation. They contain seven conserved domains out of which the two N-terminal domains, JH6 and JH7, facilitate binding of the JAKs to the receptors (Levy & Darnell, Jr., 2002). Once the JAKs become activated, or tyrosine phosphorylated, they mediate tyrosine phosphorylation of the IFN receptor tails through their catalytic, C-terminal kinase domain. This event provides docking sites for the recruitment of SH2-domain containing proteins like STAT1 (Greenlund et al., 1995).

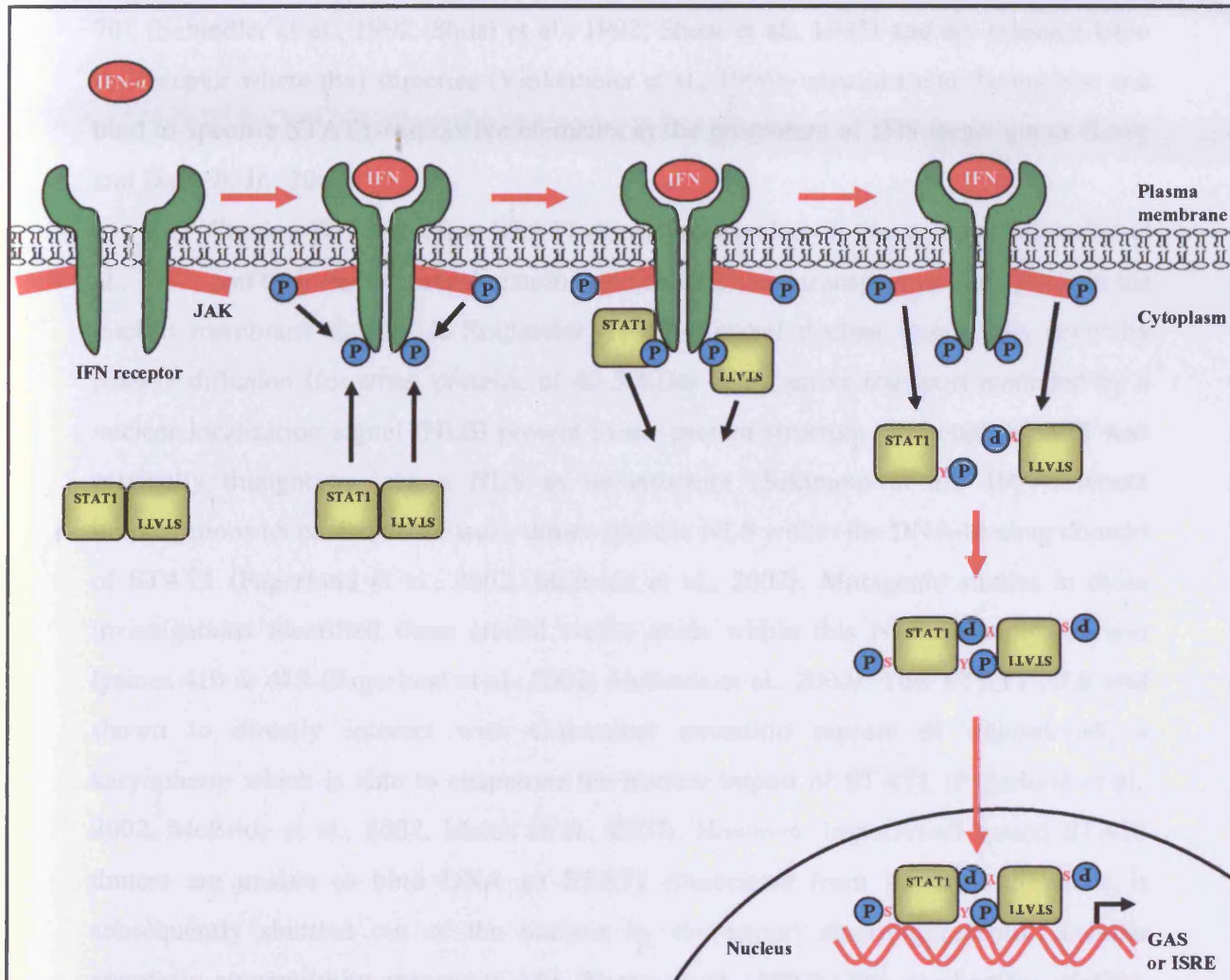


Figure 1.4 Diagram of JAK/STAT1 signalling in response to IFN

The interaction between IFN and its cognate, cell surface receptor results in the tyrosine phosphorylation of two recruited tyrosine kinases called Janus kinases (JAKs) which associate with the receptor. Once the JAKs become activated, or tyrosine phosphorylated, they mediate tyrosine phosphorylation of the IFN receptor tails through their catalytic, C-terminal kinase domain. STAT1 monomers, located in the cytoplasm as an unphosphorylated dimer, are recruited to the receptor tails and bind through their SH2 domains. Receptor-bound STAT1 monomers then become tyrosine phosphorylated at amino acid 701. The tyrosine phosphorylated STAT1 monomers are released from the receptor where they dimerise, translocate to the nucleus and bind to specific STAT1-responsive elements in the promoters of IFN target genes.

STAT1 is found latent in the cytoplasm as an unphosphorylated dimer (Lackmann et al., 1998, Braunstein et al., 2003) but docks at the IFN receptor tails as a monomer. Receptor-bound STAT1 monomers then become tyrosine phosphorylated at amino acid 701 (Schindler et al., 1992, Shuai et al., 1992, Shuai et al., 1993) and are released from the receptor where they dimerise (Vinkemeier et al., 1996), translocate to the nucleus and bind to specific STAT1-responsive elements in the promoters of IFN target genes (Levy and Darnell, Jr., 2002).

Following IFN activation STAT1 dimers accumulate in the nucleus (Schindler et al., 1992) and their nuclear translocation requires facilitated transport through pores in the nuclear membrane (Mattaj & Englmeier, 1998). General nuclear import can occur by passive diffusion (for small proteins of 40-50kDa) or by active transport mediated by a nuclear localisation signal (NLS) present in the protein structure. Although STAT1 was originally thought to lack a NLS in its structure (Sekimoto et al., 1997), recent investigations identified an unusual, dimer-specific NLS within the DNA-binding domain of STAT1 (Fagerlund et al., 2002, McBride et al., 2002). Mutagenic studies in these investigations identified three crucial amino acids within this NLS: leucine 407 and lysines 410 & 413 (Fagerlund et al., 2002, McBride et al., 2002). This STAT1 NLS was shown to directly interact with C-terminal armadillo repeats of importin- α 5, a karyopherin which is able to chaperone the nuclear import of STAT1 (Fagerlund et al., 2002, McBride et al., 2002, Melén et al., 2003). However, importin- α 5 bound STAT1 dimers are unable to bind DNA so STAT1 dissociates from importin- α 5 which is subsequently shuttled out of the nucleus by the export shuttling receptor, cellular apoptosis susceptibility protein (CAS) (Kutay et al., 1997). The mechanism of IFN-induced STAT1 nuclear import also requires energy created by the Ran GTPase enzyme which converts Ran GTP into Ran GDP (Sekimoto et al., 1996, Sekimoto et al., 1997). This creates an energy gradient that facilitates transport through pores in the nuclear membrane. In addition to this, it appears that the N-terminus of STAT1 can also provide signals for cytokine-induced nuclear translocation (Strehlow & Schindler, 1998).

The activation of STAT1 by IFN is slightly different when stimulated by type I IFNs (IFN- α , IFN- β) compared to type II IFNs (IFN- γ). This leads to the formation of different STAT1 dimer types which form DNA-bound complexes on STAT1-responsive

elements. Furthermore, the activation of STAT1 by growth factors utilises a dissimilar mechanism altogether. The following sub-sections describe the nature of STAT1 activation by type I IFNs, type II IFNs and growth factors.

1.2.3.1 STAT1 activation by Type I IFNs

The binding of type I IFN (IFN- α , IFN- β) to its receptor induces dimerisation of the IFN α R1 and IFN α R2 subunits of the receptor. The IFN α R1 subunit associates with TYK2 and the IFN α R2 subunit with JAK1, and these JAKs become subsequently tyrosine phosphorylated following receptor dimerisation (Colamonici et al., 1994, Gauzzi et al., 1996). Activated TYK2 and JAK1 then phosphorylate the IFN α R1 and IFN α R2 tails recruiting STAT1 or STAT2 monomers which become tyrosine phosphorylated at amino acids 701 and 690 respectively (Yan et al., 1996). Indeed the recruitment of STAT1 is dependent on the presence of receptor-docked STAT2 whose phosphorylation favours the binding of STAT1 (Leung et al., 1995, Meraz et al., 1996, Qureshi et al., 1996). Both tyrosine phosphorylated STAT1 and STAT2 monomers then dissociate from the receptor and form a STAT1: STAT2 heterodimer. The STAT1 component of the dimer, but not STAT2, is also serine phosphorylated at amino acid 727 which is crucial for maximal transcriptional activation (Decker & Kovarik, 2000). The heterodimer then can enter the nucleus and bind to γ -activated sequence elements (GAS) (TTNCNNNAA) in the promoters of IFN-responsive genes (Darnell et al., 1994). However, this STAT1:STAT2 heterodimer can also associate with an adaptor protein from the interferon-regulatory factor family called p48 (or IRF9) and subsequently form the heterotrimeric interferon stimulated gene factor-3 (ISGF3) complex (Fu et al., 1992). The interaction with p48 occurs through the coiled-coil domains of both STAT1 and STAT2. Following nuclear import, this ISGF3 complex initiates transcription at IFN-stimulated response elements (ISRE) (AGTTTCNNTTTCNC) located in the promoters of target genes (Fu et al., 1992, Darnell et al., 1994). The naturally truncated form of STAT1, STAT1 β , can also form part of the ISGF3 complex and frequently interchanges with STAT1 α (Horvath, 2000). Interestingly, type I IFNs also induce the formation of STAT1: STAT1 homodimers as well as STAT1:STAT3 heterodimers which both can bind to

GAS elements in target genes (Darnell et al., 1994, Zhong et al., 1994, Chatterjee-Kishore et al., 2000). The variation in STAT1 dimer formation in response to type I IFNs like IFN- α is illustrated in Figure 1.5.

1.2.3.2 STAT1 activation by Type II IFNs

The binding of type II IFN (IFN- γ) to its receptor induces dimerisation of the IFN γ R1 and IFN γ R2 subunits of the receptor. The IFN γ R1 subunit is associated with JAK1 and the IFN γ R2 subunit with JAK2 which become activated by reciprocal tyrosine phosphorylation (Igarashi et al., 1994). Activated JAK1 and JAK2 then phosphorylate the IFN γ R1 and IFN γ R2 tails recruiting latent STAT1 monomers which dock to the phosphorylated receptor via their SH2-domains. Each STAT1 monomer becomes tyrosine phosphorylated at amino acid 701 by the JAKs and dissociate to form a STAT1:STAT1 homodimer (Shuai et al., 1994). This homodimer is also serine phosphorylated at amino acid 727, and then translocates to the nucleus and binds to GAS elements in the promoters of IFN- γ responsive genes (Darnell et al., 1994). IFN- γ pre-dominantly activates the formation of STAT1:STAT1 homodimers but could potentially form other STAT1 complexes since it can also directly stimulate STAT2 tyrosine phosphorylation (Zimmerman et al., 2005).

1.2.3.3 STAT1 activation by growth factors

In contrast to cytokine receptors which bind cellular tyrosine kinases, growth factor receptors, such as the EGF or PDGF receptor, possess intrinsic tyrosine kinase activity and are able to directly phosphorylate STAT proteins themselves (Leaman et al., 1996, Vignais et al., 1996, Olayioye et al., 1999, Wang et al., 2000a). Studies have shown that STAT1 is tyrosine phosphorylated by the EGF and PDGF receptor and leads to subsequent STAT1 activation (Vignais & Gilman, 1999, Levy & Darnell, Jr., 2002). In some cases, this effect is indirect and requires the recruitment of non-receptor tyrosine kinases, such as *src*, by a ligand-independent mechanism (Levy & Darnell, Jr., 2002).

1.2.4 STAT1 post-translational modifications

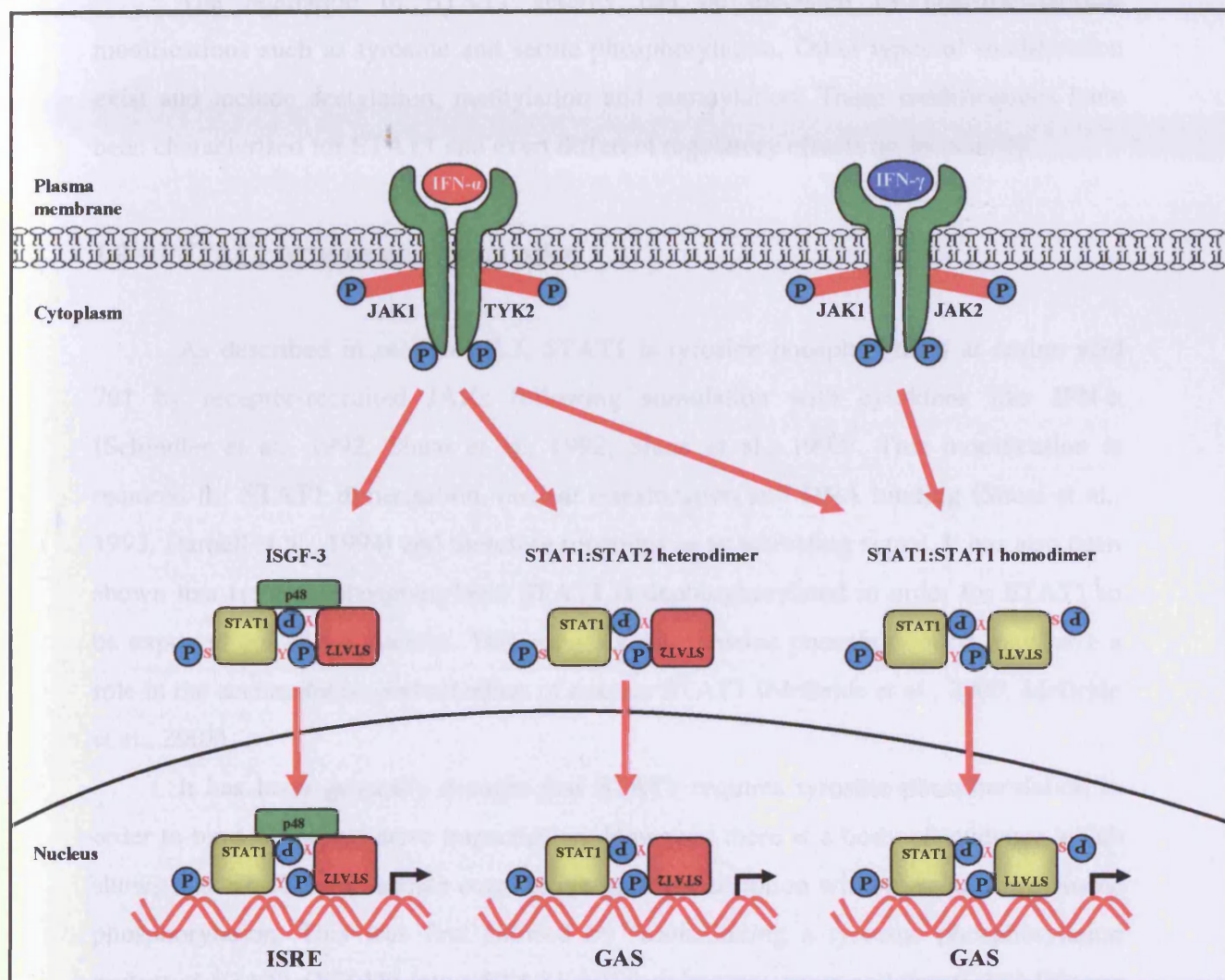


Figure 1.5 Variation in STAT1 activation by IFN-α and IFN-γ

The binding of IFN-α to its receptor causes tyrosine phosphorylation of the JAK1 and TYK2 tyrosine kinases. This event induces the activation of one of three types of STAT1 dimers: a STAT1:STAT1 homodimer, a STAT1:STAT2 heterodimer or the heterotrimer ISGF-3. These dimers bind to GAS elements in the promoters of IFN-α target genes, except ISGF-3 which binds to ISRE sites. In contrast, the binding of IFN-γ to its receptor causes tyrosine phosphorylation of the JAK1 and JAK2 tyrosine kinases. This event induces the activation and formation of a STAT1:STAT1 homodimer which binds to GAS elements in the promoters of IFN-γ responsive genes.

1.2.4 STAT1 post-translational modifications

The regulation of STAT1 activity can be mediated by post-translational modifications such as tyrosine and serine phosphorylation. Other types of modification exist and include acetylation, methylation and sumoylation. These modifications have been characterised for STAT1 and exert different regulatory effects on its activity.

1.2.4.1 STAT1 tyrosine phosphorylation

As described in section 1.2.3, STAT1 is tyrosine phosphorylated at amino acid 701 by receptor-recruited JAKs following stimulation with cytokines like IFN- α (Schindler et al., 1992, Shuai et al., 1992, Shuai et al., 1993). This modification is required for STAT1 dimerisation, nuclear translocation and DNA binding (Shuai et al., 1993, Darnell et al., 1994) and therefore functions as an activating signal. It has also been shown that tyrosine phosphorylated STAT1 is dephosphorylated in order for STAT1 to be exported out of the nucleus. This suggests that tyrosine phosphorylation may have a role in the accumulation and retention of nuclear STAT1 (McBride et al., 2000, McBride et al., 2002).

It has been generally thought that STAT1 requires tyrosine phosphorylation in order to bind DNA and drive transcription. However, there is a body of evidence which shows that STAT1 can initiate constitutive gene transcription without requiring tyrosine phosphorylation. This was first showed by reconstituting a tyrosine phosphorylation mutant of STAT1 (Y701F) into a STAT1 deficient human cancer cell line (U3A) (Kumar et al., 1997). This study demonstrated constitutive expression of the caspase 1-3 genes induced by STAT1 (Y701F) which enabled the cells to undergo apoptosis upon challenge (Kumar et al., 1997). Unphosphorylated STAT1 has also been shown to regulate the constitutive expression of other cellular genes including MHC class I antigens in T-lymphocytes (Lee et al., 1999b), low molecular weight protein-2 (LMP-2) (Chatterjee-Kishore et al., 2000), and IL-1 β (Unlu et al., 2007). In addition, it has been shown that unphosphorylated STAT1 can shuttle between the cytoplasm and nucleus in a carrier-dependent manner (Meyer et al., 2002, Marg et al., 2004). STAT1 has also been found in

the nuclei of unstimulated cells (Schindler et al., 1992), and has been identified in pre-formed STAT1:STAT2 and STAT1:STAT3 complexes in unstimulated HeLa cells (Stancato et al., 1996). This evidence along with indications that other unphosphorylated STATs, like STAT3, act similarly (Yang et al., 2005) suggests that tyrosine phosphorylation may not be vital in all cases. However, it is likely that unphosphorylated STAT1 proteins bind DNA differently to tyrosine phosphorylated STAT1 proteins and may only stimulate a sub-set of IFN-responsive genes. Either way, it is clear that unphosphorylated STAT1 proteins have a role in gene transcription.

1.2.4.2 STAT1 serine phosphorylation

STAT1 serine phosphorylation at serine 727 is crucial for maximal transcriptional activity and enhances the transcription of downstream targets, like IRF1, following IFN- γ stimulation (Wen et al., 1995, Kovarik et al., 1998, Kovarik et al., 1999, Decker & Kovarik, 2000). Although it is not essential for ISGF3 complex formation (Muller et al., 1993, Bromberg et al., 1996), a small proportion of ISGF3 complexes do contain serine phosphorylated STAT1 (Goh et al., 1999). Serine phosphorylation is thought to not affect STAT1 DNA binding (Wen & Darnell, Jr. 1997) and occurs independently of tyrosine phosphorylation (Zhu et al., 1997). It has also been shown to regulate the recruitment of the transcriptional co-activator CBP/p300 to STAT1 (Varinou et al., 2003). In addition, the conserved leucine 724 amino acid in the TAD domain of STAT1 is also required for CBP/p300 recruitment as well as for STAT1 serine phosphorylation (Sun et al., 2005).

In the literature, many serine kinases have been implicated in catalysing STAT1 serine phosphorylation in response to a range of extracellular stimuli, such as IFN- γ . In some of these cases the evidence is controversial and it is likely that more than one kinase is involved. In type I IFN induced cells, p38 mitogen-activated protein kinase (MAPK) (Goh et al., 1999) and protein kinase C- δ (PKC- δ) (Uddin et al., 2002, Kaur et al., 2005) have been identified as potential kinases whereas in IFN- γ induced cells, PKC- δ (Deb et al., 2003), Ca²⁺/calmodulin-dependent kinase II (CaMKII) (Nair et al., 2002), p38 MAPK (Goh et al., 1999) and a downstream kinase of the PI3K/Akt pathway (Nguyen et al., 2001) have all been implicated. The role of p38 MAPK in IFN- γ triggered STAT1 serine

phosphorylation has been doubted (Kovarik et al., 1999) but it is reported to be the kinase involved in response to TNF- α , lipopolysaccharide (LPS), ultraviolet (UV) irradiation and combined IL-2/IL-12 stimulation (Goh et al., 1999, Gollob et al., 1999, Zykova et al., 2005). Other potential serine kinases in response to other stimuli include extracellular signal-regulated kinase (ERK), JNK and p90 ribosomal S6 kinase 2 (RSK2) (Rahimi et al., 2005, Zykova et al., 2005). This wide collection of implicated kinases suggests further that this STAT1 modification is cell-type and stimulus-type specific.

1.2.4.3 STAT1 arginine methylation

Methylation of STAT1 at arginine 31, located in the N-terminal domain of STAT1, by the protein arginine methyltransferase PRMT1 has been reported and has been shown to enhance the DNA-binding activity of STAT1 (Mowen et al., 2001). PRMT1 has also been found to be associated with the IFN- α and IFN- β receptor (Abramovich et al., 1997, Altschuler et al., 1999) and methylates STAT1 independent of tyrosine or serine phosphorylation. Although a recent study threw doubt over whether STAT1 arginine methylation actually happens (Meissner et al., 2004), other evidence supports its presence as a regulatory modification of STAT1 (Zhu et al., 2002, Duong et al., 2004). Indeed one of these studies showed that arginine methylation can regulate the dephosphorylation of STAT1 by the T-cell protein tyrosine phosphatase (TcPTP) (Zhu et al., 2002). Furthermore, since other STATs, like STAT3 and STAT6, have been shown to be arginine methylated (Rho et al., 2001, Chen et al., 2004a), it would appear that arginine methylation has a key regulatory role in STAT activity.

1.2.4.4 STAT1 lysine acetylation

STAT1 can be also be modified by acetylation at lysines 410 and 413 (Kramer et al., 2006). Acetylated STAT1 has been shown to interact with and regulate NF- κ B activity in a human melanoma cell line (Kramer et al., 2006). The presence of this modification relies on a balance between the levels of histone deacetylases (HDACs), such as HDAC1 which can associate with STAT1 (Nusinzon & Horvath, 2003) and

histone acetyltransferases like CBP/p300. Other STATs, such as STAT3 and STAT6, are also acetylated by CBP/p300 (Shankaranarayanan et al., 2001, Wang et al., 2005, Yuan et al., 2005) and this modification has been shown to be critical for STAT3 dimerisation, DNA-binding and transcriptional regulation (Wang et al., 2005, Yuan et al., 2005). It remains to be determined whether lysine acetylation of STAT1 has a critical role in regulating its own activity and whether it modulates NF- κ B in other cell types.

1.2.4.5 STAT1 sumoylation

The protein inhibitor of activated STAT (PIAS) proteins, which are involved in the negative regulation of STAT1 (discussed in section 1.2.5), contain small ubiquitin-related modifier (SUMO) E3 ligase activity (Jackson, 2001). PIAS1 and PIASx- α have been shown to sumoylate STAT1 at lysine 703 by separate studies (Rogers et al., 2003, Ungureanu et al., 2003, Ungureanu et al., 2005) although which of these two PIAS proteins is responsible is still debatable. Ungureanu et al., showed that STAT1 is sumoylated by PIAS1 in response to IFN- γ and this negatively regulates STAT1 activity (Ungureanu et al., 2003, Ungureanu et al., 2005) whereas Rogers et al., showed that STAT1 is sumoylated by PIASx- α and that this modification does not affect STAT1 or even PIAS1 activity (Rogers et al., 2003). It would seem that sumoylation of STAT1 has some functional role in STAT1 regulation given its close proximity to tyrosine 701. However, further clarification is required.

1.2.4.6 STAT1 ubiquitination and ISGylation

STAT1 can also be modified by ubiquitination following IFN- γ induced tyrosine phosphorylation (Kim & Maniatis, 1996) which targets it for proteasomal-mediated degradation. Although ubiquitination appears to not have an important role in STAT1 regulation, certain paramyxoviruses encode viral V-proteins which ubiquitinate STAT1, resulting in its degradation, in order to evade the anti-viral effects of interferon (Horvath, 2004). Modification of STAT1 by ISGylation has also been reported (Malakhov et al., 2003, Malakhova et al., 2003). ISGylation involves the conjugation of ISG15, a

ubiquitin-like protein that is strongly induced by type I IFN, to STAT1 by the ISGylating enzyme Ubc8 (Kim et al., 2004). The role of this unique modification in the regulation of STAT1 activity is now being investigated. A study showed that ISGylation positively regulates IFN signalling (Malakhova et al., 2003) although it is not essential for IFN-mediated, anti-viral responses against vesicular stomatitis and lymphochoriomeningitis virus (Knobeloch et al., 2005, Osiak et al., 2005, Kim et al., 2006).

1.2.5 Negative regulation of STAT1

The JAK/STAT1 signalling pathway can be negatively regulated by a group of regulatory proteins which function to attenuate signal transduction. These key regulators include protein tyrosine phosphatases (PTPs), suppressors of cytokine signalling (SOCS) and the PIAS proteins. They target the JAKs or STAT1 and modulate their activity by inducing various protein modifications and thus present a biological negative feedback mechanism for this signalling pathway. The negative regulation of JAKs and STAT1 is described further below and is illustrated in Figure 1.6

PTPs, such as SH2-containing phosphatase-1 (SHP1), SHP2, CD45, PTP1B and TcPTP, can inactivate both JAKs and STAT1 by catalysing their dephosphorylation. SHP1 and SHP2 are SH2-domain containing PTPs (Neel, 1993) which are involved in the dephosphorylation of JAK1 and JAK2 (David et al., 1995, Klingmuller et al., 1995, You et al., 1999). In addition, TYK2 has been shown to be targeted for dephosphorylation by SHP1 which suggests that this PTP can negatively modulate type I IFN signalling (Yetter et al., 1995). Furthermore, SHP2 is also implicated in the dephosphorylation of STAT1 at its tyrosine 701 and serine 727 amino acid residues (Wu et al., 2002) and indicates a unique dual role for this PTP. CD45 is a receptor PTP which can bind and dephosphorylate all JAKs (Irie-Sasaki et al., 2001) and has a critical role in T- and B-lymphocyte cellular signalling (Penninger et al., 2001). PTP1B targets JAK2 and TYK2 for dephosphorylation and negatively regulates type I and II IFN responses (Myers et al., 2001). TcPTP specifically dephosphorylates JAK1, JAK3 (Simoncic et al., 2002) and its nuclear isoform, Tc45, catalyses STAT1 dephosphorylation (ten Hoeve et al., 2002).

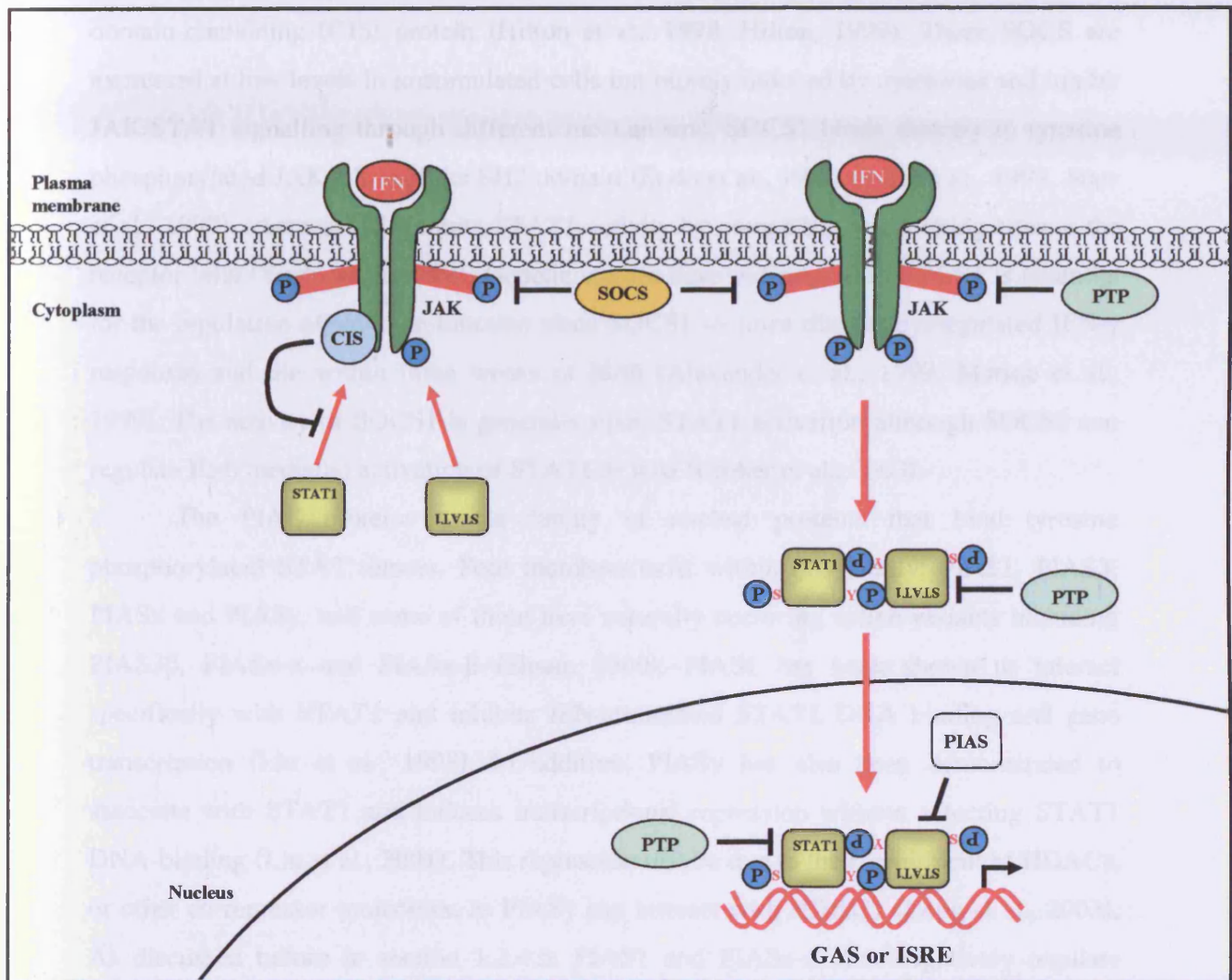


Figure 1.6 Negative regulation of IFN-stimulated JAK/STAT1 signalling

The JAK/STAT1 signalling pathway can be negatively regulated by proteins such as protein tyrosine phosphatases (PTPs), suppressors of cytokine signalling (SOCS) including cytokine inducible SH2-domain containing protein (CIS) and protein inhibitor of activated STAT (PIAS) proteins. These inhibit the pathway at different sites in the cytoplasm and nucleus and function to provide a negative feedback loop for the pathway.

The SOCS are a family of eight cytokine-induced proteins which associate with cell-membrane located receptors or JAKs and function to block downstream STAT activation. These eight members include SOCS1-SOCS7 and cytokine inducible SH2 domain-containing (CIS) protein (Hilton et al., 1998, Hilton, 1999). These SOCS are expressed at low levels in unstimulated cells but rapidly induced by cytokines and inhibit JAK/STAT signalling through different mechanisms. SOCS1 binds directly to tyrosine phosphorylated JAKs through its SH2 domain (Endo et al., 1997, Naka et al., 1997, Starr et al., 1997) whereas CIS inhibits STAT1 activity by competing for docking sites at the receptor tails (Yoshimura, 1998). Genetic studies have indicated that SOCS1 is essential for the regulation of immune function since SOCS1 *-/-* mice display dysregulated IFN- γ responses and die within three weeks of birth (Alexander et al., 1999, Marine et al., 1999). The activity of SOCS1 is generally upon STAT1 activation although SOCS3 can regulate IL-6 mediated activation of STAT1 *in vivo* (Crocker et al., 2003).

The PIAS proteins are a family of nuclear proteins that bind tyrosine phosphorylated STAT dimers. Four members exist within this family; PIAS1, PIAS3, PIASx and PIASy, and some of these have naturally occurring splice variants including PIAS3 β , PIASx- α and PIASx- β (Shuai, 2000). PIAS1 has been shown to interact specifically with STAT1 and inhibits IFN-stimulated STAT1 DNA binding and gene transcription (Liu et al., 1998). In addition, PIASy has also been demonstrated to associate with STAT1 and induces transcriptional repression without affecting STAT1 DNA-binding (Liu et al., 2001). This repression maybe due to the recruitment of HDACs, or other co-repressor molecules, as PIASy can interact with HDAC1 (Long et al., 2003). As discussed before in section 1.2.4.5, PIAS1 and PIASx- α also negatively regulate STAT1 by promoting sumoylation at lysine 703 (Rogers et al., 2003, Ungureanu et al., 2003, Ungureanu et al., 2005).

1.2.6 Function of STAT1

The generation of STAT1 knockout mice (Durbin et al., 1996, Meraz et al., 1996) enabled significant progress in understanding the biological role of STAT1 in cellular function. These STAT1 knockout mice are defective in both type I and II IFN responses

and are highly susceptible to bacterial and viral infections. Since IFNs regulate key anti-viral and immune responses, it is clear that the actions of STAT1 are closely related to the biological effects of IFNs. These STAT1 knockout mice also display defects in T-lymphocyte maturation (Fallarino & Gajewski, 1999, Lee et al., 2000a, Refaeli et al., 2002) and are susceptible to autoimmune disease due to impaired CD4⁺CD25⁺ regulatory T-lymphocyte development (Nishibori et al., 2004). This highlights a role for STAT1 in immune regulation.

It has been widely accepted that the primary role of STAT1 is in mediating the anti-viral and immune responses elicited by IFNs (Darnell et al., 1994). STAT1 is critical for the induction of MHC class I and II expression stimulated by IFN (Muller et al., 1993, Lee & Benveniste, 1996, Meraz et al., 1996). STAT1 also regulates components of the MHC class I antigen processing pathway, such as transporter of antigenic peptides-1 (TAP1) and low-molecular weight protein-2 (LMP-2) (Chatterjee-Kishore et al., 2000, Rouyez et al., 2005), and adhesion molecules like intercellular adhesion molecule-1 (ICAM-1) (Jahnke & Johnson, 1994, Naik et al., 1997, Ohmori et al., 1997, Tessitore et al., 1998) which are integral for T-lymphocyte specific immune responses. In addition, it has been shown that STAT1 protein levels are modulated within antigen-specific CD8⁺ T-lymphocytes *in vivo* during the early stages of viral infection (Gil et al., 2006). This, in particular suggests that STAT1 is an important contributor to the early regulation of CD8⁺ T-lymphocyte expansion during viral infection.

In vivo studies on STAT1 knockout mice (Durbin et al., 1996, Meraz et al., 1996), as well as *in vitro* data gathered from STAT1 deficient cells (Bromberg et al., 1996, Kumar et al., 1997) have indicated that STAT1 is anti-proliferative and pro-apoptotic. IFNs inhibit cell growth and require transcriptionally active STAT1 for this activity (Bromberg et al., 1996). For example, IFN- γ has been shown to suppress cell growth by regulating *c-myc* expression in a STAT1-dependent fashion (Ramana et al., 2000). This effect requires both tyrosine and serine phosphorylation of STAT1 although this study also revealed that IFN- γ could induce *c-myc* expression in STAT1 deficient cells and thus demonstrated the existence of a non-classical, STAT1 independent pathway in IFN- γ signalling (Ramana et al., 2000). Furthermore, STAT1 can also induce cell cycle arrest following IFN- γ stimulation by up-regulating the expression of the cell cycle inhibitor

p21^{WAF1/CIP1} (Chin et al., 1996). In addition, IFN- α can also induce growth inhibition by suppressing mRNA expression of the cell cycle protein cyclin D3 and the cyclin-dependent kinase phosphatase cdc25A (Tiefenbrun et al., 1996).

The pro-apoptotic actions of STAT1 are utilised by IFN- γ which up-regulates caspase-8 expression through a STAT1/IRF1 dependent pathway (Fulda & Debatin, 2002). IFN- γ activated STAT1 can also sensitise apoptotic-resistant cells to TNF- α induced apoptosis (Suk et al., 2001a, Suk et al., 2001b). In addition, STAT1 is also involved in the constitutive expression of the caspase 1-3 genes which are involved in TNF- α induced apoptosis (Kumar et al., 1997). STAT1 is also involved in cellular apoptosis induced by non-cytokine related stimuli such as DNA-damage (Townsend et al., 2004). The mechanism by which STAT1 promotes apoptosis appears to involve STAT1 transcription dependent and independent machinery. STAT1 DNA-binding at GAS or ISRE promoter elements and subsequent mRNA transcription in various cell types is involved in the transactivation of caspases 1, 2, 3, 7, 8 and 11 (Kumar et al., 1997, Huang et al., 2000, Sancéau et al., 2000, Suk et al., 2001b, Fulda & Debatin, 2002, Refaeli et al., 2002, Ruiz-Ruiz et al., 2004), death receptors and ligands like Fas/FasL (Lee et al., 2000b, Stephanou et al., 2000) and p21^{WAF1/CIP1} (Huang et al., 2000, Agrawal et al., 2002). However, STAT1 can also interact with the TRADD signalling complex (Wang et al., 2001b) or with p53 (Townsend et al., 2004) and promote apoptosis without initiating STAT1-specific transcription. Indeed, STAT1 enhances the transcription of pro-apoptotic p53 target genes like Bax (Townsend et al., 2004). This indicates that STAT1 can promote apoptosis by functioning as a transcriptional co-activator.

1.2.7 STAT1 in oncogenesis

STAT1 is generally classified as a tumour suppressor, since it is anti-proliferative and pro-apoptotic. Indeed STAT1 knockout mice are more prone to methylcholanthrene-induced tumours than their wild-type counterparts (Kaplan et al., 1998). Furthermore, this oncogenic susceptibility is exacerbated by crossing the STAT1 knockout mice with p53 knockout mice (Kaplan et al., 1998) which, in theory, removes the STAT1/p53 dependent apoptotic machinery. Therefore, the absence of STAT1 *in vivo* is thought to facilitate

more rapid tumour progression (Shankaran et al., 2001, Refaeli et al., 2002, Lesinski et al., 2003, Nishibori et al., 2004). This increased tumour susceptibility, in the absence of STAT1, may arise from a STAT1-dependent defect in immunosurveillance. However, a recent study challenged the classification of STAT1 as a tumour suppressor and showed that STAT1 promoted leukaemia development by sustaining high levels of MHC class I expression *in vivo* (Kovačić et al., 2006). This tumour promoting action of STAT1 was also seen *in vitro* in Wilms' tumour cells where constitutively serine phosphorylated STAT1 was shown to be pro-survival (Timofeeva et al., 2006). Furthermore, type 2 human T-lymphocyte leukaemia virus (HTLV) can induce cell growth and survival in bone marrow CD34⁺ cell line by activating STAT1 and also STAT5 (Bovolenta et al., 2002).

Constitutive activation of STAT1 is observed in several human malignancies such as breast cancer (Bowman et al., 2000), multiple myeloma (Catlett-Falcone et al., 1999), head and neck squamous carcinoma (Grandis et al., 1998) and various leukaemias including acute myelogenous leukaemia (AML) (Weber-Nordt et al., 1996). It is also been observed in some EBV-associated malignancies (described in section 1.2.8). This event is usually transduced by oncogenic tyrosine kinases, such as the fusion protein BCR-Abl and v-Abl, or by transforming oncogenes. Constitutive STAT1 activation has been detected in acute and chronic myelogenous leukaemia cells possessing the BCR-Abl tyrosine kinase (Bowman et al., 2000), and v-Abl can transform primary B-lymphocytes and subsequently activate STAT1 (Danial et al., 1995). Constitutive serine phosphorylation of STAT1, in the absence of tyrosine phosphorylation, has also been detected in chronic lymphocytic leukaemia (CLL) (Frank et al., 1997) and Wilms' tumour (Timofeeva et al., 2006). This unique form of STAT1 activation indicates that STAT1 serine phosphorylation may have a role in oncogenesis. Indeed, in the context of STAT3, it has been shown that constitutive serine phosphorylation is required for the transformation of fibroblasts by the oncogenic Src tyrosine kinase (Turkson et al., 1999).

1.2.8 STAT1 and EBV

EBV infection has been shown to induce STAT1 protein expression in both B-lymphocytes (Richardson et al., 2003, Zhang et al., 2004) and EBV-negative NPC and gastric carcinoma cell lines (Wood et al., 2007). Of all the EBV latent genes expressed LMP1 is responsible for increasing STAT1 protein expression in B-lymphocytes (Richardson et al., 2003, Zhang et al., 2004) and likewise EBNA1 in NPC and gastric carcinoma cell lines (Wood et al., 2007). Constitutive activation of STAT1 has been demonstrated in NPC tissue by immunohistochemistry (Chen et al., 2001) although it appears that STAT1 is not constitutively tyrosine phosphorylated in NPC cell lines (Chen et al., 2003). This discrepancy in defining constitutive STAT1 activation in NPC is also evident in EBV-transformed B-lymphocytes, or LCLs, which act as an *in vitro* model for PTLN. Constitutive STAT1 activation in EBV-transformed LCLs was first described by Weber-Nordt et al. in 1996, and further studies have added controversy to whether STAT1 is constitutively activated or not. Some studies have shown that STAT1 is not constitutively tyrosine phosphorylated in LCLs but is capable of being tyrosine phosphorylated in response to IFN- α (Dupuis et al. 2001, Zhang et al., 2004). However, other studies disagree with this and have reported that STAT1 is constitutively tyrosine phosphorylated in LCLs and have even described a mechanism involving an indirect autocrine loop of interferon secretion (Fagard et al., 2002, Najjar et al., 2005, Nepomuceno et al., 2002). Although this inconsistency remains to be solved, it has been shown in a few studies that EBV uses STAT1 for its own ends. Firstly, EBV-encoded LMP1 has been shown to induce expression of the glycoprotein Mucin-1 in epithelial cells through STAT1 and STAT3 (Kondo et al., 2007). Mucin-1 has been shown to have a role in tumour invasion and metastasis. Also, LMP1 induced STAT1 is believed to have role in sensitising EBV-transformed B-lymphocytes to CD95-mediated apoptosis (Le Clorennec et al., 2006). Therefore, it appears that STAT1 has a role in EBV oncogenesis although further characterisation is required in order to delineate its functional significance.

1.3 Aims of thesis

EBV induces the expression of range of cellular proteins during *in vitro* transformation of primary B-lymphocytes including STAT1. STAT1 is a transcription factor utilised by the IFN signalling pathway which has key roles in regulating IFN stimulated anti-viral and immune responses, and also cell proliferation and survival. Constitutive expression and activation of STAT1 is a feature of malignancies such as PTLN but little is known about why STAT1 is targeted by the virus. This thesis sought to investigate whether how and why EBV modulates STAT1 activity in transformed B-lymphocytes. The main objectives of this thesis are written below:-

- 1) The first objective was to analyse the post-translational modifications of STAT1 in EBV-transformed LCLs. This would enable the comparison of EBV-induced STAT1 to IFN-stimulated STAT1 in B-lymphocytes and could see if there are any qualitative differences (**Chapter 3**).
- 2) Following on from the first aim, the next objective would be to generate an *in vitro* EBV-transformed LCL model that uses the STAT1-degradative actions of the simian virus 5 V-protein (**Chapter 4**).
- 3) Finally, using the *in vitro* model established in Chapter 4, the last objective would be to investigate what function(s) STAT1 has in EBV-transformed LCLs (**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

D-MEM + L-Glutamine (Gibco BRL) was stored at 4°C.

Foetal calf serum (FCS) (Gibco BRL, Lot number 41G5961F) was stored in 50 ml aliquots at -20°C.

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

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

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.

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

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

Puromycin (Sigma) was prepared as 2.5mg/ml stock solution in sterile distilled water and stored in aliquots at 4°C.

Tetracycline (Boeringer Mannheim) was prepared as a 1mg/ml stock solution in analysis grade 100% ethanol 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 Cell lines

Cell line	Description	EBV latency/ gene expression	Reference
BL41	EBV-negative Burkitt's Lymphoma	N/A	Rowe <i>et al</i> , 1986
BL41+ B95.8	B95.8 transformed Burkitt's Lymphoma	Latency II/III	Rowe <i>et al</i> , 1986
IARC-171	EBV transformed LCL	Latency III	Rowe <i>et al</i> , 1986
IB4	EBV transformed LCL	Latency III	King <i>et al.</i> , 1980
EB	EBV transformed LCL	Latency III	Generated in house
KEM	EBV transformed LCL	Latency III	Rowe <i>et al.</i> , 1995
SP	EBV transformed LCL	Latency III	Generated in house
IB4 CAR 8	EBV transformed LCL stably expressing CAR and $\alpha\text{v}\beta 5$ integrin	Latency III	Richardson <i>et al.</i> , 2005
CV1524	STAT1 deficient EBV transformed LCL	Latency III	Chapgier <i>et al</i> , 2006
DG75	EBV-negative Burkitt's Lymphoma	N/A	Ben-Bassat <i>et al</i> , 1977
DG75 tTA LMP1	Stable DG75 cell line with inducible LMP1 expression	LMP1	Floettmann <i>et al</i> , 1996
Kit 225	IL-2 dependent leukaemic T-lymphocyte line	N/A	Hiro <i>et al</i> , 1987
911	HEK 293 cells expressing E1 backbone of adenovirus	N/A	Kindly donated by Dr. Brian McSharry*

* Dept. of Medical Microbiology, Tenovus Research Building, Cardiff University

Table 2.1 List of original cell lines used in study

2.1.3 Maintenance of cell lines

All cell lines, except 911, were cultured in growth medium comprising of RPMI-1640 medium supplemented with 10% FCS, 2mM L-glutamine and antibiotics (200U of penicillin/ml and 200µg streptomycin/ml). 911 cells were cultured in D-MEM medium supplemented with 10% FCS, 2mM L-glutamine and antibiotics (200U of penicillin/ml and 200µg streptomycin/ml). Tetracycline (1µg/ml) was used to silence tTA-responsive LMP1 expression in the DG75 tTA LMP1 cell line. The Kit225 T-lymphocyte line was cultured in medium supplemented with 20ng/ml IL-2. Puromycin (1µg/ml) was used to select for EBV-transformed LCLs expressing empty vector, simian virus 5 V-protein or HA-tagged STAT1 (WT). 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% CO₂.

Cell line	Description	EBV latency
IARC-171 pBabe	EBV transformed LCL expressing empty vector	Latency III
IARC-171 V-protein	EBV transformed LCL expressing Simian Virus 5 V-protein	Latency III
IB4 pBabe	EBV transformed LCL expressing empty vector	Latency III
IB4 V-protein	EBV transformed LCL expressing Simian Virus 5 V-protein	Latency III
CV1524 pBabe	STAT1 deficient EBV transformed LCL expressing empty vector	Latency III
CV1524 STAT1	STAT1 deficient EBV transformed LCL expressing STAT1α	Latency III

Table 2.2 List of cell lines generated in study (see Chapter 4)

2.2 Preparation of cellular protein extracts

2.2.1 Preparation of total cell lysates for SDS-PAGE

2.2.1.1 Reagents

2x Gel Sample Buffer (2xGSB) containing 100mM Tris-HCl pH6.8, 20% glycerol, 0.2M dithiotreitol (DTT), 4% sodium dodecyl sulphate (SDS) and 0.02% bromophenol blue was stored in 5ml aliquots at -20°C.

2.2.1.2 Generation of total cell lysates

Cells were counted on a haemocytometer and re-suspended in 50µl of 1x PBS per 10⁶ cells. An equal volume of 2x GSB was then added. Cells were sonicated using a W0385 sonicator (Heatsystems-Ultrasonics Inc.) and, following sonication, samples were heated at 100°C for 5 minutes on a dry heating block.

2.2.2 Preparation of cytosolic and nuclear extracts for SDS-PAGE and EMSA

2.2.2.1 Reagents

Low salt detergent lysis buffer containing 10mM HEPES pH7.9, 1.5mM MgCl₂ and 10mM KCl was stored at room temperature.

High salt buffer containing 20mM HEPES pH7.9, 420mM NaCl, 1.5mM MgCl₂, 0.2mM EDTA and 25% glycerol was stored at room temperature.

Storage buffer containing 10mM HEPES pH7.9, 25mM KCl, 0.1mM EDTA and 10% glycerol was stored at room temperature

2.2.2.2 Cytosolic and Nuclear extraction

Cytosolic and nuclear extracts were prepared using a method previously described (Brennan and O'Neill, 1995). Cytosolic extracts were prepared by lysis of cells for 5 minutes on ice in 100µl of low salt detergent lysis buffer supplemented with 1mM phenylmethosulfonylfluoride (PMSF), 1:100 dilutions of Phosphatase Inhibitor Cocktails I and II (Sigma) and 0.1% NP40 detergent. These supplements were added immediately prior to use due to their short half-lives. Following centrifugation (Heraeus Biofuge) (16,600g, 5 minutes, 4°C), the supernatant was retrieved representing the cytosolic extract. Nuclear extracts were prepared by incubating the remaining pellet for 15 minutes in 50µl of high salt buffer supplemented with 1mM PMSF and 1:100 dilutions of Phosphatase inhibitor cocktails I and II immediately prior to use. Following centrifugation (16,600g, 5 minutes, 4°C), the supernatant was collected representing the nuclear extract. For extracts prepared specifically for application in an EMSA, 50µl storage buffer was added to the nuclear extract. All extracts were then stored at -20°C.

2.2.3 Quantification of protein concentration

Protein concentration was determined using a method based on that developed by Bradford (Bradford, 1976). Protein concentration standards were generated using 1mg/ml Bovine Serum Albumin (BSA) (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 were used. Three wells containing 10µl distilled H₂O were included for the generation of the standard curve. A fixed volume (1µl) of cytosolic or nuclear extract was pipetted into the 96 well plate. Protein assay reagent (Biorad, 500-0006) was diluted 1 in 5 with distilled H₂O and 200µl was added to each standard and extract. A microplate reader (Biorad, 170-6850) was used to read the absorbance of each well at 570nm. The protein concentration of each extract was first determined by plotting a standard curve using the optical density at 570nm (OD_{570nm}) of all the protein standards on a Microsoft Excel spreadsheet. The

concentration of each sample extract was then calculated using the equation from the standard curve.

2.3 DNA affinity precipitation of STAT1 protein

The ability of nuclear STAT1 protein to bind a STAT consensus DNA sequence was investigated by DNA affinity precipitation. This technique, modified from a previously described protocol (Beadling et al, 1996), uses streptavidin-conjugated agarose beads to precipitate proteins bound to biotinylated oligonucleotide sequences from the nuclear extracts of target cells. The strong interaction between streptavidin and biotin allows the precipitation to occur and enables measurement of specific DNA-bound proteins.

2.3.1 Reagents

Dilution buffer containing 50mM Tris-HCl pH8, 0.25mM EDTA, 10mM NaF and 10% v/v Glycerol and was prepared when required.

Tris-EDTA (TE) buffer containing 10mM Tris-HCl pH8 and 1mM EDTA and was prepared and stored at room temperature.

2.3.2 Generation of double stranded GRR oligonucleotides

The oligonucleotide sequence derived from the FcγR1-GAS (GRR) was used to DNA-affinity precipitate STAT1 protein (Darnell Jr. et al., 1994). Forward and reverse single stranded GRR oligonucleotide sequences were purchased from MWG Biotech. The forward GRR oligonucleotide was supplied with 5'-biotinylation, whereas the reverse complementary sequence was not modified with biotinylation. Double stranded GRR oligonucleotide was prepared using a method previously described (Brennan & Athie-Morales, 2001a). Oligonucleotides were diluted to a concentration of 1μg/μl in TE buffer and equal volumes of the forward GRR oligonucleotide and the complementary

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°. Working dilutions were kept at 4°C.

Transcription factor	Oligonucleotide	Oligonucleotide sequence Forward (F) / Reverse (R)
STAT1	GRR	(F) 5' - BIO - GTATT <u>TCCCAGAAA</u> AGGAAC - 3' (R) 5' - GTTCCTTTTCTGGGAAATAC - 3'
NF-κB	NF-κB	5' - CAACGGCAGGGGAATCTCCCTCTCCTT - 3'

STAT consensus sequence underlined

Table 2.3 Sequences of biotinylated GRR and NF-κB oligonucleotides.

2.3.3 DNA affinity precipitation

Cytosolic and nuclear extracts were prepared from a maximum of 10 million cells on ice as described in section 2.2.2.2. 50µl of nuclear extract was mixed with 950µl of dilution buffer supplemented with a 1:200 dilution of Phosphatase inhibitor cocktails I and II, 0.5mM PMSF, 0.5mM NaVO₄ and 5mM DTT. Supplements were added immediately prior to use due to their short half-lives. Nuclear extracts were subsequently incubated (60 minutes, rotating, 4°C) with 1µg of 5'-biotinylated double stranded GRR 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-biotinylated, double stranded oligonucleotide (NF-κB) was also included in the mixture to reduce non-specific binding of proteins to the biotinylated GRR oligonucleotide. Oligonucleotide-conjugated beads were collected by centrifugation (3540g, 5 minutes, 4°C) and were washed three times using dilution buffer complemented with 0.5mM PMSF, 40mM NaCl and 5mM DTT immediately prior to use. DNA-bound 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.

2.4 Immunoprecipitation of STAT1 protein

2.4.1 Reagents

Dilution buffer containing 50mM Tris-HCl pH8, 0.25mM EDTA, 10mM NaF and 10% v/v Glycerol was prepared when required.

Cold lysis buffer containing 50mM HEPES pH 7.9, 2mM EDTA, 250mM NaCl and 0.1% NP40 was prepared when required.

2D Sample buffer containing 7M Urea (Sigma), 2M Thiourea (Sigma) and 2% w/v CHAPS (Sigma) was prepared and stored in 1 ml aliquots at -20°C for no longer than 2 months.

2.4.2 Generation of STAT1 antibody-coupled Sepharose protein G

In order to immunoprecipitate STAT1 protein from cytosolic or nuclear extracts, the presence of STAT1 antibody-coupled Sepharose protein G was required. For each sample in any experiment (10^7 cell equivalents), 40µl of Sepharose protein G beads (50% slurry in PBS; Sigma, P-3296) was coupled to 1µg of STAT1 antibody (sc-346; Santa Cruz Biotechnology). This involved first pre-washing the beads four times in cold PBS prior to resuspension in 200µl PBS containing 1µg of STAT1 antibody. This mixture was then incubated overnight at 4°C. For this preparative stage the amount of Sepharose protein G beads and antibody was increased according to the number of samples used.

2.4.3 Immunoprecipitation

Cytosolic and nuclear extracts were prepared from a maximum of 10 million cells on ice as described in section 2.2.2.2. 50µl of nuclear extract was mixed with 950µl of

dilution buffer (100µl of cytosolic extract mixed with 900µl of dilution buffer) supplemented with a 1:200 dilution of Phosphatase inhibitor cocktails I and II, 1:200 Protease cocktail inhibitor (Sigma, P-8340), 0.5mM PMSF, 0.5mM NaVO₄ and 5mM DTT. Supplements were added immediately prior to use due to their short half-lives. Nuclear or cytosolic extracts were subsequently incubated (120 minutes, rotating, 4°C) with 40µl (50% slurry in PBS) of pre-washed STAT1 antibody bound-Sepharose protein G beads. Antibody-conjugated beads were collected by centrifugation (3540g, 3-4secs, 4°C) and were washed two times using cold lysis buffer and two times using cold PBS. Antibody-bound proteins to be analysed by 1-dimensional SDS-PAGE were eluted from the antibody by the addition of 25µl PBS and 25µl 2x GSB and heating at 100°C for 5 minutes on a dry heating block. For analysis by 2-dimensional SDS-PAGE, antibody-bound proteins were eluted in 100µl 2D sample buffer.

2.5 Protein analysis by 1-dimensional (1D) Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

2.5.1 Reagents and Equipment

2.5.1.1 Reagents

Electrophoresis running buffers for pre-cast gels – MOPS and Tris-Glycine (Invitrogen; NP0001 & LC2675) 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-HCl, 192mM glycine and 20% v/v analysis grade methanol (Fisher). This 1x working solution was stored at room temperature.

Pre-stained SeeBlue Plus 2[®] molecular weight marker (Invitrogen; LC5925) was supplied as a ready-to-use protein marker and were stored in aliquots at 4°C.

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

Blocking buffer was prepared as a 1x working solution containing 0.2% I-block (Tropix. Inc) and 0.02% v/v sodium azide (NaN_3) supplemented in PBS-Tween. The blocking buffer was stored for up to 1 month at 4°C.

Alkaline phosphatase (AP) buffer was prepared as a 10x stock solution containing 1M diethanolamine (Tropix Inc.) and 10mM MgCl_2 altered to pH 9.5. 10x AP buffer was stored for up to one month at 4°C and was diluted 1:10 prior to use.

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

MESNA stripping buffer was prepared as a 1x working solution containing 62.5mM Tris-HCl pH 6.8, 2% w/v SDS and 50mM 2-mercaptoethanesulfonate (MESNA; Sigma M-1511). MESNA stripping buffer was kept for no longer than two weeks and was stored at 4°C.

2.5.1.2 Equipment

X-cell SureLock™ Mini-cell SDS-PAGE apparatus (Invitrogen)

X-cell II™ Blot module Western Blot apparatus (Invitrogen)

Hybond-P polyvinylidenedifluoride (PVDF) membrane (Amersham)

Chromatography paper 3MM Chr (Whatman)

Polyethylene lay flat film 204mm (Jencons).

P200 gel loading tips (Alpha Laboratories)

Powerpac 300 power suppliers (Biorad)

X-OMAT™ LS Kodak film 18 x 24 cm (Amersham)

Protein analysis by 1D SDS-PAGE was carried out using the NuPAGE®-Novex® Pre-cast gel system (Invitrogen). The NuPAGE®-Novex® pre-cast gels used are summarised in table 2.4.

Gel type	Running Buffer
NuPAGE 4-12% Bis-Tris 1mm x 10 well (NP 0321 BOX)	MOPS SDS
NuPAGE 4-12% Bis-Tris 1mm x 12 well (NP 0322 BOX)	MOPS SDS
Novex 4-12% Tris-Glycine 1mm x 10 well (EC6035 BOX)	Tris-Glycine

Table 2.4 NuPAGE®-Novex® Pre-cast gels (Invitrogen) used for 1D SDS-PAGE protein analysis

2.5.2 1D SDS-PAGE

Pre-cast gels (Invitrogen) were prepared by rinsing the gel cassette of its storage buffer and by carefully removing the tape covering the slot at the back of the gel cassette and the well comb. The exposed loading wells were then 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. Protein samples to be loaded onto the gels were first prepared. Whereas total cell lysates, DNA-affinity precipitation samples and immunoprecipitation samples were already made up in 2x GSB, any cytosolic and nuclear extracts were not. Following the quantification of protein concentration, an equal volume of 2x GSB was added and the samples were heated at 100°C for 5 minutes on a dry heating block. These samples along with a Pre-stained molecular weight marker and protein samples was loaded onto the gels by pipetting under the running buffer using the P200 gel loading tips. Gels were run at 200V for 50 minutes to 1 hour.

2.5.3 Western Blotting

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. Western Blotting was carried out using an X-cell IITM Blot module inserted into an X-cell SureLockTM Mini-Cell unit (Invitrogen) at 30V, 250mA for 60minutes. The blots were then washed in PBS-Tween.

2.5.4 Antibody detection

Western Blots were incubated in a sealed polyethylene bag with approximately 15ml-20ml of blocking buffer for 1 hour at room temperature on an orbital shaker (Stuart-Scientific). The blocking buffer was then replaced with 10-20ml of primary antibody diluted to the required concentration in blocking buffer (see Table 2.5). The blots were incubated with primary antibody for 1 hour at room temperature or overnight at 4°C.

Protein	Clone/Cat. #	Species	Working concentration	Citation/Supplier
Acetyl-lysine	#06-933	Rabbit	1/1000	Upstate
Actin	A-2066	Rabbit	1/1000	Sigma
Bcl-2	F7053	Mouse	1/1000	DakoCytomation
BZLF-1 (EBV)	BZ.1	Mouse	1µg/ml	Young <i>et al.</i> , 1991
Dimethylarginine	ab5394	Mouse	1/1000	AbCam
Early and late lytic Ag's	EE serum	Human	1/10,000	Rowe <i>et al.</i> , 1992
EBNA1 (EBV)	AMo serum	Human	1/1000	Rowe <i>et al.</i> , 1992
EBNA2 (EBV)	PE2	Mouse	1/50	Young <i>et al.</i> , 1989
EBNA3A (EBV)	T2.78	Mouse	1µg/ml	Rowe <i>et al.</i> , 1989
EBNA3B (EBV)	F120P	Sheep	1µg/ml	Exalpha
EBNA3C (EBV)	E3C.A10	Mouse	1µg/ml	Maunder <i>et al.</i> , 1994
phospho-ERK1/2 (Y204)	sc-7383	Mouse	200ng/ml	Santa Cruz
ERK1/2	sc-93	Rabbit	200ng/ml	Santa Cruz
IRF-1	sc-497	Rabbit	200ng/ml	Santa Cruz

LMP1 (EBV)	CS.1-4	Mouse	1µg/ml	Rowe <i>et al.</i> , 1987a
LMP1 (EBV)	R5/6	Rabbit	1µg/ml	Brennan <i>et al.</i> , 2001b
LMP2A	14B7	Rat	1/50	Fruehling <i>et al.</i> , 1996
LMP-2	sc-28809	Rabbit	200ng/ml	Santa Cruz
Mcl-1	sc-819	Rabbit	1/1000	Santa Cruz
MHC class I	HC10	Mouse	1/100	ATCC
phospho-S6 ribosomal protein (S235/S236)	#2211	Rabbit	1/1000	CST
S6 ribosomal protein	#2212	Rabbit	1/1000	CST
phospho-STAT1 (Y701)	sc-7988-R	Rabbit	200ng/ml	Santa Cruz
phospho-STAT1 (S727)	sc-16570-R	Rabbit	200ng/ml	Santa Cruz
STAT1	sc-346	Rabbit	200ng/ml	Santa Cruz
STAT2	sc-476	Rabbit	200ng/ml	Santa Cruz
phospho-STAT3 (Y705)	#9131	Rabbit	1/1000	CST
phospho-STAT3 (S727)	#9134	Rabbit	1/1000	CST
STAT3	sc-7179	Rabbit	200 ng/ml	Santa Cruz
STAT5	sc-1656	Mouse	200 ng/ml	Santa Cruz
SV5 V-protein	N/A	Mouse	1/1000	Randall <i>et al.</i> 1987
TAP1	148.3	Mouse	1/1000	Ressing <i>et al.</i> , 2005
TAP2	435.4	Mouse	1/1000	Ressing <i>et al.</i> , 2005

Santa Cruz - Santa Cruz Biotechnology; CST – Cell Signalling Technologies; Upstate – Upstate Technology; Exalpa – Exalpa Biologicals Inc.; ATCC – American Tissue Culture Collection;

Table 2.5 Primary Antibodies used in study

Post-incubation, blots were then washed three times for at least 10 minutes in PBS-Tween. Subsequently, the blots were incubated for 1 hour with 10-20ml of appropriate AP-conjugated secondary antibody diluted to 1/10,000 with blocking buffer (see Table 2.6). 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 AP buffer. Following this, the blots were incubated with CDP-Star development reagent for 10 minutes in a section of polyethylene film. Any excess reagent was removed and blots were exposed to autoradiograph film (Kodak), through a new section of polyethylene film

Specificity	Cat. #	Species	Working concentration	Supplier
Mouse IgG	170-6520	Goat	1/10,000	Biorad
Rabbit IgG	170-6518	Goat	1/10,000	Biorad
Human IgG	170-6521	Goat	1/10,000	Biorad
Rat IgG	sc-2021	Goat	1/10,000	Santa Cruz
Sheep IgG	A-5187	Goat	1/10,000	Sigma

Table 2.6 Alkaline Phosphatase-conjugated secondary antibodies used in study

2.5.5 Stripping blots for repeated antibody detection

In order to undertake antibody detection of the same Western blot with a different primary antibody, blots were stripped by incubating them 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 three times for at least ten minutes in PBS-Tween. Blots were then blocked for 1 hour at room temperature in a sealed polyethylene bag on an orbital shaker. Following this, blots were then ready for repeat antibody detection as described in section 2.5.4.

2.6 Protein analysis of STAT1 immunoprecipitates by 2D SDS-PAGE

2.6.1 Reagents and equipment

2.6.1.1 Reagents

2D sample buffer containing 7M Urea (Sigma), 2M Thiourea (Sigma) and 2 or 4% w/v CHAPS (Sigma) was 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) buffer (Amersham) was stored at 4°C.

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

NuPAGE® Sample Reducing Agent (10X) (Invitrogen; NP 0009) was stored

NuPAGE® LDS Sample Buffer (4X) (Invitrogen; NP 0008)

Iodoacetamide (Sigma) was stored at 4°C

2.6.1.2 Equipment

Ettan IPGphor II IEF system (Amersham)

7cm pH 3-10 non-linear immobiline Drystrip gels (IPG) (Amersham)

2.6.2 2D SDS-PAGE

2.6.2.1 Resolution of proteins by isoelectric focussing

7cm Immobiline Drystrip Gels (IPG) were rehydrated for 12 hours at 20°C, in Ettan IPGphor Strip Holders (Amersham), with 60µl STAT1 immunoprecipitate sample in a total volume of 125µl of 2D sample buffer supplemented with 50mM DTT, 1%

bromophenol blue and 0.5% v/v IPG buffer. Rehydration was performed using the Ettan IPGphor II IEF system. Isoelectric focusing (IEF) of the samples was then performed at 20°C (50µA/strip) 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.

2.6.2.2 SDS-PAGE

Post- isoelectric focussing, IPG strips were equilibrated prior to performing SDS-PAGE. The focused IPG strips were first reduced for 15 minutes in 1X NuPAGE[®]LDS sample buffer containing 0.5 ml 10X NuPAGE[®] sample reducing agent. The IPG strips were then alkylated for 15 minutes in 1X NuPAGE[®]LDS sample buffer containing 125mM iodoacetamide. Equilibrated IPG strips were then transferred to the IPG well of NuPAGE[®] 4-12% Bis-Tris Zoom gels (Invitrogen, NP 0330 BOX), and proteins were then separated by SDS-PAGE as described in section 2.5.2. STAT1 protein was then detected using western blotting and antibody detection as described in sections 2.5.3 and 2.5.4.

2.7. Electrophoretic Mobility Shift Assay (EMSA)

2.7.1 Reagents

Redivue adenosine 5'-[γ -³²P] triphosphate triethylammonium salt was purchased from Amersham (A0068) with relative radioactivity levels of 9.25MBq/250µCi and was stored at -20°C.

10x T4 polynucleotide kinase buffer (Promega) was stored at -20°C,

T4 polynucleotide kinase (100 units) (Promega) was stored at -20°C.

Phenol:Chloroform:Isoamyl alcohol (25:24:1) (Sigma) was stored at 4°C.

5x TBE (1L) containing 54g Tris HCl, 55g Boric acid and 7.4g EDTA in distilled H₂O was stored at room temperature.

poly(dIdC) (100 A₂₆₀ units, Amersham) was re-suspended at 1µg/µl in 5ml distilled H₂O and stored in aliquots at -20°C.

Loading dye containing 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol and 50mM EDTA in distilled H₂O was stored at 4°C.

10x binding buffer containing 40% glycerol, 10mM EDTA, 50mM DTT, 100mM Tris pH7.5, 1M NaCl, 1mg/ml nuclease-free BSA in distilled H₂O was stored in aliquots at -20°C.

Acrylamide (40%) (BDH) was stored at 4°C.

Ammonium persulphate (Sigma) was stored at room temperature.

N, N, N', N'-Tetramethylethylenediamine (TEMED) (Sigma) was stored at room temperature.

2.7.2 End-labelling of double stranded oligonucleotides with [γ -³²P]-ATP

Prior to addition of redivue adenosine 5'-[γ -³²P] triphosphate triethylammonium salt, a labelling mix was first formed in a 1.5ml eppendorf tube. This included 4µl GRR or SIE oligonucleotide (20ng/µl), 10µl 10x T₄ polynucleotide kinase buffer, 2.5µl T₄ polynucleotide kinase (25 units) and 78.5µl added to the mixture and incubated for 2.5 hours at 37°C in a perspex container. Post-incubation, the perspex box was returned to controlled radiation area. 2.5µl of 0.5M EDTA pH8 and 100µl of phenol:chloroform were added to the mix. The mixture was briefly vortexed and centrifuged at 1390g for 5 minutes in a microcentrifuge. The upper aqueous layer was removed to a fresh 1.5ml eppendorf. 4µl of 5M NaCl and 200µl of absolute ethanol were then added to the aqueous layer. The mixture was incubated at -20°C for 60 minutes in a lead container. The mixture was centrifuged at 1390g for 10 minutes in a microcentrifuge and the supernatant was removed. The pellet was allowed to air-dry before dissolving pellet in 50µl TE buffer.

Oligonucleotide	Oligonucleotide sequence
GRR	5'- GTATTTCCTCCAGAAAAGGAAC-3'
SIE*	5'-GTGCATTTCCCGTAAATCTTGTCTACA-3'

* SIE oligonucleotide was purchased from Santa Cruz Biotechnology as 500ng double stranded DNA

Table 2.7 Sequences of oligonucleotides used for EMSA

2.7.3 EMSA

2.7.3.1 Preparation of native 4% polyacrylamide gels

Gels were prepared according to the following recipe: 35ml distilled H₂O, 5x TBE, 5ml 40% acrylamide, 0.1g ammonium persulphate and 20µl TEMED. Acrylamide gel mix was then poured into gel plates and a 12-well forming comb was inserted and gels were allowed to set for 40 minutes. The combs was then removed and wells were rinsed with distilled H₂O and then filled with 0.5x TBE.

2.7.3.2 EMSA

Nuclear extracts were prepared as a reaction mix which included 1µl 10x binding buffer, 2µl poly (dIdC) and 10µg nuclear extract. The volume of the reaction mix was made up to 10µl using distilled H₂O. For supershift or cold competitor experiments, 2µg of antibody or 100ng unlabelled GRR oligonucleotide respectively was pre-incubated for 30 minutes at 4°C. 2ng of ³²P-labelled GRR or SIE oligonucleotide probe was added to the reaction mix and incubated at room temperature for 30 minutes. 2µl of loading dye was then added to the mix. Samples were loaded under the 0.5x TBE in the wells using gel loading tips. Electrophoresis apparatus (Amersham) was assembled and 0.5x TBE was added to both the upper and lower reservoirs. The gel was run at 200V for 90 minutes using a Biorad power pack and was transferred onto 3MM Whatman paper. The gel was then dried under vacuum on a gel drier (Biorad, model 583) set at 80°C for 120 minutes and then visualised by autoradiography.

2.8 Molecular Biology

2.8.1 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 (Sigma) and 1% (w/v) tryptone (Fisher) 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 agar (Fisher) 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 -80°C and were used at a final concentration of 50µg/ml.

2.8.2 Transformation of chemically competent DH5α bacterial cells

Chemically competent DH5α E-coli (Invitrogen) were thawed on ice and 100µl 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.

2.8.3 Amplification of transformed bacterial cells

A single colony of transformed bacteria, isolated from a streaked ampicillin-selective LB agar plate was added to a 1ml volume of LB broth containing ampicillin (50 μ g/ml) and incubated for 8 hours at 37°C in a rocking incubator. This 1ml culture was then supplemented to a further 250 ml of LB broth, which was again incubated at 37°C until the optical density at $\lambda=600\text{nm}$ (OD_{600nm}) reached 0.3 to 0.6 (approximately 10 to 16 hours incubation).

2.8.4 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.

The bacteria contained within 250ml ampicillin-containing LB medium were harvested by centrifugation and re-suspended in 8ml of Bacterial Resuspension Buffer (Novagen). Once the bacterial pellet was thoroughly re-suspended, 8ml 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 10,000g 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 2000g 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 10,000g and the clarified lysate 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 of Mobius Elution Buffer (Novagen) to the column. The plasmid DNA was then precipitated, by adding 3.5ml of isopropanol (Fisher) and centrifuging at 15,000g 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-centrifuged 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.

2.9 Adenoviral infection of EBV-transformed LCLs

2.9.1 Generation of functional adenovirus

Recombinant adenoviruses were grown in the 911 cell line. This cell line expresses the E1 backbone of adenovirus and enables the adenovirus to replicate following infection with replication-deficient adenovirus. The cells were seeded in ten 175cm² tissue culture flasks in the region of 70-90% confluency before being infected with recombinant adenovirus at a multiplicity of infection (MOI) of approximately 0.1 overnight at 37°C. The recombinant adenoviruses used in this process are summarised in Table 2.8. MOI, which is calculated from the titre of the adenovirus, refers to the number of recombinant adenovirus particles used per target cell. The following day, the medium was replaced with fresh growth medium and the cells were cultured at 37°C with medium replacement thereafter as required. After approximately 2-5 days, the majority of cells were loosely adhered to the tissue culture flask and the cell monolayer, from all ten flasks, was detached using a cell scraper. The cell suspension was then collected and spun down (220g, 3 minutes), the supernatant was removed and the cells were re-suspended in 10 ml PBS. The recombinant adenovirus was then extracted from the cells by the addition of an equal volume of Arklone P detergent (Basic Chemical Company), to

the preparation followed by vigorous vortexing for 30 seconds and centrifugation at 390g for 15 minutes. The upper layer containing the recombinant adenovirus was pipetted off and aliquoted into 1ml cryovials and stored at -70°C.

2.9.2 Titration of recombinant adenovirus stocks

A tissue culture infectious dose 50 (TCID₅₀ assay) was used to determine the titre of the recombinant adenoviruses in this study. Titration by TCID₅₀ assay involves infection of 911 cells with serial dilutions of recombinant adenovirus particles followed by a complete infection cycle and re-infection of neighbouring cells to generate a mature plaque.

1x10⁴ 911 cells in 200µl medium were added to ten columns (10x 8 wells) of a 96-well tissue culture plate and were grown to approximately 80% confluence. 200µl medium was added to the other columns as controls. 100µl medium was carefully withdrawn from all 96-wells and was replaced with 100µl of serial adenovirus dilutions (10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹, 10⁻¹⁰, 10⁻¹¹ and 10⁻¹²) made up in growth medium. Each dilution was added to a single row of the plate. 911 cells were then incubated with recombinant adenoviruses overnight in a 37°C incubator to allow virus particles to infect the cells. After incubation, medium was carefully removed from wells and replaced with 200µl fresh medium and the infected cells were replaced in the incubator. After 4-5 days, cells were analysed by microscopy for the formation of plaques (i.e. groups of infected cells) and any wells where no plaques were formed were re-fed with fresh medium. After more analysis by microscopy over the next 4-7 days (until no new plaques were forming), the number of wells with infected cells (i.e. with plaques) were counted for each dilution of adenovirus. The titre was then calculated (as a TCID₅₀ value) according to the method of Reed and Muench (1938) and could be converted into plaque forming units per ml (PFU/ml) when required.

2.9.3 Adenoviral infection of EBV-transformed LCLs

The adenoviral infection of EBV-transformed LCLs was performed using a previously described method (Richardson et al., 2005). 2×10^5 LCLs (in 250 μ l RPMI medium) were added to the wells of a 24-well tissue culture plate. Cells were then infected with recombinant adenoviruses at MOIs ranging from 10 to 100 by the addition of an appropriate volume of recombinant adenovirus to the wells. The plate was then incubated for 2.5 hours at 37°C on a rocking incubator at 25rpm (Stuart Scientific). After incubation, wells were topped up with 1ml of RPMI medium before being incubated at 37°C for 3 days. After 72 hours, cells were either analysed by flow cytometry (section 2.12.3) to determine the percentage of GFP positive cells or by SDS-PAGE and Western blotting (section 2.5).

Adenovirus	Transgene	Reference/Supplier
RAd5-GFP	Green Fluorescent Protein (GFP)	Kindly donated by Dr. Brian McSharry*
RAd5-SV5 V-protein	Simian virus 5 V-protein	Richardson <i>et al.</i> , 2005

* Dept. of Medical Microbiology, Tenovus Research Building, Cardiff University

Table 2.8 Recombinant adenoviruses used in study

2.10 Retroviral infection of EBV-transformed LCLs

2.10.1 Reagents

Retronectin (Takara Biomedicals, supplied by Lonza) was supplied as a 0.5mg lyophilised powder. It is reconstituted in 500 μ l sterile tissue culture grade H₂O, allowed

to rest for 30 minutes at room temperature and then topped up to 5ml with 50mM sodium carbonate buffer. It was stored in 300µl aliquots at -20°C.

Sodium Carbonate buffer (50mM, pH 9.6) containing 160mg Na₂CO₃ and 294mg NaHCO₃ in 100ml sterile tissue culture grade H₂O and pH adjusted to 9.6 was stored at 4°C.

1% BSA/PBS 1g BSA (Sigma) was dissolved in 10ml sterile tissue culture grade H₂O and filtered through a 0.2µM filter. It was stored at 4°C.

Puromycin (Sigma) was prepared as 2.5mg/ml stock solution in sterile distilled water and stored in aliquots at -20°C

2.10.1.2 Retroviral plasmid DNA

The retroviral plasmids used in the study are summarised in Table 2.9 below. The pBabe puro SV5 V-protein and pBabe puro STAT1(WT) retroviruses were kind gifts from Dr. Zara Poghosyan from the Dept. of Pathology, Cardiff University and were supplied as functional retroviral supernatant. This was done by packaging the retroviral DNA into the amphotropic ψ-crip packaging cell line followed by collection of the supernatant (Danos & Mulligan, 1988). The retroviral supernatant was also titred using an colony formation assay that was developed by the Dept. of Pathology.

Retroviral plasmid	Transgene	Selection	Reference/Supplier
pBabe puro empty vector	None	Puromycin	Morgenstern & Land, 1990
pBabe puro SV5 V-protein	Simian 5 virus V-protein	Puromycin	Kindly donated by Dr. Zara Poghosyan*
pBabe puro STAT1α (WT)	STAT1α(WT)	Puromycin	Kindly donated by Dr. Zara Poghosyan*

* Dept. of Pathology, Henry Wellcome Research Building, Cardiff University

Table 2.9 Retroviral plasmid DNA

2.10.3 2-cycle retroviral infection of EBV-transformed LCLs

Infection of EBV-transformed LCLs with retroviruses involves a 2-cycle infection protocol established within Cardiff University (Tonks et al., 2005).

Day 1 – 1st cycle of infection

150µl retronectin was added to the required number of wells of a 24-well non-tissue culture plate and rocked for 2 hours at room temperature (RT). Following this, retronectin was carefully removed, replaced with 200µl 1% BSA/PBS and allowed to rest for 30 minutes at RT. The required number of retroviral supernatant aliquots were removed from the -80°C storage facility and thawed immediately in a water bath (with extreme caution). The 1% BSA/PBS was then carefully removed and replaced with 1ml retroviral supernatant. The plate was sealed in a polyethylene bag and centrifuged for 2 hours at 3000g at RT. Following centrifugation, virus was discarded from each well in turn and immediately replaced with 2×10^5 target cells (in 1ml RPMI-1640 medium) to prevent any drying of pre-coated wells. The plate was contained in a plastic box and then slightly agitated to ensure an even distribution of target cells over the virus-coated wells. The cells were then incubated overnight at 37°C.

Day 2 – 2nd cycle of infection

The cells, incubated from the day before, were transferred to a separate 24-well tissue culture plate and replaced into the incubator. Immediately, retroviral supernatant was added to the vacant wells before any drying of the pre-coated wells could occur. The plate was again sealed in a polyethylene bag and centrifuged for 2 hours at 3000g at RT. Post-centrifugation; the retroviral supernatant was removed and replaced with the original target cells. The plate was again contained in a plastic box and then slightly agitated to ensure an even distribution of target cells over the virus-coated wells. The cells were then incubated for 3 days at 37°C.

Day5 – Puromycin selection

Following a 3 day incubation period, cells were split and re-fed with RPMI-1640 medium complemented with puromycin. Following this, puromycin-resistant cells were then grown out and cultured.

2.11 Luciferase reporter Assay

2.11.1 Reagents and Equipment

Dual-Luciferase[®] Reporter Assay System (Promega; E1910) containing 5x Passive Lysis Buffer, Luciferase Assay Reagent II (LAR II) and Stop & Glo[®] Reagent for measuring *Firefly* and *renilla* luciferase levels. This kit was stored at -20°C.

0.4mm Electroporation Cuvettes (Biorad)

Genepulser II Electroporator (Biorad)

Fluostar Optima Luminometer (Fluostar)

2.11.2 Transient transfection

Target cells were transiently transfected with luciferase reporters, summarized in table 2.10, by electroporation. Cells were washed in pre-warmed growth medium and re-suspended at a concentration between 1.5×10^7 and 2×10^7 cells per ml in RPMI-1640 medium. A specific amount of plasmid DNA, typically between 1µg and 10µg, was pipetted into a 0.4mm electroporation cuvette (Biorad) and a 0.5ml aliquot of the cell suspension was added. Cells were then electroporated at 270V and 950µF using a Biorad Genepulser II electroporator. Cells were then re-suspended in 3.5ml of fresh pre-warmed RPMI-1640 medium in a 6-well plate, and incubated for 20 hours at 37°C in a humidified atmosphere containing 5% CO₂.

Name	Description	Firefly/Renilla	Citation/Supplier
GRR(5)-luc	5x Fc- γ R1-GAS (GRR) site	Firefly	Beadling <i>et al</i> , 1996
phRL-SV40	Synthetic renilla luciferase gene downstream of a T7 promoter	Renilla	Promega; E-6261

Table 2.10 Luciferase reporters

2.11.3 Luciferase assay

To assay luciferase activity from the reporter constructs, cells were washed in chilled PBS and lysed in 100 μ l 1x Passive Lysis Buffer provided within the Dual-Luciferase[®] reporter assay kit (Promega). Lysates were clarified by centrifugation (16,600g, 1 min) and 50 μ l of the supernatant was then assayed for *Firefly* and *renilla* luciferase levels in a 96-well plate using reagents supplied with the kit. Light release was integrated for 10 seconds and measured using a Fluostar Optima luminometer.

2.12 Flow Cytometric Analysis

2.12.1 Reagents and Equipment

Propidium Iodide (PI) (Sigma) – 30x stock reconstituted in PBS and used at 50 μ g/ml final concentration. It was stored at 4°C.

4% (w/v) Paraformaldehyde (PFA) (Sigma) was prepared by dissolving 4g of paraformaldehyde in 100ml PBS at 50°C. Once dissolved, 4% PFA was stored at 4°C in the dark to prevent depolymerisation and subsequent release of the carcinogen formaldehyde. It was used in flow cytometric experiments as 2% PFA.

Becton Dickinson FACSCalibur Flow Cytometer (BD Pharmingen) – CellQuest analysis software was used to analyse data generated from this machine

2.12.2 Cell count and viability analysis with propidium iodide

Cell aliquots of between 200-500 μ l were removed from an experiment and 50 μ g/ml PI was added in order to determine the levels of cell death in each sample. Cells in the live gate only were counted for a defined time on the flow cytometer. To determine cell viability, PI staining was analysed in the FL-2 channel, acquiring data on all cells not just those found in the live gate.

2.12.3 Detection of GFP expression

The level of GFP expression in cells infected with the RAd5-GFP adenovirus was detected by one-colour flow cytometric analysis. Cells were harvested, washed in PBS and then fixed in 2% PFA. To determine the percentage of GFP positive cells, GFP expression was analysed in the FL-1 channel, acquiring data on cells found in the live gate.

2.12.4 Immunostaining for cell surface antigens

MHC class I, MHC class II, CD54 and CD19 cell surface expression on EBV-transformed LCLs was assayed using one-colour flow cytometry. The antibodies, unconjugated or fluorescein isothiocyanate (FITC)-conjugated, used for this analysis are summarized in Table 2.10. 1×10^6 cells were harvested, washed twice in chilled PBS and incubated with the appropriate antibody, generally at a dilution of 1:10 in normal human serum (NHS)/PBS, for 40 minutes at 4°C. Cells were then washed twice in chilled PBS and in experiments where an unconjugated primary antibody was used, cells were incubated with a 1:20 dilution of goat@mouse IgG:FITC secondary antibody (Sigma; F-2012) in NHS/PBS for 40 minutes at 4°C. Cells were then washed twice in chilled PBS and re-suspended in 300 μ l 2% PFA and analysed on the FACSCalibur flow cytometer.

Antibody	Description	2° Antibody	Supplier
MHC class I (W6/32)	Monoclonal IgG2a antibody specific for MHC class I (HLA-A, -B, -C) alleles	Goat@mouse-IgG:FITC	Hybridoma from ATCC
MHC class II (L243)	Monoclonal antibody specific for MHC class II	Goat@mouse-IgG:FITC	Hybridoma from ATCC
CD54:FITC	Mouse@human monoclonal IgG1 antibody for CD54	N/A	Serotec; MCA1615F
CD19:FITC	Mouse@human monoclonal IgG1 antibody for CD19	N/A	Serotec; MCA1940F

ATCC – American Tissue Culture Collection

Table 2.11 Antibodies used in immunostaining experiments

2.13 Immunofluorescence

2.13.1 Reagents and Equipment

MACS buffer containing 1mM EDTA and 0.1% BSA in PBS was stored at 4°C.

Goat anti-mouse Alexa 488-conjugated antibody (Molecular Probes, A-1101) was stored at 4°C.

12-spot (3" x 1") Shandon microscope slide (Thermo Electron Corporation)

Fluorescent microscope (Leica)

Digital Camera (Hamamatsu)

2.13.2 EBV BZLF-1 indirect immunofluorescence

1x 10⁶ cells were resuspended in 100µl MACS buffer and were applied in 10µl volumes (~1x10⁵ cells) to the spots of a 12-spot microscope slide (Thermo Electron

Corporation). These cell suspension droplets were then allowed to air dry for 30-60 minutes. Air-dried cells were then fixed in 70ml (v/v) pre-cooled acetone:methanol for 10 minutes at -20°C. Following this, the spots on the slides were rehydrated by blocking with MACS buffer supplemented with 5% normal goat serum (NGS) for 10 minutes in a moist environment. Any blocking reagent was removed and was replaced with 1µg/ml BZ.1 mouse monoclonal antibody and incubated for 90 minutes at 37°C. Following extensive washes in PBS, fixed cells were then incubated with 1:500 dilution of goat anti-mouse IgG-Alexa 488 conjugated secondary antibody supplemented with 5% NHS (EBV-seronegative) for 30 minutes at 37°C. Following more washes in PBS, BZLF-1 immunofluorescence was then analyzed and captured using a fluorescent microscope (Leica) and a digital camera (Hamamatsu).

2.14 Total RNA extraction from EBV-transformed LCLs

2.14.1 Reagents

Nucleospin® RNA II Kit (Macherey-Nagel, supplied by Abgene UK) – contains buffers RA1, RA2, MDB (all contain guanidine thiocyanate) and RA3, RNase free DNase I and RNase free H₂O. Kit was stored at room temperature except DNase I which was stored at -20°C.

β-mercaptoethanol (Sigma) was stored in a sealed container at room temperature.

2.14.2 RNA extraction

Total RNA was extracted from EBV-transformed LCLs using the Nucleospin® RNA II kit (Macherey-Nagel) according to the manufacturers instructions below:-

2x10⁶ cells were pelleted by centrifugation (9840g, 1 minute) and lysed in 350µl buffer RA1 supplemented with 3.5µl β-mercaptomethanol, which was vortexed

vigorously. The lysate was filtered through a Nucleospin[®] Filter unit by centrifugation (11,000g, 1 minute) to reduce viscosity. 350µl ethanol (70%) was added to the homogenised lysate, to adjust RNA binding conditions, and was mixed by vortexing. The lysate was then passed through a Nucleospin[®] RNA II column by centrifugation (8,000g, 30 seconds) to allow RNA to bind to the column. The silica membrane within the RNA column was de-salted by passing 350µl MDB (Membrane Desalting Buffer) through the column by centrifugation (11,000g, 1 minute). This was performed to enhance DNA digestion in the next step. 95µl DNase I reaction mixture was incubated for 15 minutes at room temperature in the centre of the silica membrane. The silica membrane was then washed by passing 200µl buffer RA2 through the column by centrifugation (8,000g, 30 seconds). Buffer RA2 also inactivates the DNase I. The column was then washed a second time, by centrifugation (8,000g, 30 seconds), using 600µl buffer RA3. After discarding the flow-through, the column was washed through a third time with 250µl buffer RA3 and centrifuged for 2 minutes at 11,000g. RNA was then eluted by passing 60µl RNase free H₂O through the column by centrifugation (11,000g, 1 minute). An aliquot was used to quantify the yield and purity of RNA obtained. This aliquot was diluted 1:100 in RNase free H₂O 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.

2.15 LCL recognition T-lymphocyte assay

2.15.1 Reagents and Equipment

Coating buffer containing 0.1M Na₂HPO₄, adjusted to pH 9 with 0.1M NaH₂PO₄ was stored at room temperature.

Blocking buffer containing 1%BSA and 0.05% Tween-20 detergent (v/v) in PBS was stored at 4°C.

PBS-Tween (0.05%) containing 0.05% Tween-20 detergent (v/v) in 1x PBS was prepared and stored at room temperature.

Recombinant IFN- γ was stored at -80°C.

Anti-human IFN- γ antibody (BD Pharmingen; 18891D) was stored as 1mg/ml stock solution at 4°C and used at 2 μ g/ml.

Biotinylated anti-human IFN- γ antibody (BD Pharmingen; 18902D) was stored as 0.5mg/ml stock solution at 4°C and used at 1 μ g/ml.

ExtrAvidin[®]-peroxidase (Sigma; E2886) was stored at 4°C and used as a 1:1000 dilution.

3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma) was stored at 4°C.

Microwell plate reader (Biorad)

2.15.2 LCL recognition T-lymphocyte assay

LCL recognition assays were performed to measure the recognition of the IARC-171 pBabe and IARC-171 V-protein LCLs by two EBV-specific CD8⁺ T-lymphocyte clones, summarised in Table 2.12 (Long et al., 2005). These T-lymphocyte clones were kindly provided by Tracey Haigh, Cancer Research UK Institute for Cancer Studies, University of Birmingham. Firstly, the IARC-171 pBabe and V-protein LCLs, in 100 μ l RPMI-1640 medium, were applied to the wells of a V-bottomed 96 well plate in a range of cell numbers. For the assay using the EBNA1 HPV T-lymphocyte clone, this range included 10⁵, 5x10⁴, 2.5x10⁴, 1.25x10⁴ and 0.625x10⁴ LCLs, and for the EBNA3B T-lymphocyte clone this range included 4x10⁴, 2x10⁴ and 1x10⁴ LCLs. Triplicate wells were also used for each set of cell numbers. For each assay a number of controls were also included. These controls included T-lymphocyte clone alone, HLA-matched LCL for each T-lymphocyte clone and HLA-mismatched LCL for each T-lymphocyte clone. These HLA-matched or mismatched LCLs, also kindly provided by Tracey Haigh, Cancer Research UK Institute for Cancer Studies, University of Birmingham, were exposed to a 1 hour pre-incubation at 37°C with 5 μ M epitope peptide or an equivalent volume of DMSO (LCL without peptide). Epitope peptides were also provided by Tracey Haigh, Cancer Research UK Institute for Cancer Studies, University of Birmingham. Following pre-incubation with epitope peptide or DMSO, these LCLs were washed (x4) in RPMI-1640 medium and were then added to triplicate wells in 100 μ l volume. For the

assay using the EBNA1 HPV T-lymphocyte clone, these controls contained 10^5 LCLs and for the EBNA3B AVF T-lymphocyte clone these controls contained 4×10^4 LCLs. After addition of LCLs to specific wells, a standard number of T-lymphocyte clones (500/well for EBNA1 HPV and 200/well for EBNA3B AVF) in 100 μ l RPMI-1640 were added to the wells. The 96-well plate was then centrifuged (166g, 4 minutes) to localise the LCLs with the T-lymphocytes and the plate was then incubated at 37°C for approximately 18 hours.

T-lymphocyte clone	HLA restriction	Epitope co-ordinates	Reference
EBNA1 HPV	HLA-B35	407-417	Blake <i>et al.</i> , 1997
EBNA3B AVF	HLA-A11	399-408	Gavioli <i>et al.</i> , 1993

Table 2.12 EBV-specific CD8⁺ T-lymphocyte clones used in LCL recognition T-lymphocyte assays

The supernatant medium was harvested post-incubation and was assayed for IFN- γ release by enzyme linked immunosorbent assay (ELISA). An IFN- γ ELISA plate was set-up the night before by coating the wells of a 96-well plate with 2 μ g/ml anti-human IFN- γ antibody diluted in coating buffer. 50 μ l were applied to each well, the plate was sealed with a lid and incubated overnight at 4°C. Following this, the coating solution was removed and replaced with 200 μ l/well blocking buffer. The plate was sealed and incubated for 2 hours at room temperature. The plate was washed (x5) with PBS-Tween and 50 μ l of the test culture supernatants were then added. In addition to this, a range of standards were set-up using doubling dilutions of recombinant IFN- γ . These dilutions ranged from 2000pg/ml to 31.25 pg/ml and also included a value for 0pg/ml. The supernatants and standards were incubated for 2-4 hours at room temperature. Following this incubation period, the plate was washed (x6) in PBS-Tween and then 1 μ g/ml biotinylated anti-human IFN- γ antibody, diluted in 50 μ l blocking buffer, was then added to each well. The plate was sealed and incubated for 1 hour at room temperature. After

this, the plate was washed (x6) in PBS-Tween and 50µl ExtrAvidin peroxidase (1/1000 dilution) was added to the wells and was incubated for 30 minutes at room temperature. The plate was the washed (x10) in PBS-Tween and 100µl TMB substrate solution was added to all wells. The reaction of the TMB substrate with the peroxidise-conjugated avidin turns the wells blue and this reaction was then stopped with 100µl H₂SO₄. The acid stops the reaction and the colour of the wells turns a bright yellow. These wells were then analysed using a microplate reader set to read at the 450nm wavelength. A standard curve for IFN-γ was assembled using the results for the recombinant standards and the values of the test culture supernatants were read off this curve.

CHAPTER 3

Analysis of STAT1 post-translational modifications and transcriptional activity in EBV-transformed B-lymphocytes

3.1 Introduction

Constitutive activation of STAT1 is observed in many cancers including acute myeloid leukaemia and EBV-associated malignancies (Weber-Nordt et al., 1996, Fagard et al., 2002, Nepomuceno et al., 2002,). However, in EBV-transformed LCLs, the post-translational modifications of the STAT1-DNA binding complex remain controversial. Some studies have shown that STAT1 is not constitutively tyrosine phosphorylated in LCLs but is capable of being tyrosine phosphorylated in response to IFN- α (Dupuis et al. 2001, Zhang et al., 2004). However, other studies disagree with this and have reported that STAT1 is constitutively tyrosine phosphorylated in LCLs and have even described a mechanism involving an indirect autocrine loop of interferon secretion (Fagard et al., 2002, Najjar et al., 2005, Nepomuceno et al., 2002). Since STATs are capable of regulating the expression of apoptotic and cell cycle proteins such as Bcl-X_L and cyclin D1 (Bowman et al., 2000), the role of STAT1 in the progression of EBV-associated malignancy may be vital. This is highlighted by recent evidence suggesting that STAT1 acts as a tumour promoter rather than a tumour suppressor in the development of leukaemia (Kovacic et al., 2006). Therefore, the regulation of STAT1 by specific post-translational modifications may provide a key insight into its activity in EBV-associated malignancy.

This objective of this chapter was to investigate the post-translational modifications of STAT1 in EBV-transformed LCLs. Given the conflicting literature surrounding the status of STAT1 tyrosine phosphorylation in these cells, this chapter focussed on this modification and on the DNA-binding activity of STAT1. To add to this, other post-translational modifications, such as serine phosphorylation and lysine

acetylation were also measured in EBV-transformed LCLs in order to analyse the molecular nature of STAT1 in these cells compared to IFN-stimulated B-lymphocytes.

3.2 EBV induces STAT1 protein expression through LMP1

EBV has been shown to induce STAT1 protein expression in B-lymphocytes in a process involving NF- κ B (Richardson et al., 2003, Zhang et al., 2004). Of all the latent genes expressed when EBV transforms B-lymphocytes, LMP1 is responsible for increasing STAT1 protein expression (Richardson et al., 2003, Zhang et al., 2004). To demonstrate this effect, STAT1 protein expression was assayed in a set of B-cell lines which are essentially genetically similar but differ in their EBV gene expression profile. These B-cell lines, which were derived from the same patient, are BL41, an EBV-negative Burkitt's lymphoma; BL41+B95.8, the same line that has been infected *in vitro* with the B95.8 strain of EBV; and IARC-171, an EBV-transformed LCL (Rowe et al., 1986). Total cell lysates were generated from cells that were either untreated or stimulated with IFN- α for 30 minutes. These lysates were then analysed by SDS-PAGE and Western Blotting using specific antibodies to pan-STAT1, pan-STAT2, LMP1 and actin. The results in Figure 3.1 demonstrate that EBV induces STAT1 protein expression; a phenotype not seen with STAT2. In the BL41 cell line, which is EBV negative, very little STAT1 protein expression is seen. However, when this line is infected with the B95.8 strain of EBV, STAT1 protein expression increases and correlates with the expression of LMP1. However this expression in both STAT1 and LMP1 is lower than that seen in the IARC-171 LCL. The intermediate pattern of STAT1 and LMP1 expression seen in the BL41+B95.8 cell line can be explained by the fact that it does not display a classical Latency III phenotype as seen with IARC-171.

To demonstrate the role of LMP1 in the up-regulation of STAT1 protein, a stable transfectant of an EBV-negative Burkitt's lymphoma cell line where LMP1 expression can be controlled by a tetracycline-regulated LMP1 expression vector was used (Floettmann et al., 1996). This cell line, DG75 tTA LMP1, allows LMP1 to be induced in an EBV-negative B-cell system through removal of tetracycline. Nuclear extracts were generated of DG75 tTA LMP1 cells cultured in the presence or absence of tetracycline

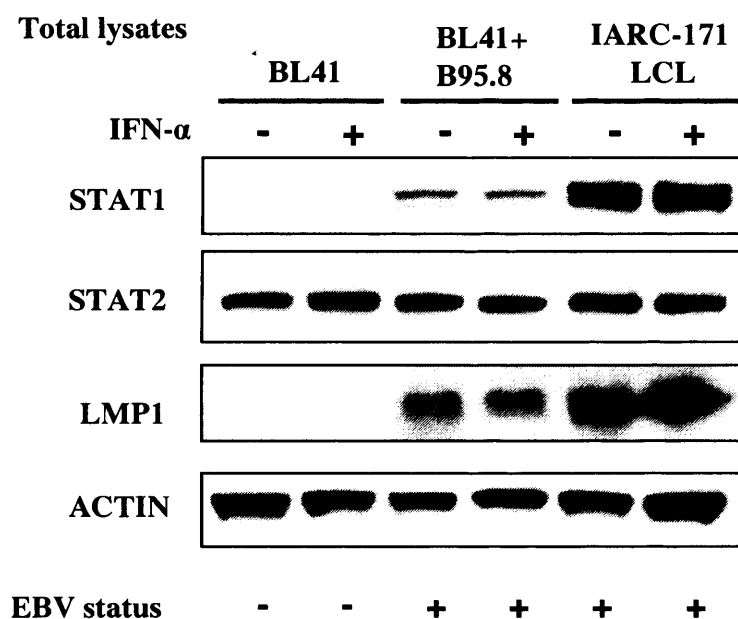


Figure 3.1 EBV induces STAT1 protein expression in B-lymphocytes

Total cell lysates were generated from the BL41, BL41+B95.8 and IARC-171 cell lines. These cells were either untreated or stimulated with IFN- α (1,000 IU) for 30 minutes. These lysates were analysed by SDS-PAGE and Western Blotting using antibodies specific to pan-STAT1, pan-STAT2, LMP1 and actin. Typically 2×10^5 cells were applied in each lane of the gel. This result is representative of at least four experiments.

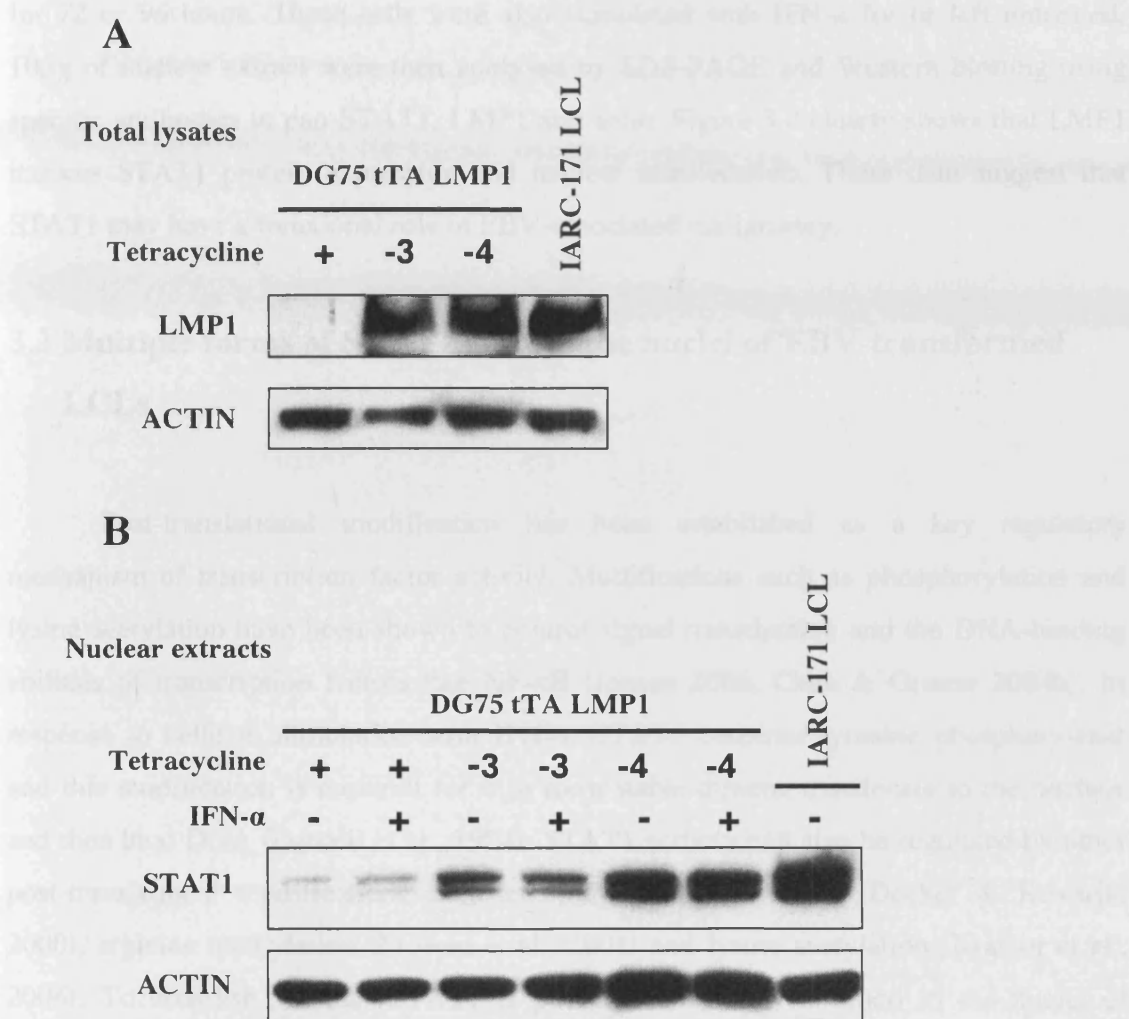


Figure 3.2 LMP1 induces STAT1 protein expression and nuclear translocation

(A) LMP1 expression was measured in stable DG75 transfectants which contain inducible LMP1 expression following removal of 1 μ g/ml tetracycline. Total lysates were generated from cells that were washed five times in RPMI-1640 media and re-cultured in the presence of tetracycline (+) or absence of tetracycline for either 72 hours (-3) or 96 hours (-4). IARC-171 LCLs were used as a positive control for LMP1. These lysates were analysed by SDS-PAGE and Western Blotting using antibodies specific to LMP1 and actin. Typically 2×10^5 cells were applied in each lane of the gel. These results are representative of three experiments. (B) STAT1 nuclear expression was measured in stable DG75 transfectants with inducible LMP1 expression. These cells were re-cultured in the presence of tetracycline (+) or absence of tetracycline for either 72 hours (-3) or 96 hours (-4). Cells were also incubated with IFN- α (1,000 IU) for 30 minutes or left unstimulated. STAT1 tyrosine phosphorylation and nuclear expression was also measured in unstimulated IARC-171 LCLs. Nuclear extracts were generated and were then analysed by SDS-PAGE and Western blotting using antibodies specific to phospho-STAT1 (Y701), pan-STAT1 and actin. 10 μ g of nuclear extract was applied to each lane of the gel. These results are representative of three experiments.

for 72 or 96 hours. These cells were also stimulated with IFN- α for or left untreated. 10 μ g of nuclear extract were then analysed by SDS-PAGE and Western blotting using specific antibodies to pan-STAT1, LMP1 and actin. Figure 3.2 clearly shows that LMP1 induces STAT1 protein expression and nuclear translocation. These data suggest that STAT1 may have a functional role in EBV-associated malignancy.

3.3 Multiple forms of STAT1 exist in the nuclei of EBV-transformed LCLs

Post-translational modification has been established as a key regulatory mechanism of transcription factor activity. Modifications such as phosphorylation and lysine acetylation have been shown to control signal transduction and the DNA-binding abilities of transcription factors like NF- κ B (Jensen 2006, Chen & Greene 2004b). In response to cellular stimulation with IFN- α , STAT1 becomes tyrosine phosphorylated and this modification is required for it to form stable dimers, translocate to the nucleus and then bind DNA (Darnell et al., 1994). STAT1 activity can also be regulated by other post-translational modifications such as serine phosphorylation (Decker & Kovarik, 2000), arginine methylation (Mowen et al., 2001) and lysine acetylation (Kramer et al., 2006). To establish whether STAT1 is post-translationally modified in the nuclei of EBV-transformed LCLs, immunoprecipitated STAT1 from nuclei of IARC-171 and IB4 LCLs was analysed by 2D-electrophoresis (Figure 3.3). Immunoprecipitated proteins were first subject to isoelectric focussing using a pH 3-10 non-linear immobilised pH gradient and then separated in the second dimension by SDS-PAGE. STAT1 protein expression was analysed by Western Blotting using a specific antibody to pan-STAT1. The results in Figure 3.3 indicate that STAT1, located in the nuclei of EBV-transformed LCLs, exists in multiple forms. This suggests that EBV-induced STAT1 is being post-translationally modified by some means.

3.4 STAT1 is tyrosine phosphorylated in LCLs following IFN- α stimulation

To determine whether the multiple forms of STAT1 exist in EBV-transformed

LCLs, the next step was to analyze what post-translational modifications of STAT1 are found in these cells. Grady and colleagues [1995] have analysed STAT1 tyrosine phosphorylation in a variety of cell types.

For 30 weeks, the cells were treated with IFN- α (1000 IU/ml) and then analysed by Western blotting using an antibody specific to phospho-STAT1 (p-STAT1).

Figure 3.4 shows the results of this analysis. The cells were treated with IFN- α for 30 weeks and then analysed by Western blotting using an antibody specific to phospho-STAT1 (p-STAT1).

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Figure 3.3 Multiple forms of STAT1 exist in the nucleus of EBV-transformed LCLs

STAT1 immunoprecipitates were generated from the nuclear extracts of IB4 (A) and IARC-171 (B) LCLs and were analysed by 2D-electrophoresis and Western Blotting using an antibody specific to pan-STAT1.

Figure 3.3 shows the results of this analysis. The cells were treated with IFN- α for 30 weeks and then analysed by Western blotting using an antibody specific to phospho-STAT1 (p-STAT1).

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3.4 STAT1 is tyrosine phosphorylated in LCLs following IFN- α stimulation

Having demonstrated that multiple forms of STAT1 exist in EBV-transformed LCLs, the next step was to analyse what post-translational modifications of STAT1 are found in these cells. Given the conflicting literature, we first analysed STAT1 tyrosine phosphorylation in a range of LCLs that were either untreated or stimulated with IFN- α for 30 minutes. Total cell lysates were generated from 3 previously described EBV-transformed LCLs; IARC-171, KEM and IB4 (King et al. 1980, Rowe et al., 1986, Rowe et al., 1995), and 2 EBV-transformed LCLs; EB and SP, which were generated in the laboratory. These lysates were analysed by SDS-PAGE and Western blotting using specific antibodies to phospho-STAT1 (Y701), pan-STAT1 and actin. Figure 3.4 illustrates that in all five LCLs, STAT1 was tyrosine phosphorylated only after stimulation with IFN- α , and agrees with data that has been previously reported (Dupuis et al., 2001, Zhang et al., 2004). These data suggest that EBV is inducing STAT1 protein expression and nuclear translocation without triggering tyrosine phosphorylation.

3.5 STAT1 is capable of binding DNA in the absence of tyrosine phosphorylation

Transcriptional regulation by STAT1 is dependent on its ability to bind DNA in the nucleus. To investigate whether EBV-induced STAT1 can bind DNA, STAT1 DNA-binding was measured in the BL41, BL41+B95.8 and IARC-171 cell lines by DNA-Affinity precipitation (AP). STAT1 DNA binding was measured by precipitating the biotinylated GRR oligonucleotide with nuclear extracts from untreated or IFN- α stimulated cells. Proteins bound to the GRR oligonucleotide were analysed by SDS-PAGE Western blotting using specific antibodies to phospho-STAT1 (Y701) and pan-STAT1 (Figure 3.5A). STAT1 DNA binding was observed in the BL41 and BL41+B95.8 cell lines only after IFN- α stimulation whereas in the IARC-171 LCL it was seen in both untreated and IFN- α stimulated cells.

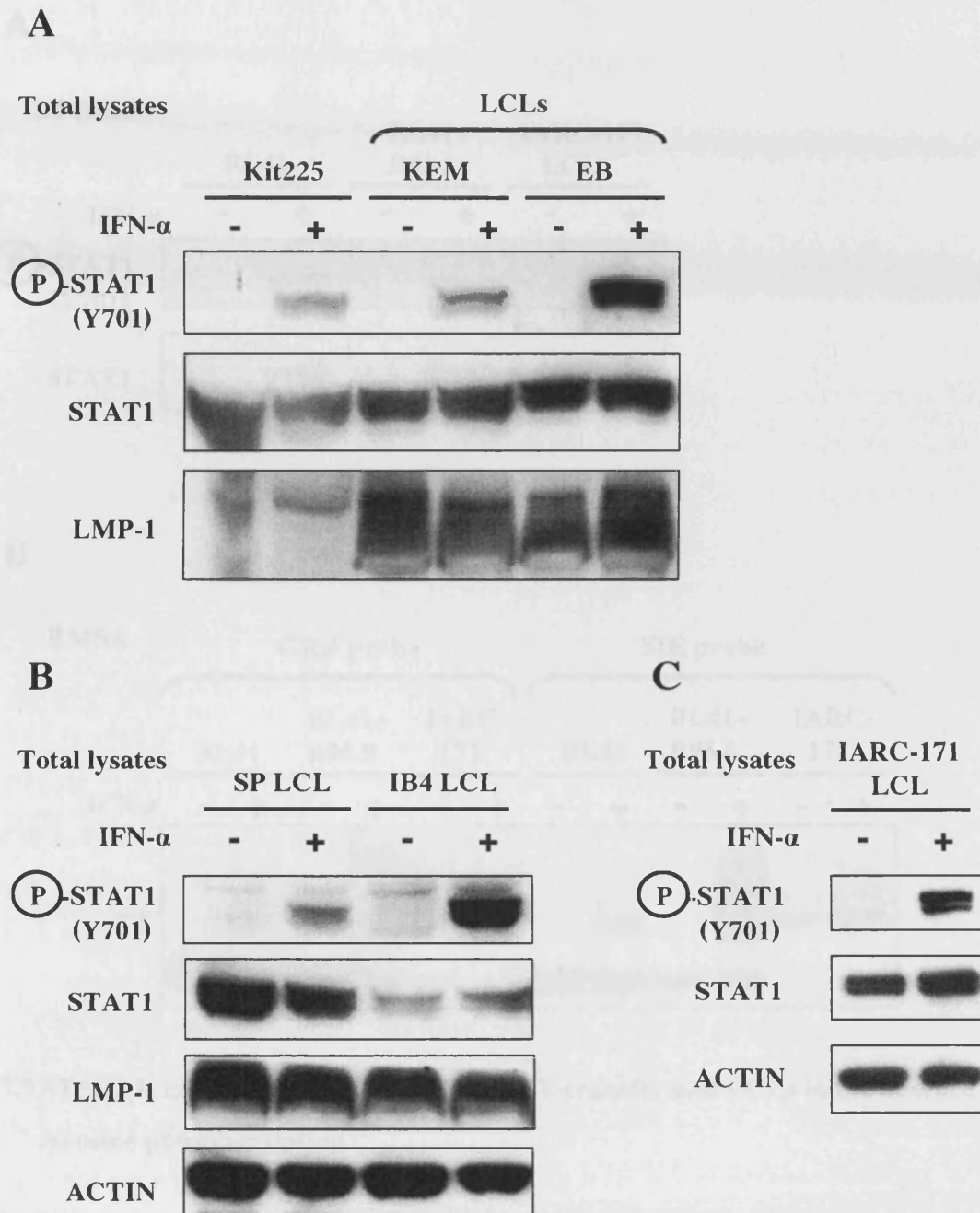
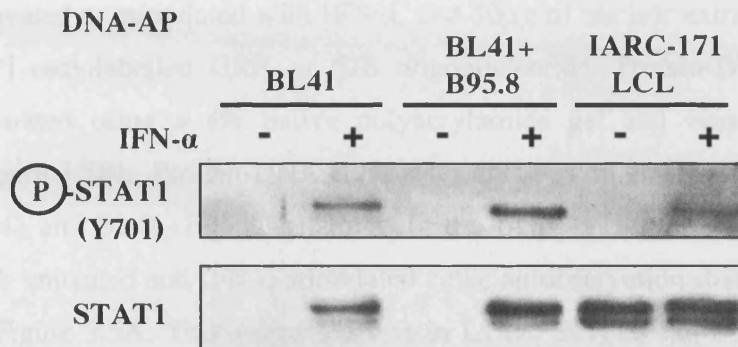


Figure 3.4 STAT1 is tyrosine phosphorylated in LCLs following IFN- α stimulation

Total cell lysates were generated from five cell lines; Kit 225, KEM-LCL, EB-LCL (A), SP LCL and IB4 LCL (B). These cell lines were either untreated or stimulated with IFN- α (1,000 IU) for 30 minutes. These lysates were analysed by SDS-PAGE and Western blotting using antibodies specific to phospho-STAT1 (Y701), pan-STAT1, LMP1(R5/6 or CS1-4) and actin. The Kit 225 T-cell line was used as a positive control for the presence of tyrosine phosphorylated STAT1 following IFN- α stimulation and LMP-1 detection was used as a positive marker for EBV. (C) Nuclear extracts were generated from untreated and IFN- α stimulated IARC-171 LCLs. 10 μ g of nuclear extract was analysed by SDS-PAGE and Western blotting using antibodies specific to phospho-STAT1 (Y701), pan-STAT1 and actin.

A



B

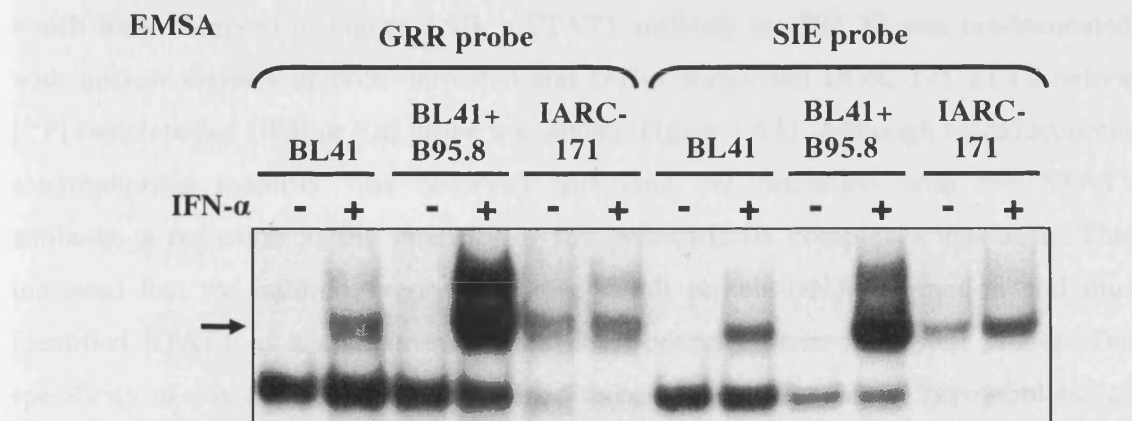


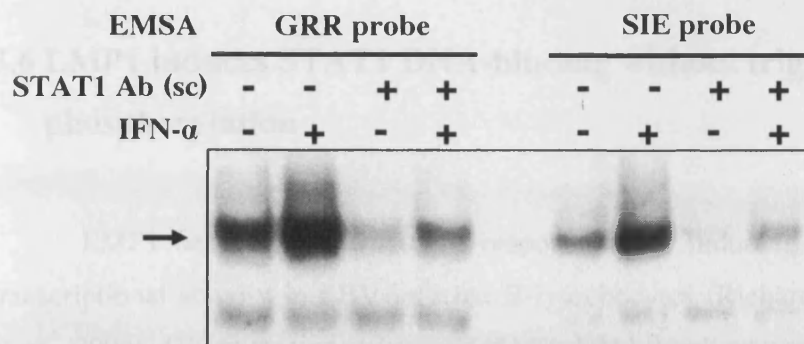
Figure 3.5 STAT1 is capable of binding DNA in EBV-transformed LCLs in the absence of tyrosine phosphorylation

(A) STAT1 DNA binding was measured in the BL41, BL41+B95.8 and IARC-171 cell lines using a DNA-Affinity precipitation (AP) assay. These cell lines were either untreated or stimulated with IFN-α (1,000 IU) for 30 minutes. DNA bound proteins were analysed by SDS-PAGE and Western blotting using antibodies specific to phospho-STAT1 (Y701) and pan-STAT1. Typically 1×10^7 cell equivalents were applied to each lane of the gel. These results are representative of four separate experiments. (B) STAT1 DNA binding was measured in the BL41, BL41+B95.8 and IARC-171 cell lines using an Electrophoretic Mobility Shift Assay (EMSA). These cell lines were either untreated or stimulated with IFN-α (1,000 IU) for 30 minutes. 10μg of nuclear extract was then incubated with either 2 ng [32 P] radiolabelled GRR oligonucleotide or SIE oligonucleotide probe. Protein-DNA complexes were separated using a 4% native polyacrylamide gel and visualized by autoradiography. Only protein-DNA complexes are shown as free probe has been removed from the figure. Arrow indicates specific protein-DNA complex. The results shown are representative of three separate experiments.

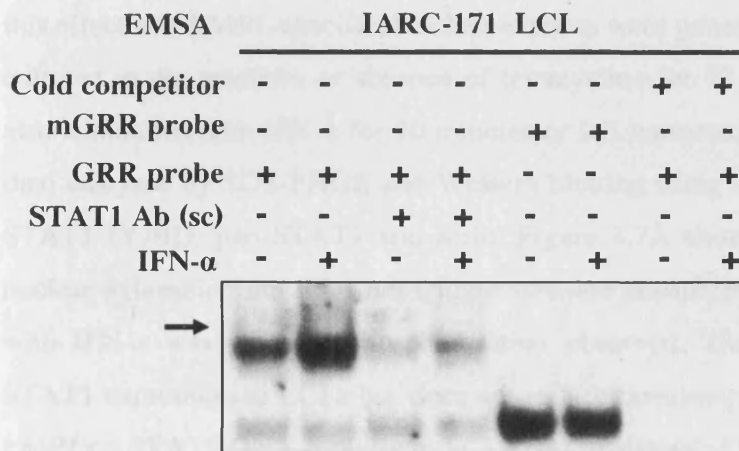
To confirm this constitutive binding, we investigated STAT1 DNA binding in the same cell lines by electrophoretic mobility shift assay (EMSA). Cells were either untreated or stimulated with IFN- α , and 10 μ g of nuclear extract was incubated with 2ng [32 P] radiolabelled GRR or SIE oligonucleotide. Protein-DNA complexes were then separated using a 4% native polyacrylamide gel and visualized by autoradiography (Figure 3.5B). Protein-DNA complexes are seen on both probes in the IFN- α stimulated BL41 and BL41+B95.8 cell lines. In the IARC-171 LCL, these complexes are seen in both untreated and IFN- α stimulated cells, an observation that is consistent with the data in Figure 3.5A. This suggested that in LCLs, STAT1 can bind DNA in the absence of detectable tyrosine phosphorylation. This observation was consistent with previously published data which demonstrated LMP1-induced STAT1 DNA-binding (Richardson et al., 2003).

To elucidate the identity of the protein-DNA complexes in IARC-171 LCLs which were observed in Figure 3.5B, a STAT1 antibody (sc-592 X) was pre-incubated with nuclear extracts of both untreated and IFN- α stimulated IARC-171 LCLs before [32 P] radiolabelled GRR or SIE probe was added (Figure 3.6A). Although no reduction in electrophoretic mobility was observed following pre-incubation with the STAT1 antibody, a reduction in the intensity of the protein-DNA complexes was seen. This indicated that the antibody was preventing a full protein-DNA interaction and thus identified STAT1 as a component of the DNA-bound protein with both probes. The specificity of this complex was ascertained through incubation of a [32 P] radiolabelled mutant GRR probe and through competitive binding with an excess of cold GRR competitor probe (Figure 3.6B). In order to fully confirm that the protein-DNA complex contained STAT1, untreated and IFN- α stimulated IARC-171 LCLs were pre-incubated with a different STAT1 antibody (610199; BD Transduction Laboratories) before [32 P] radiolabelled GRR probe was added (Figure 3.6C). The supershift seen with the STAT1 antibody reiterated its presence in the DNA-binding complex.

A



B



C

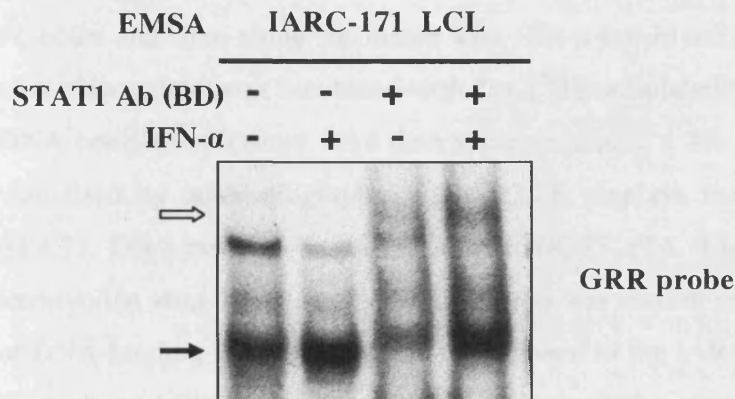


Figure 3.6 STAT1 forms part of the constitutive DNA-binding complex in IARC-171 LCLs

Supershift analysis of protein-DNA complexes was measured in untreated and stimulated IARC-171 LCLs. 10 μ g of nuclear protein was pre-incubated for 30 minutes with 2 μ g STAT1 supershift antibody (sc) (A) or with 2 μ g STAT1 supershift antibody (BD) (C) prior to incubation with 2ng [32 P] radiolabelled GRR oligonucleotide (A,C) or SIE oligonucleotide probe (A). Protein-DNA complexes were then separated using a 4% native polyacrylamide gel and visualized by autoradiography. Arrows indicate specific protein-DNA and supershifted protein-DNA complexes. The results shown are representative of two separate experiments. (B) The specificity of STAT1-DNA complexes was measured in untreated and IFN- α stimulated IARC-171 LCLs. 10 μ g of nuclear protein was pre-incubated for 30 minutes with 2 μ g STAT1 supershift antibody (sc) or 100ng cold GRR oligonucleotide prior to incubation with 2ng [32 P] radiolabelled GRR oligonucleotide or mGRR oligonucleotide probe. Protein-DNA complexes were then separated using a 4% native polyacrylamide gel and were visualized by autoradiography.

3.6 LMP1 induces STAT1 DNA-binding without triggering tyrosine phosphorylation

LMP1 has been shown to be responsible for inducing STAT1 expression and transcriptional activity in EBV-infected B-lymphocytes (Richardson et al., 2003, Zhang et al., 2004). Given that constitutive STAT1 DNA binding was seen in the absence of tyrosine phosphorylation in IARC-171 LCLs (Figure 3.5), it was investigated whether this effect was LMP1-specific. Nuclear extracts were generated of DG75 tTA LMP1 cells cultured in the presence or absence of tetracycline for 72 or 96 hours. These cells were also stimulated with IFN- α for 30 minutes or left untreated. 10 μ g of nuclear extract were then analysed by SDS-PAGE and Western blotting using specific antibodies to phospho-STAT1 (Y701), pan-STAT1 and actin. Figure 3.7A shows that LMP1 induces STAT1 nuclear expression but does not trigger tyrosine phosphorylation. Only after stimulation with IFN- α was tyrosine phosphorylation observed. This shows that LMP1 elevates STAT1 expression in LCLs but does not induce tyrosine phosphorylation. The impact of LMP1 on STAT1 DNA-binding in LCLs was investigated using an EMSA (Figure 3.7B). DG75 tTA LMP1 cells were cultured in the presence or absence of tetracycline for 72 or 96 hours and were either incubated with IFN- α for 30 minutes or left unstimulated. 10 μ g of nuclear extract was incubated with 2ng [32 P] radiolabelled GRR probe and any protein-DNA complexes formed were then separated using a 4% native polyacrylamide gel and visualized by autoradiography. Figure 3.7B displays the emergence of a constitutive STAT1 DNA-binding complex in the DG75 tTA LMP1 cells in the absence of tetracycline after 96 hours. This observation was seen in unstimulated cells and the levels of DNA-binding were comparable to that seen in the IARC-171 LCL. These data clearly show that LMP1 alone is sufficient for inducing a constitutive STAT1 DNA-binding complex that is unphosphorylated at tyrosine Y701.

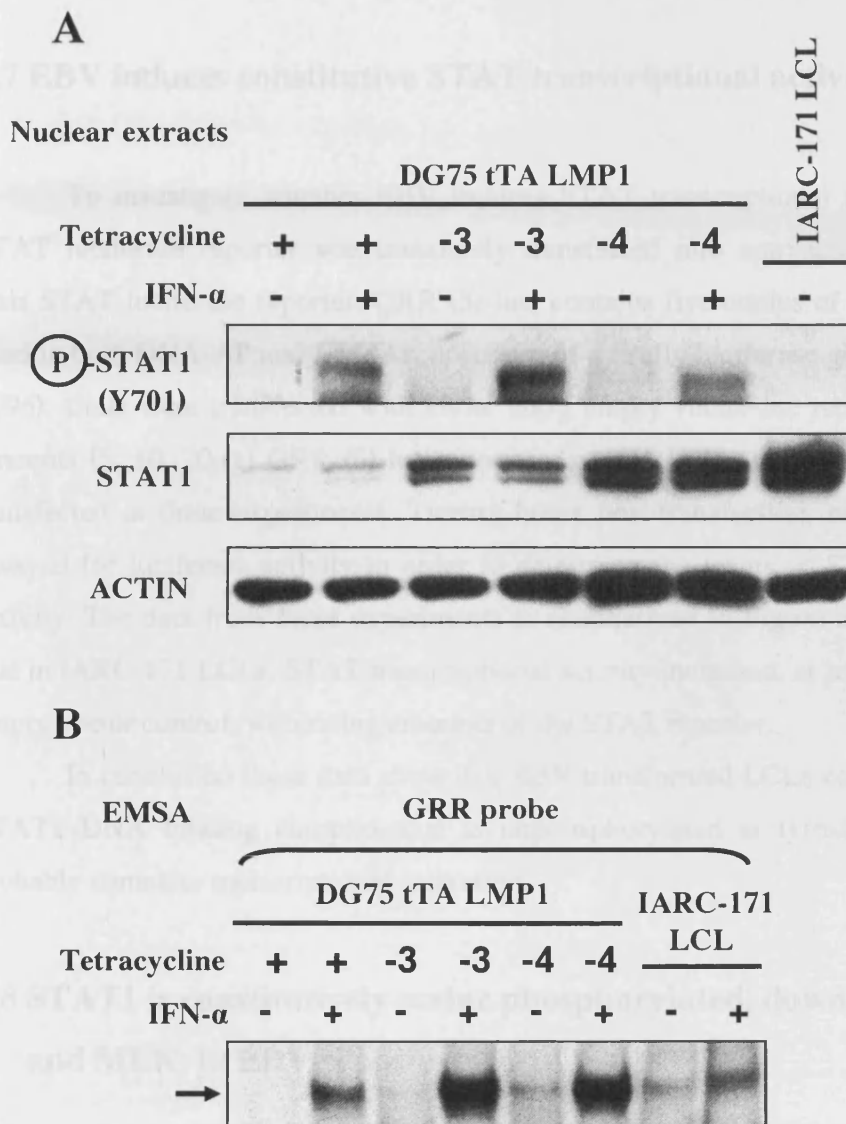


Figure 3.7 LMP1 induces STAT1 protein expression, nuclear translocation and DNA-binding without triggering tyrosine phosphorylation

STAT1 tyrosine phosphorylation, nuclear expression and DNA-binding was measured in stable DG75 transfectants with inducible LMP1 expression. These cells were re-cultured in the presence of tetracycline (+) or absence of tetracycline for either 72 hours (-3) or 96 hours (-4). Cells were also incubated with IFN- α (1,000 IU) for 30 minutes or left untreated. STAT1 tyrosine phosphorylation and nuclear expression was also measured in untreated IARC-171 LCLs and DNA-binding in untreated and IFN- α stimulated IARC-171 LCLs. (A) STAT1 tyrosine phosphorylation and nuclear expression was analysed by SDS-PAGE and Western blotting using antibodies specific to phospho-STAT1 (Y701), pan-STAT1 and actin. 10 μ g of nuclear extract was applied to each lane of the gel. These results are representative of three experiments. (B) STAT1 DNA binding was measured by EMSA. 10 μ g of nuclear extract was incubated with 2ng [32 P] radiolabelled GRR oligonucleotide probe. Protein-DNA complexes were separated using a 4 % native polyacrylamide gel and visualized by autoradiography. Only the protein-DNA complexes are shown as free probe has been removed from the figure. Arrow indicates specific protein-DNA complex. The results shown are representative of three separate experiments

3.7 EBV induces constitutive STAT transcriptional activation

To investigate whether EBV induces STAT transcriptional activity in LCLs, a STAT luciferase reporter was transiently transfected into untreated IARC-171 LCLs. This STAT luciferase reporter, GRR (5)-luc, contains five copies of the GRR sequence, used in both DNA-AP and EMSAs, upstream of a firefly luciferase gene (Beadling et al., 1996). Cells were transfected with either 20 μ g empty vector-luc reporter, or increasing amounts (5, 10, 20 μ g) GRR (5)-luc reporter. 1 μ g phRL-SV40-luc reporter was also co-transfected in these experiments. Twenty hours post-transfection, cells were lysed and assayed for luciferase activity in order to determine the levels of STAT transcriptional activity. The data from these experiments is summarized in Figure 3.8. The data shows that in IARC-171 LCLs, STAT transcriptional activity increased, at levels higher than the empty vector control, with rising amounts of the STAT reporter.

In conclusion these data show that EBV transformed LCLs contain a constitutive STAT1 DNA binding complex that is unphosphorylated at tyrosine 701 which can probably stimulate transcriptional activation.

3.8 STAT1 is constitutively serine phosphorylated, downstream of PI3K and MEK, in EBV-transformed LCLs

STAT1 may also be serine phosphorylated at the C-terminal residue 727, and this type of modification is proposed to be important for full transactivation potential (Decker & Kovarik, 2000). To investigate whether STAT1 is serine phosphorylated in EBV-transformed LCLs, STAT1 was immunoprecipitated from the nuclei of IARC-171 LCLs and an EBV-negative Burkitt's lymphoma line, DG75 (Ben-Bassat et al., 1977). Nuclear extracts were generated from untreated or IFN- α stimulated cells and incubated with 1 μ g STAT1 antibody pre-coupled to Sepharose protein G. Immunoprecipitated proteins were analysed by SDS-PAGE and Western blotting using specific antibodies to phospho-STAT1 (S727) and pan-STAT1. Figure 3.9 shows that STAT1 is constitutively serine

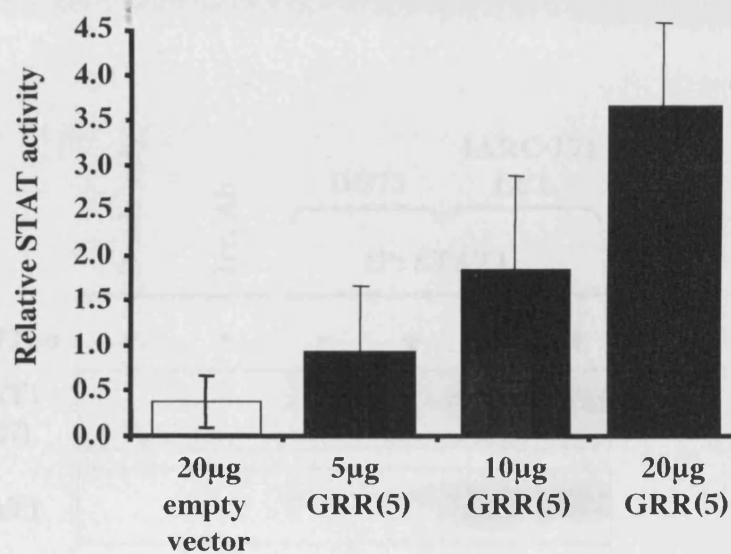


Figure 3.8 EBV induces constitutive STAT transcriptional activation

STAT transcriptional activation was measured in IARC-171 LCLs using a STAT reporter assay. 1×10^7 cells were transfected with either 20µg empty vector-luc reporter, 5µg GRR (5)-luc reporter, 10µg GRR (5)-luc reporter or 20µg GRR (5)-luc reporter. 1µg phRL SV40 reporter was also co-transfected and luciferase activity was assayed 20hrs post-transfection. Relative luciferase activity was calculated as a ratio of firefly over renilla luciferase. The results are mean values representative of at least three experiments. Error bars indicate one sample error from the mean.

phosphorylated STAT1 (P-STAT1) levels, although at a much higher level in the IARC-171 LCL. This reflects the high levels of STAT1 in this line.

The observation of constitutive STAT1 serine phosphorylation in one LCL model led to the question of which serine kinase regulates this process and whether it can be inhibited. Many kinases have been implicated in other cell models including JAKs, tyrosine-activated protein kinases (p38, MAPK, c-Src) (e.g. Zelenka et al., 2002), extracellular signal-regulated kinase (ERK) (Zelenka et al., 2002), protein kinase C (PKC) (Liu et al., 2002, Kane et al., 2005, Ueffing et al., 2002), calcineurin-NFAT (Kawada et al., 2002) and phosphatidylinositol 3-OH kinase (PI3K) (Sprecher et al., 2001, Hattori et al., 2002).

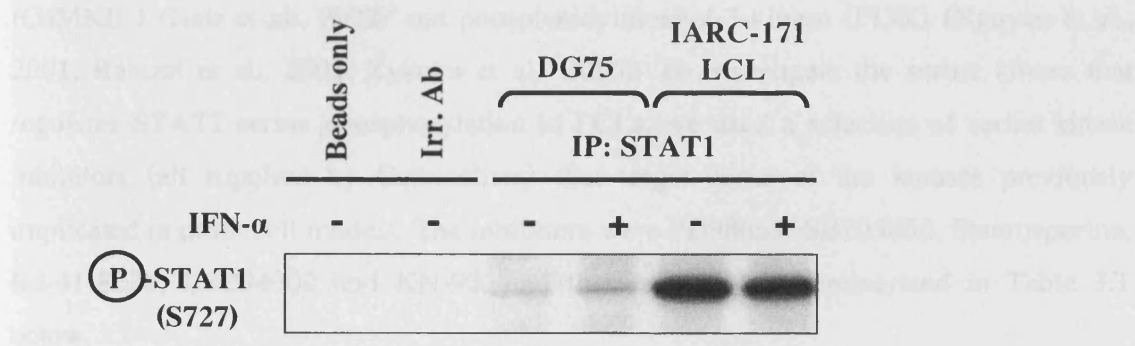


Figure 3.9 STAT1 is constitutively serine phosphorylated in IARC-171 LCLs

STAT1 immunoprecipitates were generated from two B-cell lines, DG75 and IARC-171 LCL. These cell lines were either untreated or incubated with IFN- α (1,000 IU) for 30 minutes. A beads only and irrelevant antibody (ATF-3) control were also incubated with nuclear extracts of untreated IARC-171 LCLs. STAT1 immunoprecipitates were then analysed by SDS-PAGE and Western blotting using antibodies specific to phospho-STAT1 (S727) and pan-STAT1. Typically, 5×10^6 cell equivalents were loaded in each lane of the gel. These results are representative of three separate experiments.

Cell Line	Cell Line	Cell Line
1717-171	1717-171	1717-171
1717-171	1717-171	1717-171
1717-171	1717-171	1717-171
1717-171	1717-171	1717-171

Table 3.1 List of serum-derived monoclonal antibodies used in the study

phosphorylated in both cell lines, although at a much higher level in the IARC-171 LCL. This reflects the higher levels of STAT1 in this line.

The observation of constitutive STAT1 serine phosphorylation in our LCL model led to the question of which serine kinase(s) regulate this process and whether it can be inhibited. Many kinases have been implicated in other cell models including p38 mitogen-activated protein kinase (p38 MAPK) (Goh et al., 1999, Zykova et al., 2005), extracellular signal-regulated kinase (ERK) (Zykova et al., 2005), protein kinase C- δ (PKC- δ) (Deb et al., 2003, Kaur et al., 2005, Uddin et al., 2002), calmodulin kinase II (CaMKII) (Nair et al., 2002) and phosphatidylinositol-3-kinase (PI3K) (Nguyen et al., 2001, Rahimi et al., 2005, Zykova et al., 2005). To investigate the serine kinase that regulates STAT1 serine phosphorylation in LCLs, we used a selection of serine kinase inhibitors (all supplied by Calbiochem) that target some of the kinases previously implicated in other cell models. The inhibitors were PD98059, SB203850, Staurosporine, Ro-31-8220, LY294002 and KN-93, and their actions are summarised in Table 3.1 below.

Inhibitor name	Method of action	Source
PD98059	MEK inhibitor	Calbiochem
SB203850	p38 MAPK inhibitor	Calbiochem
Staurosporine	broad spectrum serine/threonine kinase inhibitor	Calbiochem
Ro-31-8220	PKC inhibitor	Calbiochem
LY294002	PI3K inhibitor	Calbiochem
KN93	CaMKII inhibitor	Calbiochem
Rottlerin	PKC- δ inhibitor	Calbiochem

Table 3.1 List of serine/threonine kinase inhibitors used in the study

Total lysates were generated from IARC-171 LCLs, treated for 1 hour with each of these inhibitors, and were analysed by SDS-PAGE and Western Blotting using antibodies specific to phospho-STAT1 (S727), pan-STAT1, phospho-ERK1/2 (Y204), pan-ERK1/2, phospho-S6 (S235/S236) and pan-S6. Figure 3.10 shows that the serine phosphorylation of STAT1 is only sensitive to the broad spectrum inhibitor Staurosporine after one hour. This suggested that a longer incubation time would be required with a number of the other, more specific inhibitors. To investigate the effects of these inhibitors over a longer time period, total lysates were generated from IARC-171 LCLs treated for 8 hours with a selection of the inhibitors used in Figure 3.10 and Rottlerin, a PKC- δ inhibitor. These lysates were again analysed by SDS-PAGE and Western blotting using antibodies specific to phospho-STAT1 (S727), pan-STAT1, phospho-ERK1/2 (Y204), pan-ERK1/2, phospho-S6 (S235/S236) and pan-S6. Figure 3.11 shows that all the inhibitors had varying effects in abrogating the serine phosphorylation of STAT1 with the broad spectrum inhibitor Staurosporine having the greatest effect.

To rule out that some the effects seen with the inhibitors were due to a loss in cell viability, IARC-171 LCLs were treated for 24 hours with all the inhibitors used in Figure 3.11 and cell viability was analysed by flow cytometry using propidium iodide staining. Figure 3.12 demonstrates that a number of the inhibitors, namely Staurosporine, Ro-31-8220 and Rottlerin were highly cytotoxic. However, two of the remaining inhibitors PD98059 and LY294002 reduced STAT1 serine phosphorylation but did not cause dramatic cell death. To investigate the effect of PD98059 and LY294002 on STAT1 serine phosphorylation further, total lysates were generated from IARC-171 LCLs treated for 1 hour or 24 hours with different combinations of these inhibitors. These lysates were analysed by SDS-PAGE and Western Blotting using antibodies specific to phospho-STAT1 (S727), pan-STAT1, phospho-ERK1/2 (Y204), pan-ERK1/2, phospho-S6 (S235/S236), pan-S6 and actin. As LY294002 has been shown to inhibit the phosphorylation of S6 ribosomal protein (Breslin et al., 2005), phospho-S6 detection was used to demonstrate the actions of LY294002. Phospho-ERK1/2 detection was used as to demonstrate the actions of PD98059. Figure 3.13 shows that PD98059 and LY294002, in combination, further reduced the levels of STAT1 serine phosphorylation compared to that seen with the two inhibitors incubated alone. This effect was manifested after 24

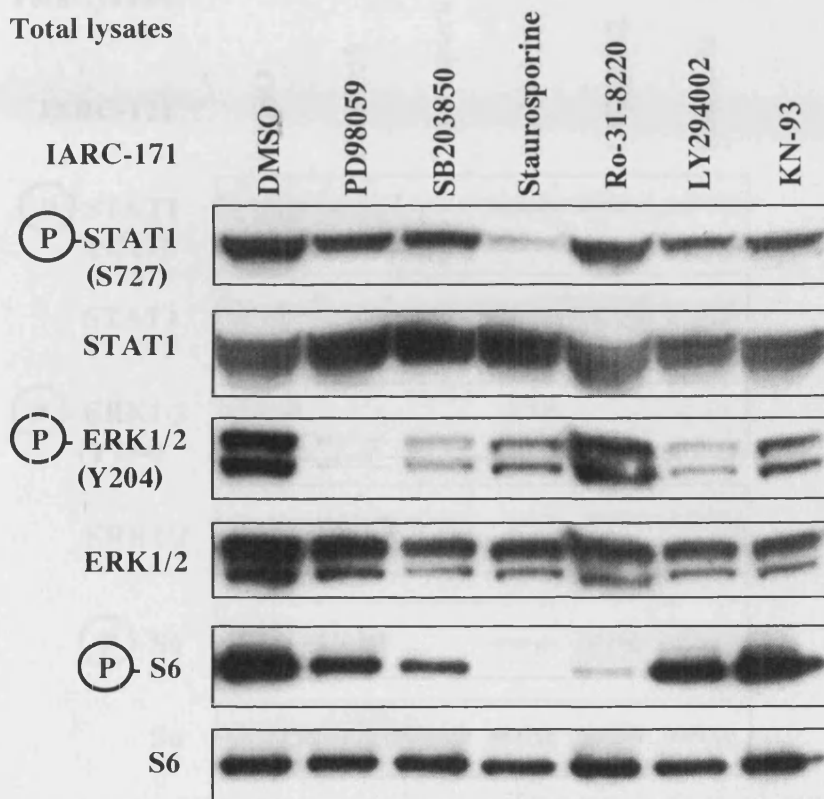


Figure 3.10 STAT1 serine phosphorylation is sensitive to staurosporine after 1 hour

Total cell lysates were generated from IARC-171 LCLs incubated for 1 hour with different serine kinase inhibitors. These inhibitors were PD98059 (50 μ M), SB203850 (50 μ M), Staurosporine (2 μ M), Ro-31-8220 (5 μ M), LY294002 (20 μ M) and KN-93 (5 μ M). DMSO was incubated 1 hour as a control. These lysates were then analysed by SDS-PAGE and Western Blotting using antibodies specific to phospho-STAT1 (S727), pan-STAT1, phospho-ERK1/2 (Y204), pan-ERK1/2, phospho-S6 and pan-S6. Typically 5 x 10⁵ cells were applied in each lane of the gel. These results are representative four separate experiments.

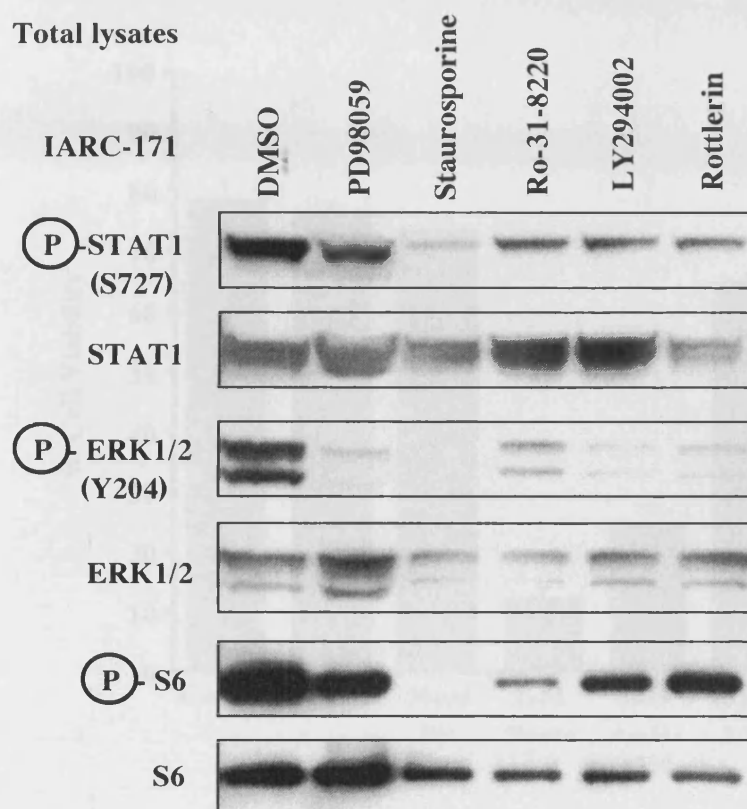


Figure 3.11 Multiple serine kinase inhibitors reduce STAT1 serine phosphorylation in LCLs after 8 hours

Total cell lysates were generated from IARC-171 LCLs incubated for 8 hours with different serine kinase inhibitors. These inhibitors were PD98059 (50 μ M), Staurosporine (2 μ M), Ro-31-8220 (5) LY294002 (20 μ M) and Rottlerin (5 μ M). DMSO was incubated for 8 hours as a control. These lysates were then analysed by SDS-PAGE and Western Blotting using antibodies specific to phospho-STAT1 (S727), pan-STAT1, phospho-ERK1/2 (Y204), pan-ERK1/2, phospho-S6 and pan-S6. Typically 5×10^5 cells were applied in each lane of the gel. These results are representative of six separate experiments.

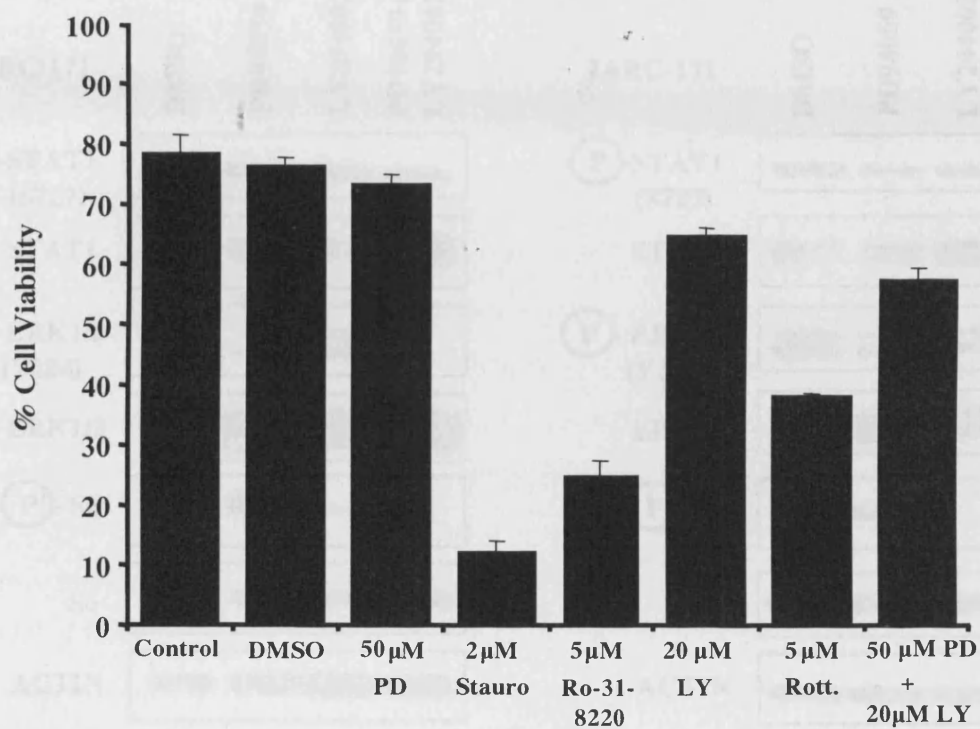


Figure 3.12 Analysis of cell viability following 24 hour incubation with a panel of serine kinase inhibitors

IARC-171 LCLs were incubated for 24hrs with either PD98059 (50µM), Staurosporine (2µM), Ro-31-8220 (5µM), LY294002 (20µM), Rottlerin (5µM) or PD98059 (50µM) + LY294002 (20µM). Cells were then analyzed for viability by flow cytometry. The data represents the percentage of cells that were not positive for propidium iodide. Error bars indicate one sample error from the mean.

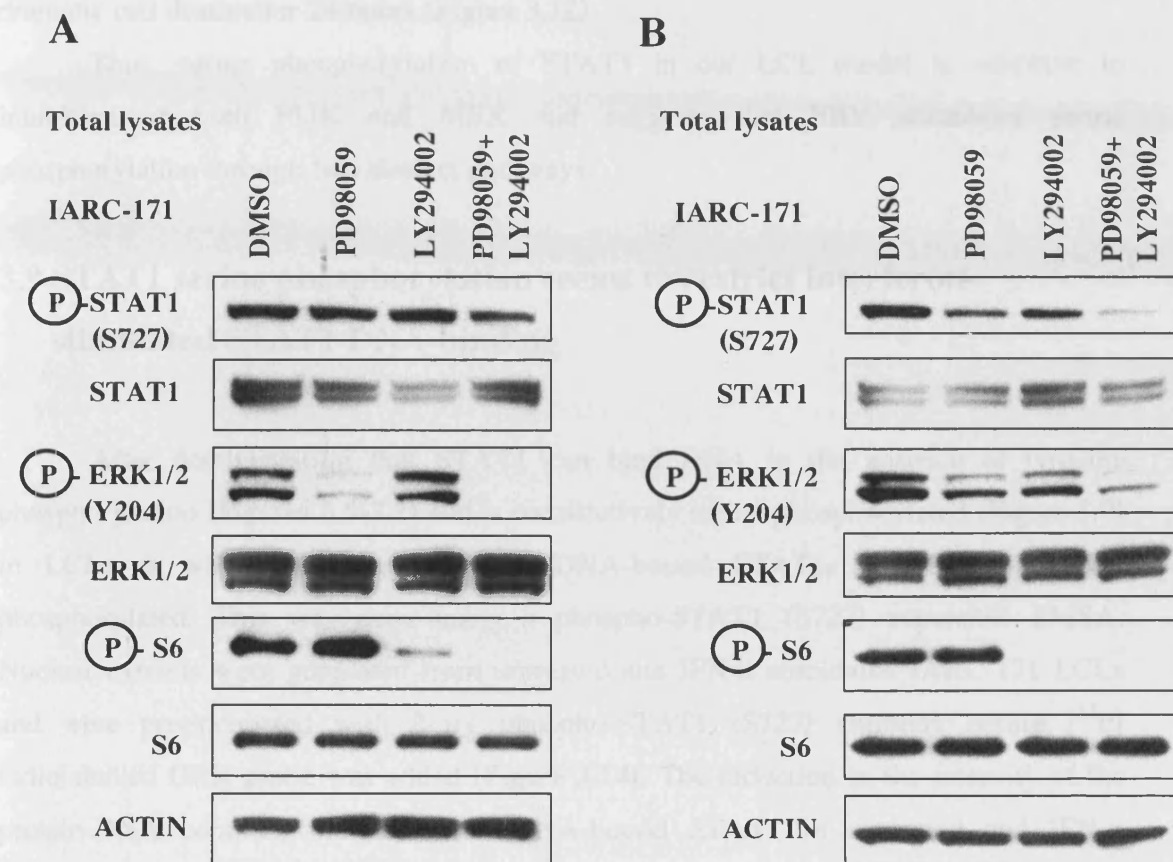


Figure 3.13 STAT1 is serine phosphorylated, downstream of PI3K and MEK

Total cell lysates were generated from IARC-171 LCLs incubated for 1 hour (A) or 24 hours (B) with different combinations of PD98059 and LY294002. These combinations were PD98059 (50 μ M) alone, LY294002 (20 μ M) alone, and PD98059 (50 μ M) + LY294002 (20 μ M). DMSO was incubated for 1 or 24 hours as a control. These lysates were then analysed by SDS-PAGE and Western Blotting using antibodies specific to phospho-STAT1 (S727), pan-STAT1, phospho-ERK1/2 (Y204), pan-ERK1/2, phospho-S6, pan-S6 and actin. Typically 5×10^5 cells were applied in each lane of the gel. These results are representative of four separate experiments.

hours but was not seen after 1 hour. Also, both inhibitors in combination did not cause dramatic cell death after 24 hours (Figure 3.12).

Thus, serine phosphorylation of STAT1 in our LCL model is sensitive to inhibition of both PI3K and MEK and suggests that EBV stimulates serine phosphorylation through two distinct pathways.

3.9 STAT1 serine phosphorylation seems to restrict interferon-stimulated STAT1 DNA-binding

After demonstrating that STAT1 can bind DNA in the absence of tyrosine phosphorylation (Figures 3.5-3.7) and is constitutively serine phosphorylated (Figure 3.9) in LCLs, it was investigated whether DNA-bound STAT1, in LCLs, is serine phosphorylated. This was done using a phospho-STAT1 (S727) supershift EMSA. Nuclear extracts were generated from untreated and IFN- α stimulated IARC-171 LCLs and were pre-incubated with 2 μ g phospho-STAT1 (S727) antibody before [32 P] radiolabelled GRR probe was added (Figure 3.14). The reduction in the intensity of the protein-DNA complex indicated that DNA-bound STAT1 in untreated and IFN- α stimulated IARC-171 LCLs is serine phosphorylated.

After showing that DNA-bound STAT1 in LCLs is serine phosphorylated, the next step was to investigate whether inhibiting STAT1 serine phosphorylation in EBV-transformed LCLs affected its DNA-binding capabilities. STAT1 DNA binding was analysed in IARC-171 LCLs that were either untreated, incubated with a combination of PD98059 and LY294002 for 24hrs and/or stimulated with IFN- α for 30 minutes by DNA-AP (Figure 3.15A) and EMSA (Figure 3.15B). The results show that inhibition of serine phosphorylation did not abrogate STAT1 DNA binding in untreated or IFN- α stimulated IARC-171 LCLs. In fact, surprisingly, inhibition of serine phosphorylation actually increased the amount of STAT1 DNA binding in IFN- α treated cells. Interestingly, IFN- α treatment did not increase the amount of STAT1 found in nuclear extracts. This effect is attributed to the high levels of STAT1 that already exist in the nucleus of an LCL and shows that IFN- α does not stimulate further STAT1 nuclear translocation.

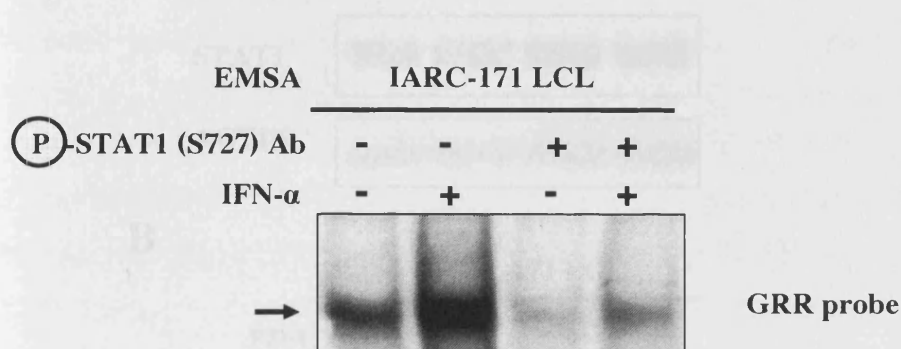


Figure 3.14 DNA-bound STAT1 is serine phosphorylated in LCLs

The presence of serine-phosphorylated STAT1 in DNA-bound protein complexes was measured in unstimulated and IFN- α treated IARC-171 LCLs by EMSA. 10 μ g of nuclear protein was pre-incubated for 30 minutes with 2 μ g phospho-STAT1 (S727) antibody prior to incubation with 2ng [32 P] radiolabelled GRR oligonucleotide probe. Protein-DNA complexes were separated using a 4 % native polyacrylamide gel and visualized by autoradiography. Arrows indicate specific protein-DNA complexes. The results shown are representative of two separate experiments.

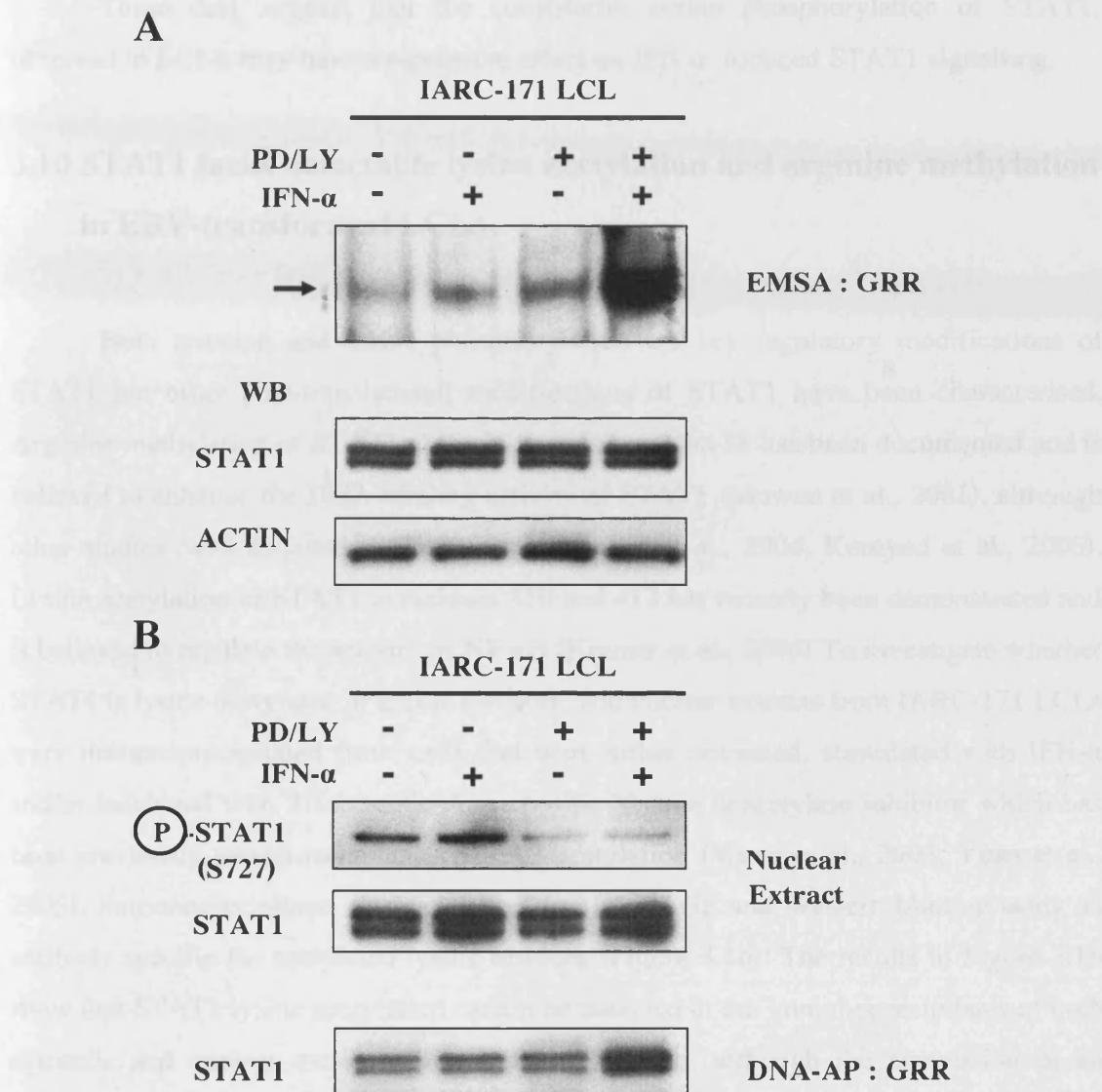


Figure 3.15 STAT1 serine phosphorylation, downstream of PI3K and MEK, seems to restrict interferon-stimulated STAT1 DNA binding in LCLs

The effect of serine phosphorylation on STAT1 DNA binding was measured in IARC-171 LCLs using an EMSA (A) and DNA-AP (B). These cells were either unstimulated, treated with a combination of PD98059 (50 μ M) and LY294002 (20 μ M) for 24hrs and/or incubated with IFN- α (1,000 IU) for 30 minutes. (A) For EMSA, 10 μ g of nuclear extract was incubated with 2 ng [32 P] radiolabelled GRR oligonucleotide probe. Protein-DNA complexes were separated using a 4% native polyacrylamide gel and visualized by autoradiography. 10 μ g of nuclear extract was also analysed by SDS-PAGE and Western Blotting using antibodies specific to STAT1 and actin. This demonstrates that the nuclear levels of STAT1 were equal in each sample analysed. The results shown are representative of five separate experiments. (B) For DNA-AP, nuclear extracts from 1×10^7 cells were precipitated with 1 μ g biotinylated GRR oligonucleotide probe and 30 μ l streptavidin-coated agarose beads. Nuclear extracts and DNA bound proteins were analysed by SDS-PAGE and Western blotting using antibodies specific to phospho-STAT1 (S727) and pan-STAT1. The results shown are representative of three separate experiments.

These data suggest that the constitutive serine phosphorylation of STAT1, observed in LCLs, may have a repressive effect on IFN- α - induced STAT1 signalling.

3.10 STAT1 lacks detectable lysine acetylation and arginine methylation in EBV-transformed LCLs

Both tyrosine and serine phosphorylation are key regulatory modifications of STAT1 but other post-translational modifications of STAT1 have been characterised. Arginine methylation of STAT1 at the N-terminal residue 31 has been documented and is believed to enhance the DNA-binding activity of STAT1 (Mowen et al., 2001), although other studies have disputed these claims (Meissner et al., 2004, Komyod et al., 2005). Lysine acetylation of STAT1 at residues 410 and 413 has recently been demonstrated and is believed to regulate the activity of NF- κ B (Kramer et al., 2006) To investigate whether STAT1 is lysine acetylated in LCLs, cytosolic and nuclear extracts from IARC-171 LCLs were immunoprecipitated from cells that were either untreated, stimulated with IFN- α and/or incubated with Trichostatin A, a specific histone deacetylase inhibitor which has been previously shown to enhance STAT3 acetylation (Wang et al., 2005, Yuan et al., 2005). Immunoprecipitates were analysed by SDS-PAGE and Western blotting using an antibody specific for acetylated lysine residues (Figure 3.16). The results in Figure 3.16 show that STAT1 lysine acetylation cannot be detected in the immunoprecipitates of both cytosolic and nuclear extracts from IARC-171 LCLs, although the acetylation of an unidentified protein was observed in standard cytosolic and nuclear extracts.

Having shown a lack of detectable lysine acetylation of STAT1 in the cells, it was investigated whether STAT1 is arginine methylated in LCLs. STAT1 was immunoprecipitated from the cytosolic and nuclear extracts of the EBV-negative Burkitt's lymphoma line, BL41, and IARC-171 LCL. These cells were either untreated or stimulated with IFN- α for 30 minutes. Immunoprecipitates were analysed by SDS-PAGE and Western blotting using an antibody specific for methylated arginine residues (Figure 3.17A). The results in Figure 3.17A show that STAT1 arginine methylation cannot be detected in the immunoprecipitates of both cytosolic and nuclear extracts from IARC-171

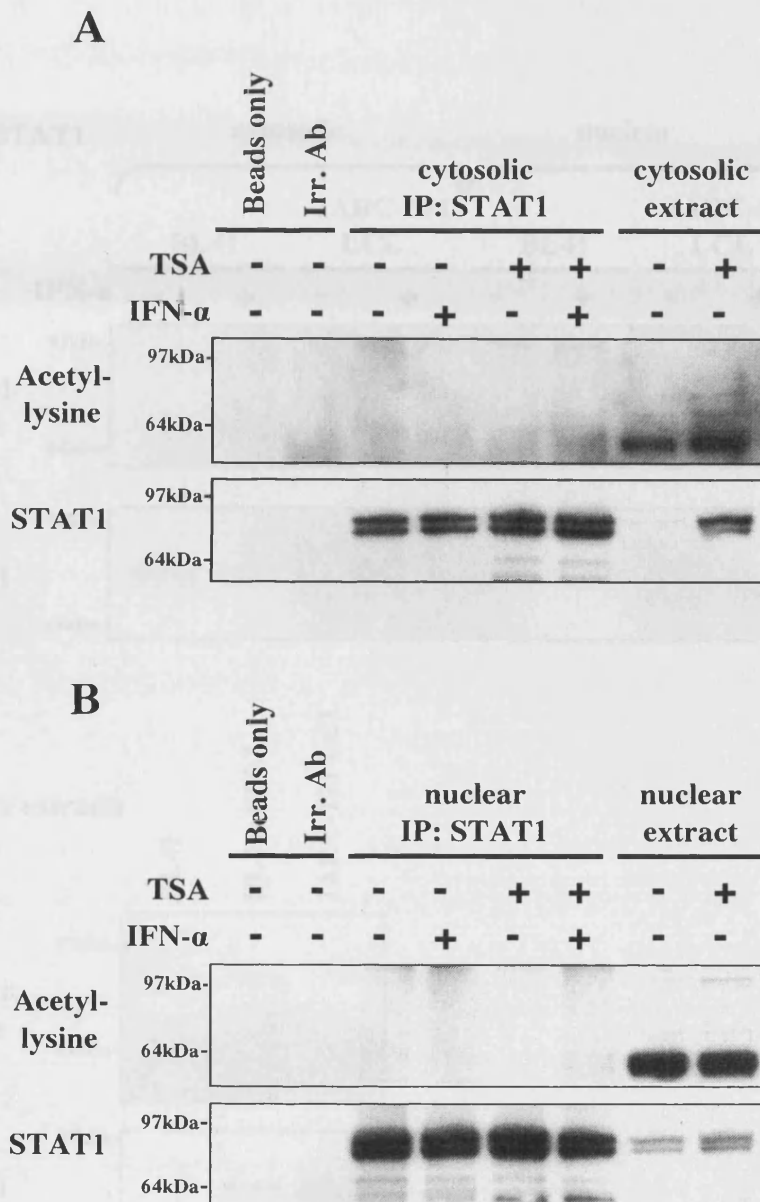
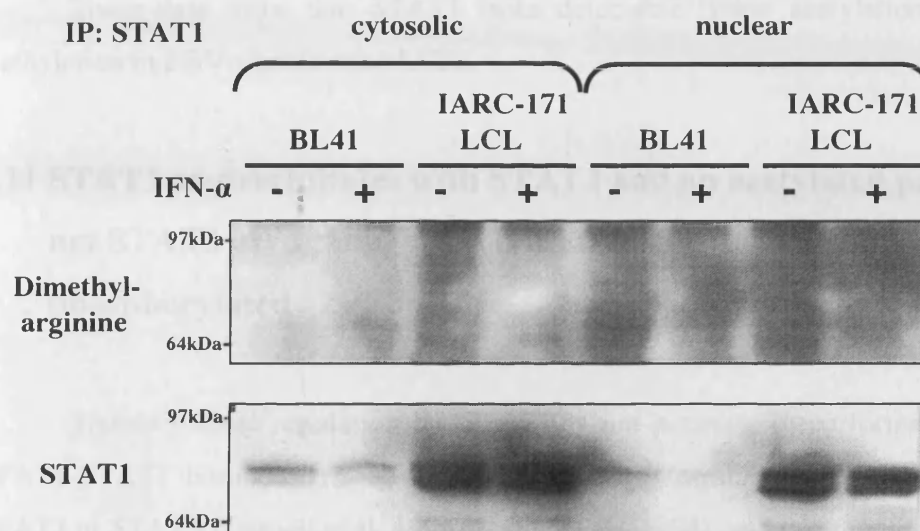


Figure 3.16 STAT1 lacks detectable lysine acetylation in LCLs

STAT1 immunoprecipitates were generated from cytosolic (A) or nuclear extracts (B) of IARC-171 LCLs that were either unstimulated, incubated with IFN- α (1,000 IU) for 30 minutes and/or treated with trichostatin A (TSA) (2 μ M) for 24 hrs. STAT1 immunoprecipitates were then analysed by SDS-PAGE and Western blotting using antibodies specific to acetyl-lysine and pan-STAT1. Typically, 5×10^6 cell equivalents were loaded in lanes 1-6 of the gel and 2.5×10^5 cell equivalents of nuclear extracts from unstimulated and TSA-treated IARC-171 LCLs were loaded in lanes 7 and 8 as controls. These results are representative of four separate experiments.

A



B

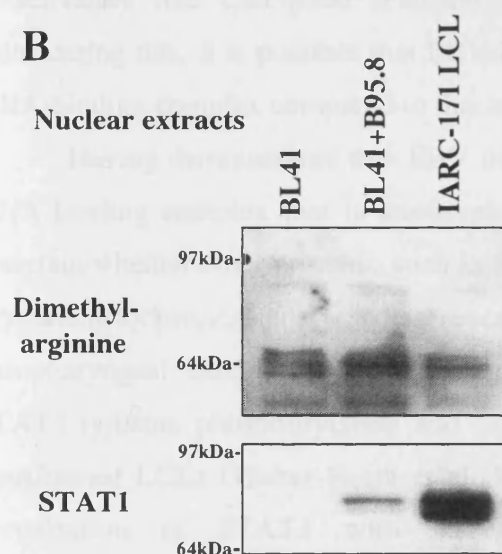


Figure 3.17 STAT1 lacks detectable arginine methylation in LCLs

(A) STAT1 immunoprecipitates were generated from cytosolic and nuclear extracts of BL41 and IARC-171 LCL lines. Cells were either untreated or incubated with IFN- α (1,000 IU) for 30 minutes. STAT1 immunoprecipitates were then analysed by SDS-PAGE and Western blotting using antibodies specific to dimethylarginine and pan-STAT1. Typically, 5×10^6 cell equivalents were loaded in each lane of the gel. These results are representative of two separate experiments. (B) Nuclear extracts were generated from BL41, BL41+B95.8 and IARC-171 LCL lines. $2\mu\text{g}$ of nuclear extract was analysed by SDS-PAGE and Western blotting using antibodies specific to dimethylarginine and pan-STAT1. The presence of a methylated protein in the nuclear extracts of all three cell lines acts as a positive control for the antibody.

LCLs. However the methylation of an unknown protein was observed in nuclear extracts of both cell lines (Figure 3.17B).

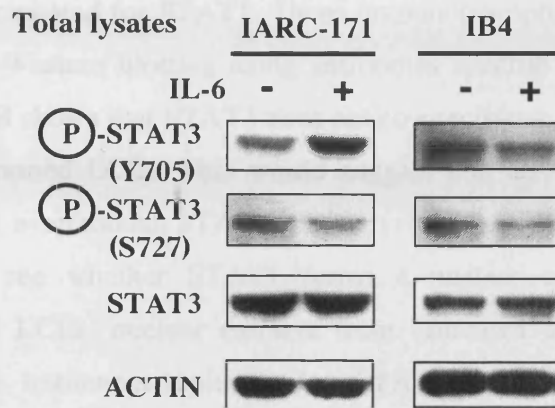
These data show that STAT1 lacks detectable lysine acetylation and arginine methylation in EBV-transformed LCLs.

3.11 STAT1 co-precipitates with STAT2 and an acetylated protein, but not STAT3 even though it is both tyrosine and serine phosphorylated

Transcriptional regulation by STAT1 is not necessarily performed by classical STAT1-STAT1 homodimers. STAT1 is capable of forming heterodimers with either STAT2 or STAT3 (Darnell et al. 1994, Zhong et al., 1994), and can recruit transcriptional co-activators like CBP/p300 (Paulson et al., 1999) to sites of gene transcription. Considering this, it is possible that EBV-induced STAT1 is capable of forming a unique DNA-binding complex compared to that seen following IFN- α stimulation.

Having demonstrated that EBV transformed LCLs contain a constitutive STAT1 DNA binding complex that is unphosphorylated at tyrosine 701, the next step was to ascertain whether other proteins, such as STAT2 or STAT3, are included in this complex. Immunohistochemical analysis has revealed that STAT3 is constitutively activated in nasopharyngeal carcinoma and Hodgkin's disease (Chen et al., 2001). Constitutive STAT3 tyrosine phosphorylation and DNA-binding has also been described in EBV-transformed LCLs (Weber-Nordt et al. 1996, Nepomuceno et al., 2002). Since heterodimerisation of STAT3 with STAT1 requires tyrosine phosphorylation, the phosphorylation status of STAT3 was determined in two EBV-transformed LCLs, IARC-171 and IB4. Total lysates were generated from cells that were either untreated or stimulated with IL-6 for 30 minutes. These lysates were analysed by SDS-PAGE and Western blotting using antibodies specific to phospho-STAT3 (Y705), phospho-STAT3 (S727), pan-STAT3 and actin. Figure 3.18A shows that, in both LCLs, STAT3 is constitutively tyrosine and serine phosphorylated.

A



B

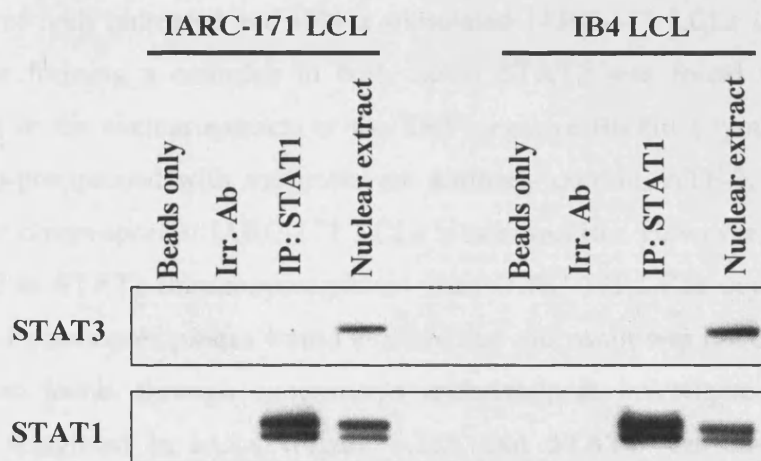


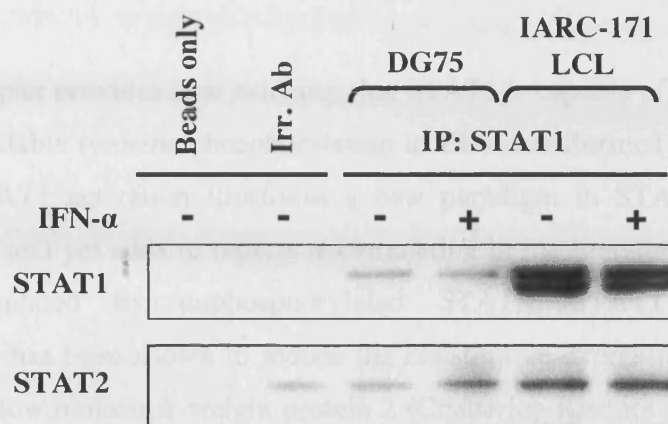
Figure 3.18 STAT3 is constitutively tyrosine and serine phosphorylated in LCLs but does not co-precipitate with nuclear STAT1

(A) Total cell lysates were generated from two LCLs, IARC-171 and IB4. These cell lines were either untreated or stimulated with 10 ng/ml interleukin-6 (IL-6) for 30 minutes. These lysates were analysed by SDS-PAGE and Western blotting using antibodies specific to phospho-STAT3 (Y705), phospho-STAT3 (S727), pan-STAT3 and actin. Typically 5×10^5 cells were applied in each lane of the gel. These results are representative of three experiments. (B) STAT1 immunoprecipitates were generated from nuclear extracts of untreated IARC-171 and IB4 LCLs. A beads only and irrelevant antibody (ATF-3) control were also incubated with nuclear extracts of untreated IARC-171 and IB4 LCLs. Immunoprecipitates were then analysed by SDS-PAGE and Western blotting using antibodies specific to pan-STAT3 and pan-STAT1. Typically, 2×10^6 cell equivalents were loaded for each immunoprecipitate. Also, 2×10^5 cell equivalents of nuclear extract from untreated IARC-171 and IB4 LCLs were loaded as positive controls. These results are representative of two separate experiments.

To investigate whether STAT1 complexes with STAT3 in the nuclei of EBV-transformed LCLs, nuclear extracts from untreated IARC-171 and IB4 LCLs were immunoprecipitated for STAT1. These immunoprecipitates were then analysed by SDS-PAGE and Western blotting using antibodies specific to pan-STAT3 and pan-STAT1. Figure 3.18B shows that STAT3 does not co-precipitate with STAT1 in the nuclei of both EBV-transformed LCLs. This would suggest that STAT3 was not forming a complex with STAT1 even though STAT3 is both tyrosine and serine phosphorylated.

To see whether STAT1 forms a nuclear complex with STAT2 in EBV-transformed LCLs, nuclear extracts from untreated and IFN- α stimulated IARC-171 LCLs were immunoprecipitated for STAT1. These immunoprecipitates were then analysed by SDS-PAGE and Western blotting using antibodies specific to pan-STAT2 and pan-STAT1. Figure 3.19A shows that STAT2 co-precipitates with STAT1 in the nuclei of both untreated and IFN- α stimulated IARC-171 LCLs indicating that STAT2 may be forming a complex in both cases. STAT2 was found to co-precipitate with STAT1 in the nuclear extracts of the EBV-negative Burkitt's lymphoma line DG75 but also co-precipitated with the irrelevant antibody control, ATF-3, which would suggest that the observation in IARC-171 LCLs is non-specific. However, the presence of more STAT2 in STAT1 immunoprecipitates from IARC-171 LCLs compared to that seen in ATF-3 immunoprecipitates would indicate that this result was not entirely non-specific. It was also found, through experiments undertaken to investigate whether STAT1 was lysine acetylated in LCLs (Figure 3.16), that STAT1 was co-precipitating with an acetylated protein of roughly 14-15kDa in size in IFN- α stimulated and/or TSA-treated IARC-171 LCLs (Figure 3.19B). This protein, given its molecular weight, could possibly be one of the histone proteins. From the data in Figure 3.19B, it seems that this acetylated histone protein is complexing with STAT1 in IFN- α stimulated cells rather than in untreated cells, even though it does reside in the nuclear extracts of untreated cells. This indicates a phenotypic difference in the STAT1 protein complex induced by EBV compared to that seen following IFN- α stimulation.

A



B

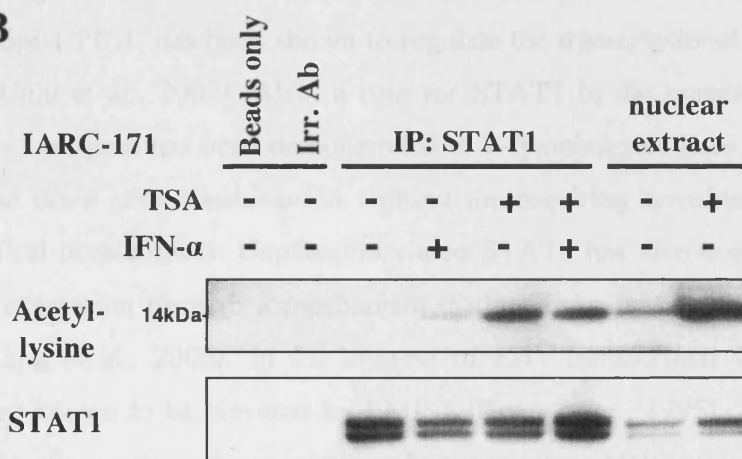


Figure 3.19 STAT1 co-precipitates with STAT2 and with an acetylated protein in LCLs

(A) STAT1 immunoprecipitates were generated from nuclear extracts of two B-cell lines, DG75 and IARC-171 LCL. These cell lines were either untreated or incubated with IFN- α (1,000 IU) for 30 minutes. A beads only and irrelevant antibody (ATF-3) control were also incubated with nuclear extracts of untreated IARC-171 LCLs. STAT1 immunoprecipitates were then analysed by SDS-PAGE and Western blotting using antibodies specific to pan-STAT1 and pan-STAT2. Typically, 5×10^6 cell equivalents were loaded in each lane of the gel. These results are representative of two separate experiments. (B) STAT1 immunoprecipitates were generated from nuclear extracts of IARC-171 LCLs that were either unstimulated, incubated with IFN- α (1,000 IU) for 30 minutes and/or treated with trichostatin A (TSA) (2 μ M) for 24 hrs. STAT1 immunoprecipitates were then analysed by SDS-PAGE and Western blotting using antibodies specific to acetyl-lysine and pan-STAT1. Typically, 5×10^6 cell equivalents were loaded in lanes 1-6 of the gel and 2.5×10^5 cell equivalents of nuclear extracts from unstimulated and TSA-treated IARC-171 LCLs were loaded in lanes 7 and 8 as controls. These results are representative of three experiments.

3.12 Discussion

This chapter provides new evidence that STAT1 is capable of binding DNA in the absence of detectable tyrosine phosphorylation in EBV-transformed LCLs. This form of constitutive STAT1 activation illustrates a new paradigm in STAT signalling in the context of EBV and yet adds to reports accumulating in the literature that describe gene expression regulated by unphosphorylated STATs. STAT1 lacking tyrosine phosphorylation has been shown to induce the constitutive expression of genes in other cells, including low molecular weight protein 2 (Chatterjee-Kishore et al., 2000), and the caspase 1-3 genes (Kumar et al., 1997). Unphosphorylated STAT1, in a complex with IRF8 and Spi-1/PU.1, has been shown to regulate the transcriptional induction of the IL-1 β gene (Unlu et al., 2007). Also, a role for STAT1 in the constitutive expression of MHC class I antigens has been demonstrated in T-lymphocytes (Lee et al., 1999b). Thus, STAT1 can drive gene transcription without the requiring tyrosine phosphorylation to form classical homodimers. Unphosphorylated STAT3 has also been shown to activate oncogene expression through a mechanism distinct from that used by classical STAT3 dimers (Yang et al., 2005). In the context of EBV-transformed LCLs, MHC class I antigens are known to be elevated by LMP-1 (Rowe et al., 1995). Thus LMP1-induced STAT1 may play a role in the regulation of genes such as MHC class I.

Although this study has characterised a STAT1 DNA-binding complex lacking tyrosine phosphorylation in EBV-transformed LCLs, this complex does not seem to exist in untreated BL41+B95.8 cells (Figure 3.5). This observation conflicts with previously published data which shows DNA-bound STAT1 in untreated BL41+B95.8 cells (Richardson et al., 2003). However, this contrast reflects the variable expression of LMP1 in these cells as clones high in LMP1 display DNA-bound STAT1 whereas those low in LMP1 lack detectable STAT1 DNA-binding. The role of LMP1 in this STAT1-DNA-binding complex is evident from the data displayed in Figure 3.7. LMP1 is sufficient to induce a constitutive STAT1 DNA-binding complex that lacks tyrosine phosphorylation. However, this hypothesis could be strengthened by depleting LMP1 from an LCL by RNA interference. Other STATs, such as STAT2 or STAT3, may comprise part of this complex since STAT1 is capable of forming STAT1-STAT2 and STAT1-STAT3

heterodimers as well as forming classical STAT1 homodimers (Darnell et al., 1994, Zhong et al., 1994). In regards to STAT3, it was found, by immunoprecipitation, that STAT3 did not co-precipitate with STAT1 in the nucleus of IARC-171 LCLs even though it was both tyrosine and serine phosphorylated (Figure 3.18). STAT2 was found to co-precipitate with STAT1 in the nuclei of EBV-transformed LCLs, although this effect was also observed in an EBV-negative B-cell line (Figure 3.19A) which would suggest that it is a feature of B-cell malignancy rather than an EBV-specific phenotype. Other, non-STAT related proteins could be involved also since an acetylated, low molecular weight protein was found to co-precipitate with STAT1 in IFN- α stimulated LCLs (Figure 3.19B). Only with further characterisation will the function of this STAT1 complex in EBV-transformed LCLs be elucidated.

Constitutive serine phosphorylation of STAT1 has been observed in malignancies such as CLL (Frank et al., 1997) and Wilms' tumour (Timofeeva et al., 2006). This chapter provides further evidence that STAT1 is constitutively serine phosphorylated in EBV infected cells agreeing with reports in the literature (Zhang et al., 2004). The data suggests that this is not EBV specific since constitutive serine phosphorylation was also observed in the EBV-negative Burkitt's lymphoma, DG75 (Figure 3.9). However, constitutive STAT1 DNA binding was seen in IARC-171 LCLs, but not in the DG75 cells (Figure 3.7B) even though both cell lines exhibit serine phosphorylated STAT1 (Figure 3.9). This would suggest that DNA-bound serine phosphorylated STAT1 is a feature of EBV-transformed LCLs. Also, since constitutive serine phosphorylation is absent in normal peripheral blood B-lymphocytes (Frank et al., 1997), this suggests that constitutive serine phosphorylation of STAT1 may be a feature of B-cell malignancy in general. Recent evidence has shown that serine phosphorylated STAT1 promotes cell survival through the up-regulation of two known pro-survival genes, *mcl-1* and *HSP-27* (Timofeeva et al., 2006), indicating why malignant B-cells may accumulate this molecular change.

Lysine acetylation of STAT1 has recently been demonstrated (Kramer et al., 2006) and could regulate its transcriptional abilities as lysine acetylation of STAT3 has been shown to be vital for its DNA-binding and transcriptional capacity (Wang et al., 2005, Yuan et al., 2005). The evidence displayed in this chapter suggests that STAT1 is

not lysine acetylated in EBV-transformed LCLs (Figure 3.16). This observation would suggest that this modification is not necessary for STAT1 function in LCLs although we do not rule out the possibility that we cannot detect it with the technology at our disposal. Arginine methylation of STAT1 has also been characterised and is believed to enhance the DNA-binding activity of STAT1 (Mowen et al., 2001). However, the data supplied in this chapter suggests that STAT1 is not arginine methylated in EBV-transformed LCLs (Figure 3.17). More specific antibodies for both lysine-acetylated and arginine-methylated STAT1 may provide a different answer but, at present, do not exist commercially.

Many serine kinase(s) have been implicated in catalyzing STAT1 serine phosphorylation in various cell systems. The data supplied in this chapter shows that the constitutive serine phosphorylation of STAT1 in LCLs is abrogated following long term treatment with inhibitors of PI3K and MEK (Figure 3.14b). Both of these enzymes have also been implicated by other studies (Nguyen et al., 2001, Rahimi et al., 2005, Zykova et al., 2005). Long term treatment was necessary to ensure sufficient inhibition of STAT1 serine phosphorylation as shorter incubation times yielded very little or no effect (Figure 3.14a). It is possible that this may reflect some form of indirect mechanism or perhaps just a slow inhibitory effect by PD98059 and LY294002 in combination. This is highlighted by the fact that staurosporine, a broad spectrum serine/threonine kinase inhibitor, caused rapid inhibition after only one hour (Figure 3.10). By inhibiting STAT1 serine phosphorylation in LCLs, through use of the combined incubation of PD98059 and LY294002, increased STAT1 DNA-binding in LCLs stimulated with IFN- α was shown (Figure 3.15). This provides evidence that the constitutive serine phosphorylation of STAT1 in LCLs may have a repressive effect on IFN- α - induced STAT1 signalling. Repression of STAT signalling has been linked to serine phosphorylation and suggests that its role is more complex than previously thought (Bowman et al., 2000). STAT3 serine phosphorylation has been shown to prevent tyrosine phosphorylation and DNA-binding through either a direct influence or an indirect negative interaction upon upstream tyrosine kinases (Chung et al., 1997, Jain et al., 1998, Sengupta et al., 1998). The data in this chapter agrees with these findings in that serine phosphorylation seems to

repress STAT1 DNA-binding in IFN- α stimulated LCLs. This effect could explain why no constitutive STAT1 tyrosine phosphorylation is seen in our LCLs (Figure 3.4).

In summary, this chapter builds on previous reports by being the most complete survey of post-translational modifications of STAT1 in EBV-transformed LCLs. This work illustrates three key advances in our knowledge. Firstly, this work has demonstrated that LMP1-induced STAT1 lacks tyrosine phosphorylation, lysine acetylation and arginine methylation but is capable of binding DNA. Secondly, this work has shown, for the first time in EBV-transformed LCLs, that the serine phosphorylation of STAT1 is regulated by two distinct pathways, PI3K and MEK. Thirdly and most surprisingly, this modification appears to repress the DNA binding of interferon stimulated STAT1. This indicates that STAT1 may be subject to some form of viral re-programming by EBV during cellular transformation.

CHAPTER 4

Generation of an *in vitro* model to study the function of STAT1 in EBV-transformed LCLs

4.1 Introduction

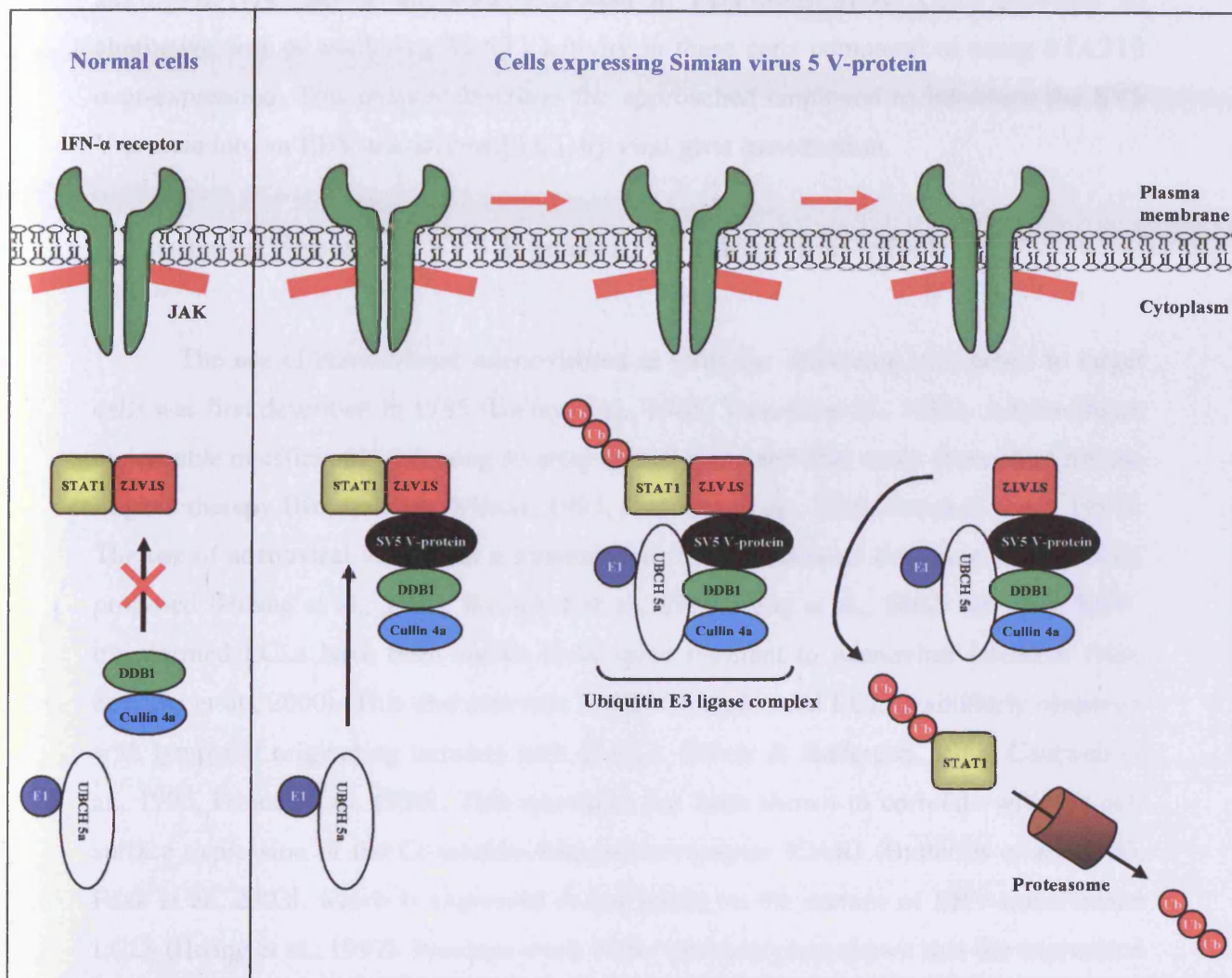
The mechanism for how EBV induces STAT1 protein expression and transcriptional activity has been intensively studied. Chapter 3 of this thesis demonstrates that EBV induces a distinct form of DNA-bound STAT1 that may have a functional role in EBV-associated malignancy. However, in the context of EBV-transformed B-lymphocytes, there has been little investigation into the function of STAT1 in these cells. This is most likely due to the lack of commercially available pharmacological inhibitors of STAT1 at present. However, other methods have been employed to inhibit STAT1 activity in EBV-transformed B-lymphocytes. Fludarabine, which is a nucleoside analogue used in the treatment of haematological malignancies like CLL (Plunkett et al., 1993), has demonstrated inhibitory effects on STAT1 signalling in peripheral blood mononuclear cells and EBV-transformed LCLs (Frank et al., 1999, Fagard et al., 2002). Fludarabine has a wide range of actions including inhibition of DNA and RNA synthesis. Given that high concentrations were required over a long time period in order to see inhibition of STAT1 (Fagard et al., 2002), it is likely that these effects were not STAT1-specific. Over-expression of STAT1 β , a naturally inactive isoform of STAT1, has also been used to inhibit STAT1 activity in LCLs (Le Clorennec et al., 2006). This method indicated that STAT1 may be involved in CD95-mediated apoptosis, in conjunction with NF- κ B and p53 (Le Clorennec et al., 2006).

Techniques, such as STAT1 RNA interference which can be used to reduce STAT1 protein expression, depend on achieving high transfection efficiency. However,

EBV-transformed LCLs, along with B-lymphocytes in general, are resistant to genetic modification. It has been shown that transfection of plasmid DNA into EBV-transformed LCLs generally results in a transfection efficiency of less than 5% (White et al., 2002). In retrospect, it would seem that only one study, at present, has found a viable way to inhibit STAT1 in EBV-transformed LCLs (Le Clorennec et al., 2006).

The objective of this chapter was to generate an *in vitro* model that could be used to evaluate the function of STAT1 in EBV-transformed B-lymphocytes. In order to do this, this work focussed on utilising the actions of the V-protein from the simian virus type 5 (SV5). SV5 belongs to a family of paramyxoviruses which have evolved evasive mechanisms to the anti-viral effects of interferon (Horvath, 2004). These paramyxoviruses, which are sub-divided into several genera including *Rubulavirus*, *Henipavirus*, *Morbillivirus*, and *Respirovirus*, encode viral V-proteins which are able to directly interact with STAT proteins (Horvath, 2004). These V-proteins, interestingly, act in different ways within each individual genera. For example, the V-proteins from rubulaviruses like SV5, type II human parainfluenza virus and mumps virus target STATs for poly-ubiquitination and subsequent proteasomal degradation (Didcock et al., 1999, Parisien et al., 2001, Andrejeva et al., 2002, Nishio et al., 2002, Ulane et al., 2003). However, V-proteins from Henipaviruses like Nipah and Hendra sequester both STAT1 and STAT2 in cytoplasmic complexes without inducing degradation whereas the V-protein from the Measles virus, a Morbillivirus, prevents IFN-induced STAT1 and STAT2 nuclear translocation (Rodriguez et al., 2002, Rodriguez et al., 2003, Palosaari et al., 2003). These disruptive effects on interferon signalling are not just restricted to paramyxoviruses. Human cytomegalovirus, rabies virus and the hepatitis C virus all encode viral proteins which interfere with STAT1 activity and help evade the anti-viral response in infected cells (Vidy et al., 2005, Lin et al., 2005, Lan et al., 2006, Paulus et al., 2006)

Of all the rubulavirus V-proteins, the SV5 V-protein solely targets STAT1 for proteasomal degradation (Didcock et al., 1999) and represents a virus-evolved STAT1 inhibitor. Its mechanism of action, which is displayed in Figure 4.1, involves poly-ubiquitination of STAT1 by a ubiquitin E3 ligase complex and subsequent degradation by the proteasome. Interestingly, STAT2 is vital to this process, acting as a linker between



Adapted from Precious *et al.*, 2005 J.Virol

Figure 4.1 Degradation of STAT1 by the Simian Virus 5 V-protein

Schematic of how the V-protein from the simian virus 5 degrades STAT1. In normal cells, the ubiquitin E3 ligase complex does not associate with STAT1-STAT2 heterodimers. In cells expressing the simian virus 5 V-protein, the ubiquitin E3 ligase complex physically associates with STAT2 and catalyzes the polyubiquitination of STAT1. The V-protein itself is the physical linker between STAT2 and the ubiquitin E3 ligase complex. Polyubiquitinated STAT1 dissociates from STAT2 and is degraded by the proteasome.

STAT1 and the ubiquitin E3 ligase complex without being degraded itself (Precious et al., 2005). The use of the SV5 V-protein in EBV-transformed LCLs provides an alternative way of inhibiting STAT1 activity in these cells compared to using STAT1 β over-expression. This chapter describes the approach employed to introduce the SV5 V-protein into an EBV-transformed LCL by viral gene transduction.

4.2 Introduction of the SV5 V-protein by adenoviral infection

The use of recombinant adenoviruses as tools for delivering transgenes to target cells was first described in 1985 (Ballay et al., 1985, Yamada et al., 1985). Adenoviruses are capable of efficiently infecting an array of cell types and thus make them ideal for use in gene therapy (Kosarsky & Wilson, 1993, Haddada et al., 1995, Kovesdi et al., 1997). The use of adenoviral vectors as a treatment for EBV-associated malignancies has been proposed (Huang et al., 1997, Westphal et al., 1999, Feng et al., 2002) although EBV-transformed LCLs have been shown to be quite resistant to adenoviral infection (Von Seggern et al., 2000). This characteristic of EBV-transformed LCL is similarly observed with lymphoid originating tumours such as CLL (Silver & Anderson, 1988, Cantwell et al., 1996, Prince et al., 1998). This resistance has been shown to correlate with the cell surface expression of the Coxsackie-Adenovirus receptor (CAR) (Butteries et al., 2000, Fuxe et al., 2003), which is expressed at low levels on the surface of EBV-transformed LCLs (Huang et al., 1997). Previous work in the laboratory has shown that the expression of $\alpha\beta 5$ integrin is also a co-factor in the susceptibility of EBV-transformed LCLs to adenoviral infection (Richardson et al., 2005). Considering this, an EBV-transformed LCL, IB4 CAR 8, was generated with high levels of CAR and $\alpha\beta 5$ integrin expression which made it more susceptible to adenoviral type 5 (Ad5) infection (Richardson et al., 2005). This cellular model thus represents a useful tool for genetic modification of EBV-transformed LCLs by recombinant adenoviruses.

To demonstrate the susceptibility of this model to adenoviral infection, IB4 LCLs (parental LCL) and IB4 CAR 8 LCLs were infected with a recombinant Ad5-green fluorescent protein (GFP) adenovirus, kindly donated by Dr. Brian McSharry (Dept. of Medical Microbiology, Cardiff University), for 72 hours at multiplicities of infection

(MOI) of 10, 30, 50 and 100. A control infection was also performed where IB4 and IB4 CAR 8 LCLs were incubated with an equivalent volume of PBS. Post-infection, cells were harvested, fixed and analysed for GFP fluorescence by flow cytometry. Figure 4.2A shows that, at an MOI of 50, IB4 LCLs contain 9.3% GFP positive cells whereas IB4 CAR 8 LCLs contain 62.7% GFP positive cells. The results from all other samples are summarised in Figure 4.2B. These data clearly demonstrate that IB4 CAR 8 LCLs are more susceptible to adenoviral infection compared to the parental IB4 LCL and confirm what was previously shown (Richardson et al., 2005).

The IB4 CAR 8 LCL was now used to introduce the SV5 V-protein by adenoviral infection. The SV5 V-protein-encoding adenovirus (Ad5 SV5), a kind gift from Dr. Rick Randall (University of St. Andrews), was used to infect IB4 CAR 8 LCL at MOIs of 30, 50 and 100. 72 hours post-infection, the cells were harvested as total lysates and analysed by SDS-PAGE and Western blotting using antibodies specific to pan-STAT1 (Figure 4.3B). The infection efficiency of this experiment was also analysed by infecting IB4 CAR 8 LCLs with Ad5-GFP at an MOI of 30. Figure 4.3A shows that 51% of the cells were GFP positive indicating that 51% of the cells in the experiment were adenovirally infected. Figure 4.3B shows that Ad5 SV5 produced a small reduction in STAT1 protein expression at MOIs 50 and 100. This data agrees with that already published (Richardson et al., 2005) and shows that the V-protein is reducing STAT1 expression in an EBV-transformed LCL. However, the effect is weak and could be due to the fact that only half of the cells are infected. Also, the reduction in STAT1 expression seen in Figure 4.3B was not observed in all the experiments and appeared to be dependent on the percentage of adenovirally infected cells. Considering this, it seemed that a more efficient and longer lasting method of viral gene transduction would be required in order to truly produce an *in vitro* model for studying the function of STAT1 in EBV-transformed LCLs. Not only that, this adenoviral LCL model only analyses the effects of the SV5 V-protein in one EBV-transformed LCL, which has already been genetically modified in order to permit adenoviral gene transduction (Richardson et al., 2005). Therefore, to introduce the SV5 V-protein into an EBV-transformed LCL by a more permanent means, retroviral infection was chosen. Retroviruses are able to stably integrate into the host genome and this ability

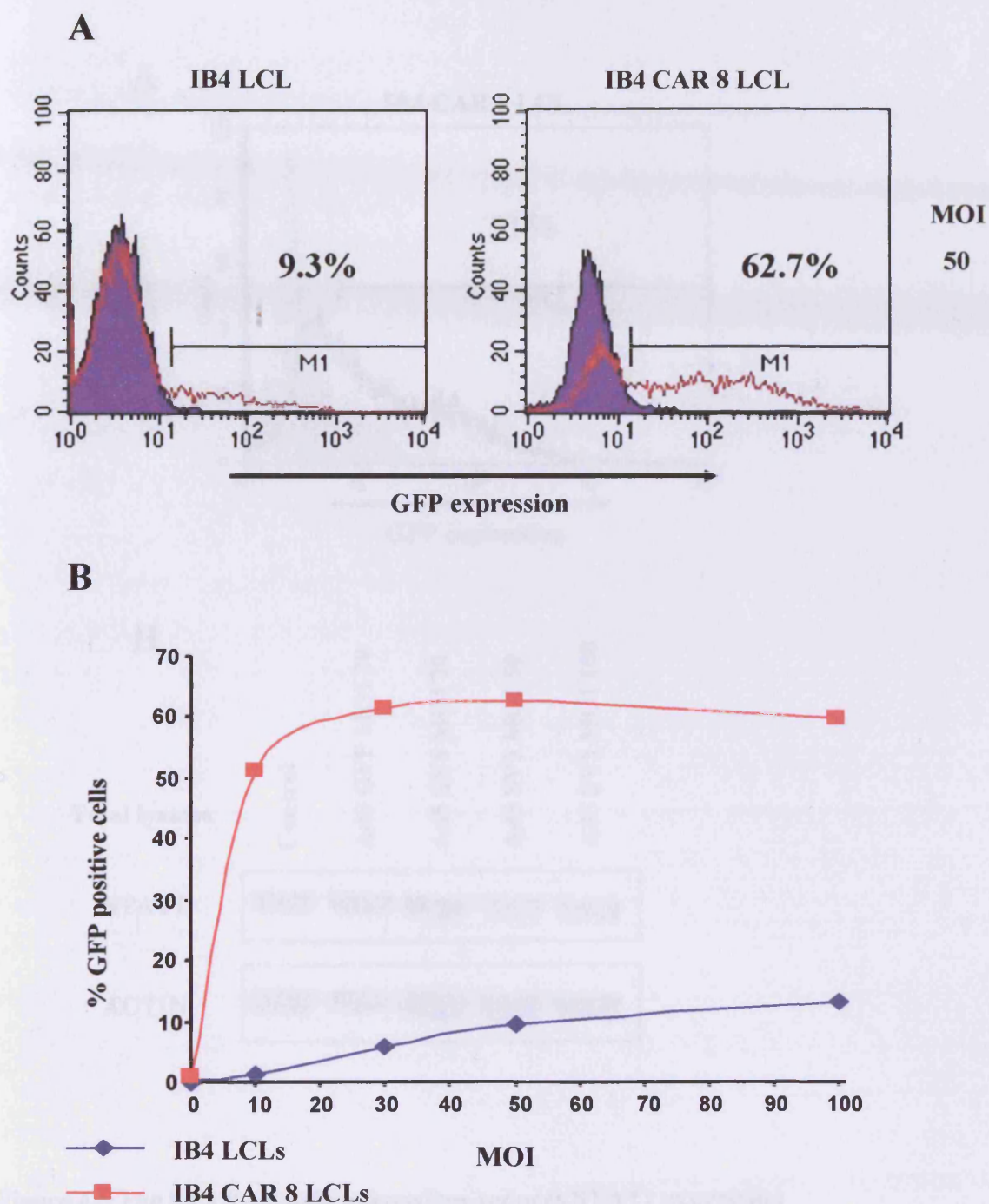


Figure 4.2 IB4 CAR 8 LCLs are more susceptible to adenoviral infection

IB4 and IB4 CAR8 LCLs were infected with a recombinant Ad5-GFP adenovirus at a range of MOIs (MOIs 10, 30, 50 and 100) for 72 hours. Post-infection, cells were analysed for GFP fluorescence by flow cytometry. A representative flow cytometric analysis is shown in (A). For both cell lines, blue shading indicates a control infection and the red outline indicates cells infected with Ad5 GFP at MOI 50. All the results from the adenoviral infection are displayed in the graph in (B). The graph is representative of at least two experiments.

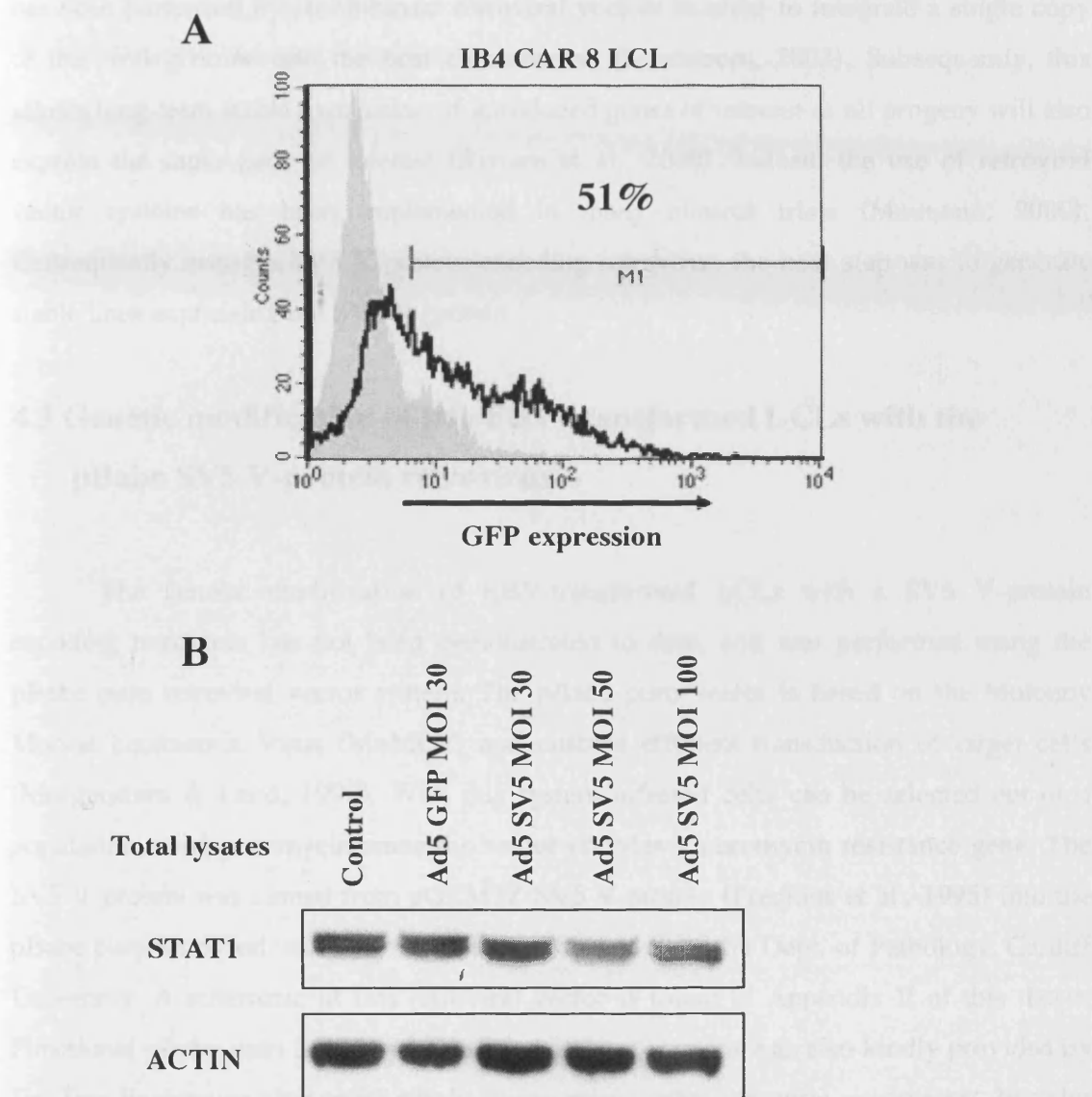


Figure 4.3 The SV5 V-protein adenovirus reduces STAT1 expression

IB4 CAR8 LCLs were infected with Ad5 GFP (MOI 30) and Ad5 SV5 (MOIs 30,50 and 100) adenoviruses for 72 hours. Cells infected with Ad5 GFP were first analysed for GFP fluorescence by flow cytometry (**A**). Light grey shading indicates a control infection and the black outline indicates cells infected with Ad5 GFP. Cells from all infections were then harvested as total lysates. These lysates were analysed by SDS-PAGE and Western Blotting using antibodies specific to pan-STAT1 and actin. Typically 2×10^5 cells were applied in each lane of the gel.

has been harnessed by recombinant retroviral vectors in order to integrate a single copy of the viral genome into the host chromosome (Lundstrom, 2003). Subsequently, this allows long-term stable expression of introduced genes of interest as all progeny will also express the same gene of interest (Riviere et al., 2000). Indeed, the use of retroviral vector systems has been implemented in many clinical trials (Mountain, 2000). Consequently, using a SV5 V-protein encoding retrovirus, the next step was to generate stable lines expressing the SV5 V-protein.

4.3 Genetic modification of two EBV-transformed LCLs with the pBabe SV5 V-protein retrovirus

The genetic modification of EBV-transformed LCLs with a SV5 V-protein encoding retrovirus has not been demonstrated to date, and was performed using the pBabe puro retroviral vector system. The pBabe puro vector is based on the Moloney Murine Leukaemia Virus (MoMLV) and enables efficient transduction of target cells (Morgenstern & Land, 1990). With this system infected cells can be selected out of a population using puromycin since the vector encodes a puromycin resistance gene. The SV5 V-protein was cloned from pGEM3Z SV5 V-protein (Precious et al., 1995) into the pBabe puro retroviral vector by Dr. Zara Poghosyan from the Dept. of Pathology, Cardiff University. A schematic of this retroviral vector is found in Appendix II of this thesis. Functional pBabe puro SV5 V-protein retroviral supernatant was also kindly provided by Dr. Zara Poghosyan along with pBabe puro empty vector retroviral supernatant. In order to retrovirally infect EBV-transformed LCLs, a previously described 2-cycle retroviral infection protocol was adopted (Tonks et al., 2005). This protocol involves the centrifugation of retrovirus onto retronectin-coated tissue culture plates prior to the application of target cells. Retronectin is a commercially available chimeric peptide of recombinant human fibronectin fragments which has been shown to enhance retroviral gene transfer in mammalian cells (Kimizuku et al., 1991, Hanenberg et al., 1996, Hanenberg et al., 1997, Stockschrader et al., 1999, Relander et al., 2001). Two EBV-transformed LCLs, IARC-171 and IB4, were chosen for retroviral infection so that the effects of the V-protein could be observed in more than just one LCL. The wells of a 24-

well tissue culture plate were pre-coated with retronectin for two hours. Following this, retroviral supernatant from the pBabe puro empty vector and pBabe puro SV5 V-protein viruses was added to the wells and centrifuged for two hours at 3000g. A mock infection was also set up where RPMI-1640 medium was added to a well instead of retroviral supernatant. After centrifugation, the retroviral supernatant or medium was removed and replaced with 2×10^5 IARC-171 LCLs or IB4 LCLs. The 24-well plate was then incubated at 37°C overnight. The following day, the second cycle of infection was performed involving removing the target cells, centrifuging more retroviral supernatant before replacing the original target cells. From here the 24-well plate was then incubated at 37°C for 3 days.

After 3 days incubation, puromycin selection was started in order to select out puromycin-resistant LCLs (i.e. retrovirally infected cells). To ascertain what dose to use for selection, a puromycin titration was performed on both uninfected IARC-171 and IB4 LCLs. Both IARC-171 and IB4 LCLs were treated for 72 hours with a range of doses of puromycin. These doses were 0.1, 1, 2.5, 5 and 10 µg/ml. Cell viability was then analysed by flow cytometry using propidium iodide staining. Figure 4.4 demonstrates that a minimum dose of 1 µg/ml puromycin was required to kill 100% of IARC-171 or IB4 LCLs. This dose was then used to grow out puromycin-resistant cells. Cells were analysed by flow cytometry to determine the percentage of live cells (i.e. percentage of genetically modified cells). This was done by calculating the percentage of cells in the live gate. Figure 4.5A shows that IARC-171 LCLs infected with pBabe puro empty vector virus (termed IARC-171 pBabe LCL) were almost 83% viable and IARC-171 LCLs infected with pBabe puro SV5 V-protein virus (termed IARC-171 V-protein LCL) were 84% viable. Figure 4.6A demonstrates that IB4 LCLs infected with pBabe puro empty vector virus (termed IB4 pBabe LCL) were 68% viable and IB4 LCLs infected with pBabe puro SV5 V-protein virus (termed IB4 V-protein LCL) were 62% viable. The mock infections with both LCLs showed 0% cell viability (Figures 4.5A and 4.6A). These data indicate that IARC-171 and IB4 LCLs have been successfully modified with the pBabe puro empty vector and pBabe puro SV5 V-protein retroviruses.

In order to demonstrate the phenotype of IARC-171 and IB4 V-protein LCLs, total lysates were generated from IARC-171 LCL, IARC-171 pBabe LCL, IARC-171 V-

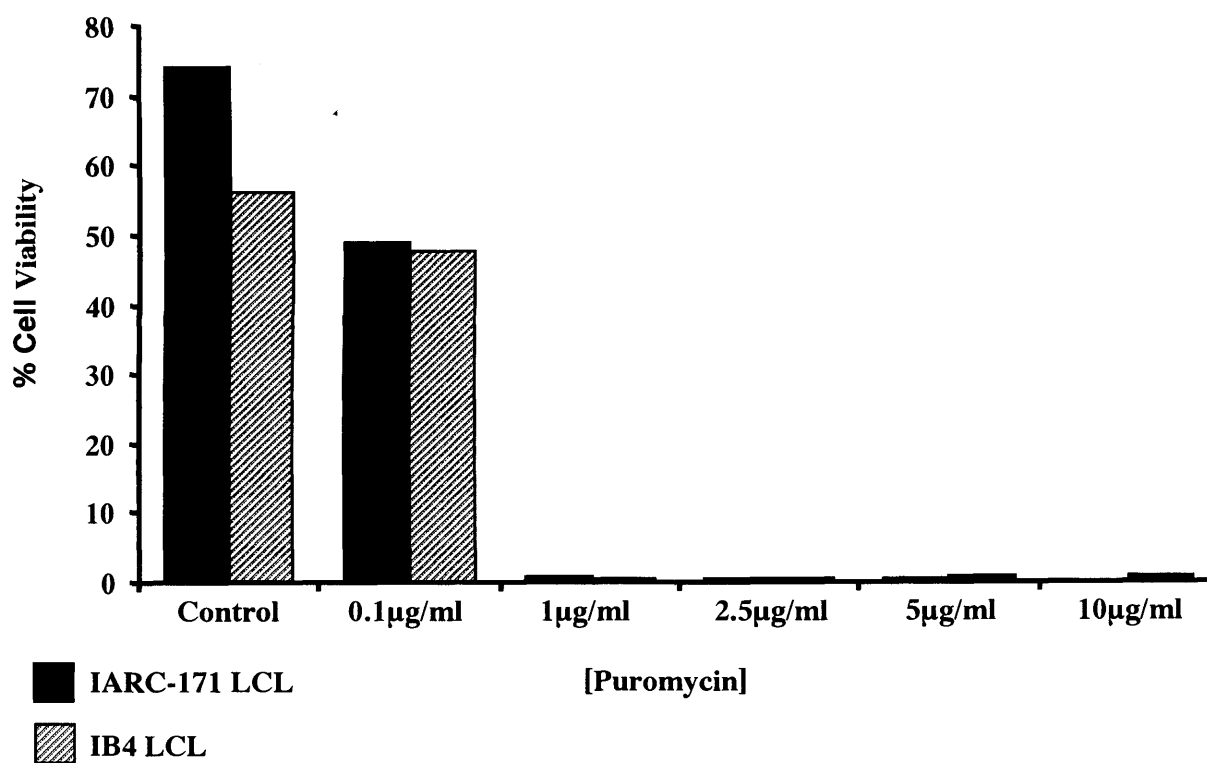
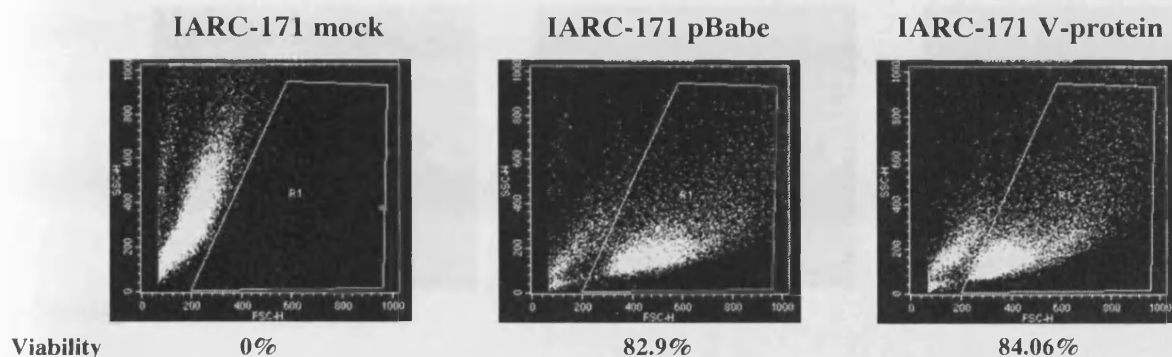


Figure 4.4 Titration of puromycin on IARC-171 and IB4 LCLs

IARC-171 and IB4 LCLs were treated with a range of doses of puromycin for 72hrs. Cells were then tested for viability by flow cytometry using propidium iodide staining. The data represents the percentage of cells that were not propidium iodide positive. The results indicate that a dose of 1µg/ml is sufficient to kill 100% of cells.

A



B

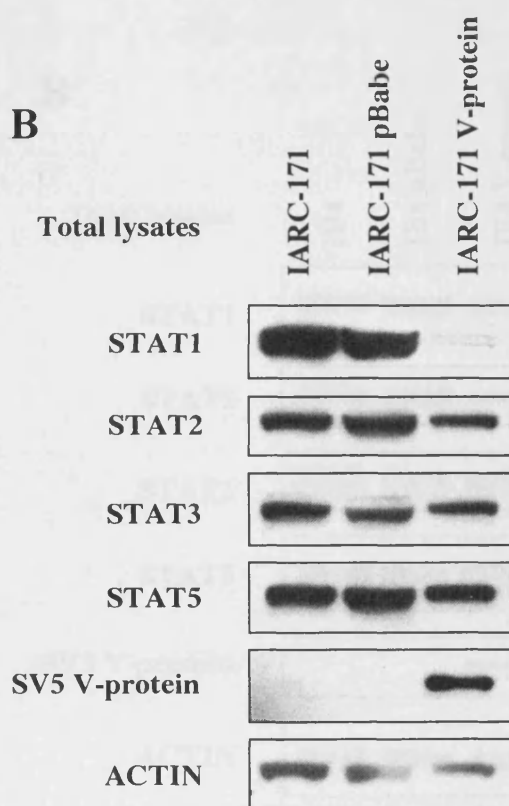
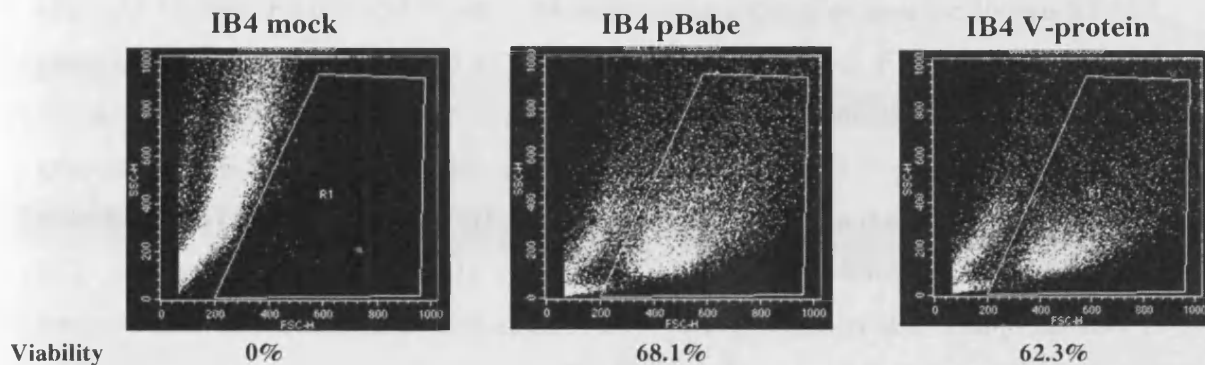


Figure 4.5 Genetic modification of IARC-171 LCLs with pBabe SV5 V-protein retrovirus

IARC-171 LCLs were infected in a 2-cycle retroviral infection with RPMI-1640 only, pBabe puro empty vector retrovirus or pBabe puro SV5 V-protein retrovirus to generate IARC-171 pBabe LCL (empty vector) and IARC-171 V-protein LCL (SV5 V-protein). These cell lines were cultured with puromycin (1 μ g/ml) and monitored regularly for cell viability by flow cytometry. **(A)** Forward and side scatter dot plot results after 11 days under selection (58 days for IARC-171 V-protein). The R1 gate was used to determine the percentage of viable cells within the population. **(B)** Total lysates were generated from IARC-171 LCL, IARC-171 pBabe LCL and IARC-171 V-protein LCL. These lysates were analysed by SDS-PAGE and Western Blotting using antibodies specific to pan-STAT1, pan-STAT2, pan-STAT3, pan-STAT5, SV5 V-protein and actin. Typically 2 \times 10⁵ cells were applied in each lane of the gel.

A



B

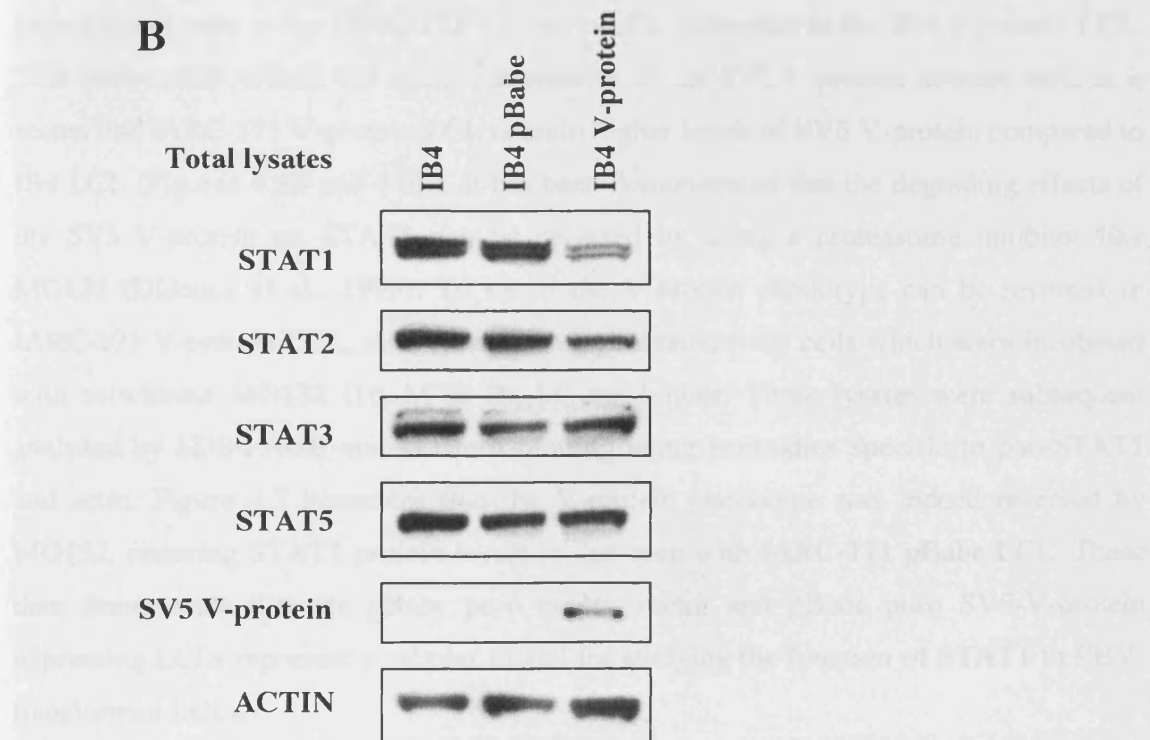


Figure 4.6 Genetic modification of IB4 LCLs with pBabe SV5 V-protein retrovirus

IB4 LCLs were infected in a 2-cycle retroviral infection with RPMI-1640 only, pBabe puro empty vector retrovirus or pBabe puro SV5 V-protein retrovirus to generate IB4 pBabe LCL (empty vector) and IB4 V-protein LCL (SV5 V-protein). These cell lines were cultured with puromycin (1 μ g/ml) and monitored regularly for cell viability by flow cytometry. **(A)** Forward and side scatter dot plot results after 11 days under selection. The R1 gate was used to determine the percentage of viable cells within the population. **(B)** Total lysates were generated from IB4 LCL, IB4 pBabe LCL and IB4 V-protein LCL. These lysates were analysed by SDS-PAGE and Western Blotting using antibodies specific to pan-STAT1, pan-STAT2, pan-STAT3, pan-STAT5, SV5 V-protein and actin. Typically 2x10⁵ cells were applied in each lane of the gel.

protein LCL, IB4 LCL, IB4 pBabe LCL and IB4 V-protein LCL. These lysates were analysed by SDS-PAGE and Western blotting using antibodies specific to pan-STAT1, pan-STAT2, pan-STAT3, pan-STAT5, SV5 V-protein and actin. Figures 4.5B and 4.6B illustrate that IARC-171 and IB4 V-protein LCLs display a reduction in STAT1 protein expression which correlates with the presence of the SV5 V-protein. A marginal reduction in STAT2, STAT3 and STAT5 expression was seen in the IARC-171 V-protein LCL although this effect is likely to reflect lower protein loading as indicated by the levels of actin protein. A small reduction in STAT2 expression is also seen in the IB4 V-protein LCL but this result is not representative of all the analysed data. However, the reduction in STAT1 expression in both IARC-171 and IB4 V-protein LCL verifies the STAT1-specific V-protein phenotype. Interestingly, a greater reduction in STAT1 protein expression is seen in the IARC-171 V-protein LCL compared to the IB4 V-protein LCL. This observation reflects the relative expression of the SV5 V-protein in these cells as it seems that IARC-171 V-protein LCL contain higher levels of SV5 V-protein compared to IB4 LCL (Figures 4.5B and 4.6B). It has been demonstrated that the degrading effects of the SV5 V-protein on STAT1 can be reversed by using a proteasome inhibitor like MG132 (Didcock et al., 1999). To see if the V-protein phenotype can be reversed in IARC-171 V-protein LCL, total lysates were generated from cells which were incubated with or without MG132 (10 μ M or 20 μ M) for 1 hour. These lysates were subsequent analysed by SDS-PAGE and Western blotting using antibodies specific to pan-STAT1 and actin. Figure 4.7 illustrates that the V-protein phenotype was indeed reversed by MG132, restoring STAT1 protein levels to that seen with IARC-171 pBabe LCL. These data demonstrate that the pBabe puro empty vector and pBabe puro SV5-V-protein expressing LCLs represent a cellular model for studying the function of STAT1 in EBV-transformed LCLs.

4.4 Use of a STAT1-deficient LCL to study the function of STAT1 in EBV-transformed LCLs

Complete human STAT1 deficiency is a very rare clinical condition that was first

reported by Dupont et al. in 1993 [Dupont et al., 2003]. The two infant patients reported in this study suffered from

an inherited defect in the gene for STAT1 and were found to be more susceptible

to all described opportunistic infections. In these STAT1-deficient cells, development

of post-EBV-transformed LCLs was severely impaired [Dupont et al., 2003]. During our infection EBV

transformed LCLs were generated from IARC-171 V-protein LCLs and were shown to be STAT1

deficient. In order to study the function of STAT1 in EBV-transformed B

lymphocytes, we generated LCLs from a STAT1-deficient LCL and a STAT1

transfected LCL. In order to study the function of STAT1 in EBV-transformed B

lymphocytes, we generated LCLs from a STAT1-deficient LCL and a STAT1

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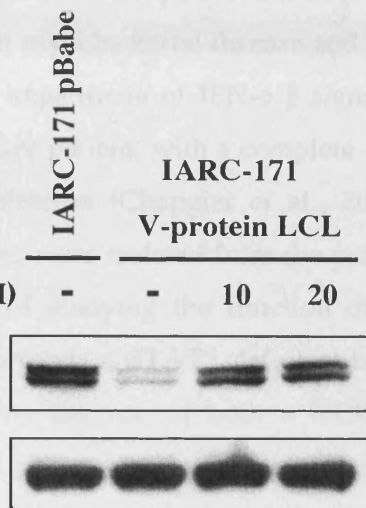
transfected LCL. In order to study the function of STAT1 in EBV-transformed B

lymphocytes, we generated LCLs from a STAT1-deficient LCL and a STAT1

transfected LCL. In order to study the function of STAT1 in EBV-transformed B

Figure 4.7 The proteasome inhibitor MG132 reverses the V-protein phenotype

Total lysates were generated from IARC-171 V-protein LCLs treated with or without 10 μ M or 20 μ M MG132 for 1 hour. Total lysates were also generated from IARC-171 pBabe LCLs as a control. These lysates were analysed by SDS-PAGE and Western Blotting using antibodies specific to pan-STAT1 and actin. Typically 2x10⁵ cells were applied in each lane of the gel. This result is representative of at least two experiments.



4.4 Use of a STAT1 deficient LCL to study the function of STAT1 in EBV-transformed LCLs

Complete human STAT1 deficiency is a very rare clinical condition that was first reported by Dupuis et al. in 2003 (Dupuis et al., 2003). The two infant patients reported in this study suffered from mycobacterial disease and were found to be more susceptible to viral disease due to an impairment of IFN- α/β signalling. A recent paper by Chapgier et al. described a third infant patient, with a complete STAT1 deficiency, who developed a post-transplant EBV infection (Chapgier et al., 2006). During this infection, EBV-transformed B-lymphocytes were isolated from the patient and were shown to be STAT1 deficient. In the context of studying the function of STAT1 in EBV-transformed B-lymphocytes, these cells provide a STAT1 deficient background in which STAT1 could be reconstituted. Therefore, the use of both a STAT1 deficient LCL and a STAT1 reconstituted LCL would potentially be a better system for studying the function of STAT1 in these cells than the V-protein expressing LCLs. Using a retrovirus expressing wild-type STAT1, the next step was to reconstitute STAT1 into this LCL.

The STAT1 deficient LCL, CV1524, was kindly provided by Dr. Peter Arkwright (University of Manchester). To demonstrate the phenotype of this STAT1 deficient LCL, total lysates were generated from IARC-171 LCL, IB4 LCL and CV1524 LCL and were analysed by SDS-PAGE and Western blotting using antibodies specific to pan-STAT1, pan-STAT2, pan-STAT3, pan-STAT5 and actin. Figure 4.8 shows that the CV1524 LCL is clearly STAT1 deficient but is positive for STAT2, STAT3 and STAT5. The levels of STAT2, STAT3 and STAT5 are not comparable to that seen in other LCLs and could reflect excess protein loading or possibly STAT protein compensation. It could be that the CV1524 LCL has higher levels of other STAT proteins in order to counteract the absence of STAT1.

In order to reconstitute STAT1 into the CV1524 LCL, a pBabe puro retrovirus expressing HA-tagged STAT1 α (WT) was used. HA-tagged STAT1 α (WT) was cloned from pEFHASTAT1 α (WT) into the pBabe puro retroviral vector by Dr. Zara Poghosyan. A schematic of this retroviral vector is found in Appendix II of this thesis. Functional pBabe puro STAT1 α (WT) retroviral supernatant was generated and was also kindly

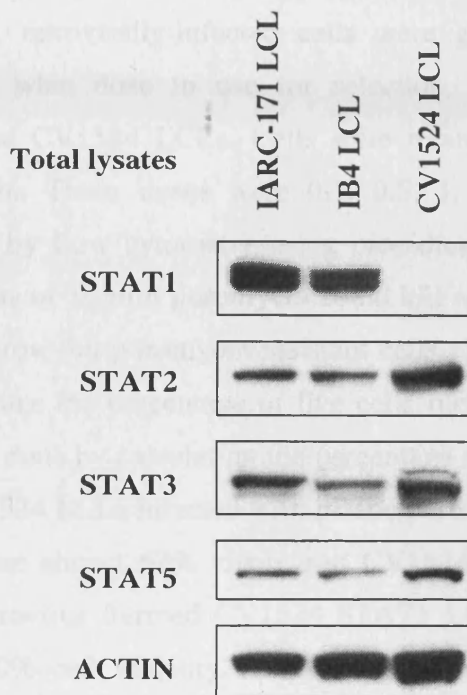


Figure 4.8 CV1524 LCLs are STAT1-deficient

Total lysates were generated from IARC-171, IB4 and CV1524 LCLs. These lysates were analysed by SDS-PAGE and Western Blotting using antibodies specific to pan-STAT1, pan-STAT2, pan-STAT3, pan-STAT5 and actin. Typically 2×10^5 cells were applied in each lane of the gel.

provided by Dr. Zara Poghosyan. CV1524 LCLs were retrovirally infected with either pBabe puro empty vector retrovirus or pBabe puro STAT1 α (WT) retrovirus using an adapted 2-cycle infection protocol (see Chapter 2). A mock infection was also set up where RPMI-1640 medium was used instead of retroviral supernatant. 3 days post-infection, retrovirally-infected cells were grown out using puromycin selection. To ascertain what dose to use for selection, a puromycin titration was performed on uninfected CV1524 LCLs. Cells were treated for 72 hours with a range of doses of puromycin. These doses were 0.1, 0.5, 1, 2.5 and 5 μ g/ml. Cell viability was then analysed by flow cytometry using propidium iodide staining. Figure 4.9 demonstrates that a dose of 1 μ g/ml puromycin could kill >95% of CV1524 LCLs. This dose was then used to grow out puromycin-resistant cells. Cells were then analysed by flow cytometry to determine the percentage of live cells (i.e. percentage of genetically modified cells). This was done by calculating the percentage of cells in the live gate. Figure 4.10A shows that CV1524 LCLs infected with pBabe puro empty vector virus (termed CV1524 pBabe LCL) were almost 62% viable and CV1524 LCLs infected with pBabe puro STAT1 α (WT) retrovirus (termed CV1524 STAT1 LCL) were 53% viable. The mock infection showed 0% cell viability. These data indicate that CV1524 LCLs were successfully modified with the pBabe puro empty vector and pBabe puro STAT1 α (WT) retroviruses

In order to demonstrate the restoration of STAT1 α expression in the CV1524 STAT1 LCL, total lysates were generated from CV1524 LCL, CV1524 pBabe LCL, CV1524 STAT1 LCL and IARC-171 LCL and were analysed by SDS-PAGE and Western Blotting using antibodies specific to pan-STAT1, pan-STAT2, pan-STAT3, pan-STAT5 and actin. IARC-171 LCL was used as a positive control for STAT1 protein expression in an EBV-transformed LCL. Figure 4.10B shows that that STAT1 α was successfully reconstituted into CV1524 LCL as displayed by CV1524 STAT1 LCL. However, although it was successful, the level of STAT1 protein expression was much less than that seen in a normal EBV-transformed LCL.

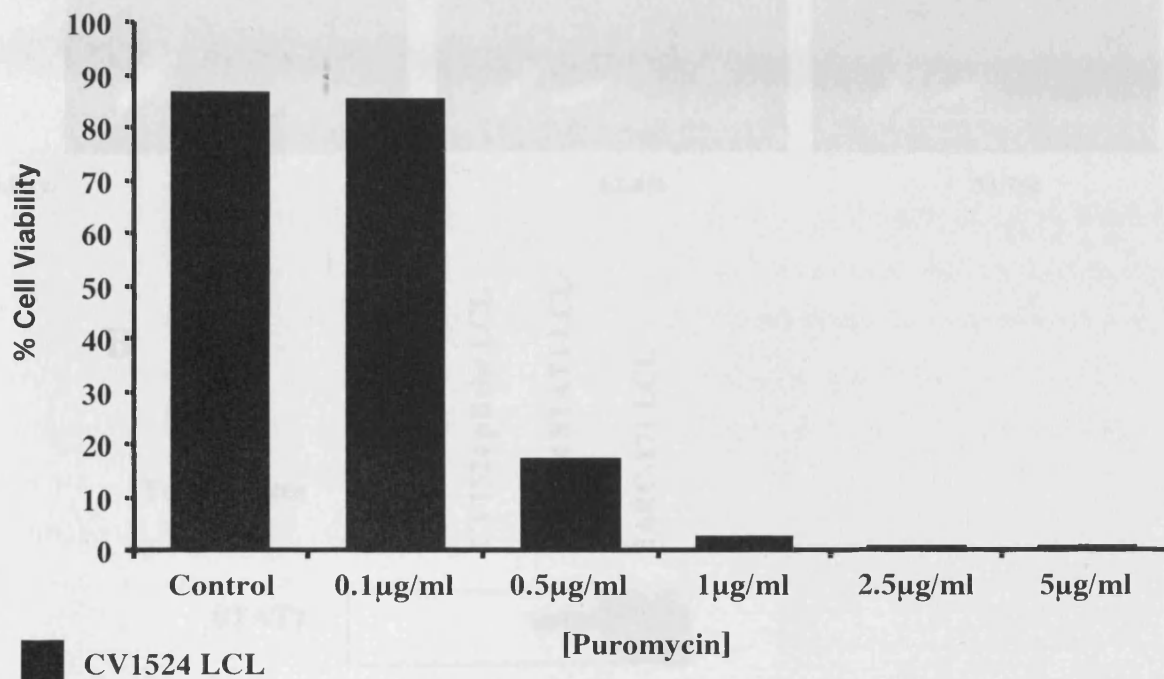
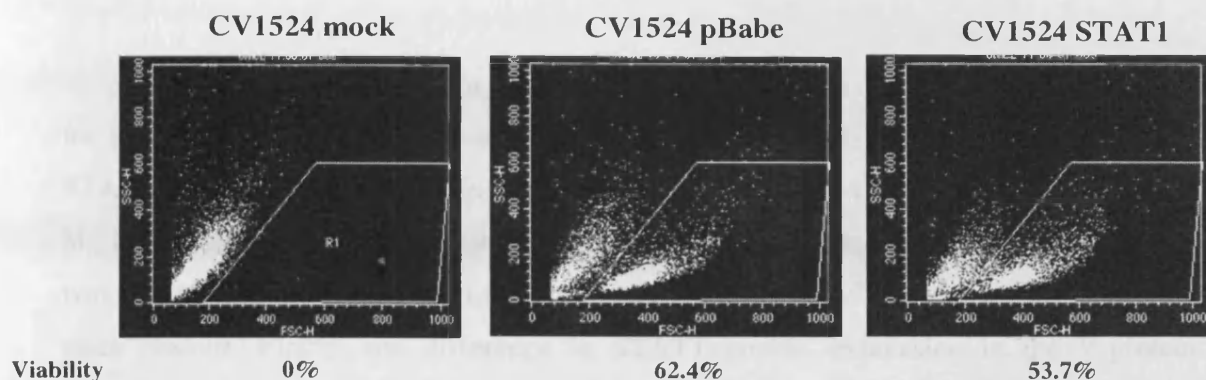


Figure 4.9 Titration of puromycin on CV1524 LCLs

CV1524 LCLs were treated with a range of doses of puromycin for 72hrs. Cells were then tested for viability by flow cytometry using propidium iodide staining. The data represents the percentage of cells that were not propidium iodide positive. The results indicate that a dose of 1µg/ml is sufficient to kill 100% of cells.

A



B

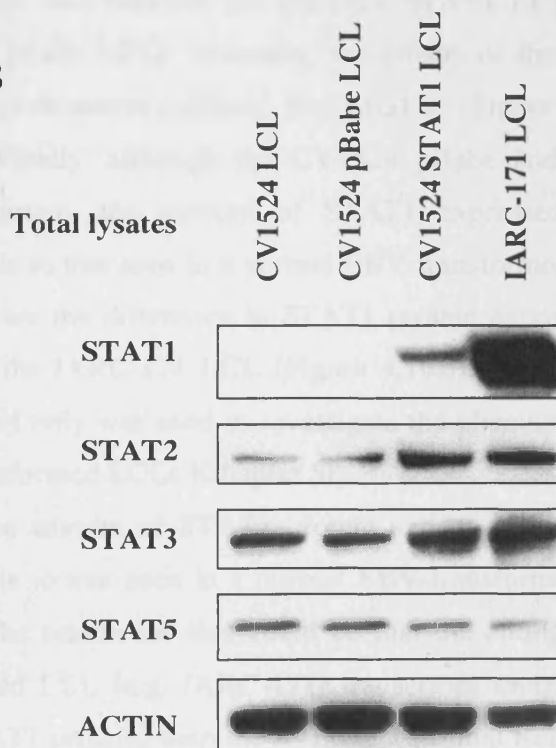


Figure 4.10 Reconstitution of STAT1 α in CV1524 LCL using a pBabe STAT1 α retrovirus

CV1524 LCLs were infected in a 2-cycle retroviral infection with RPMI-1640 only, pBabe puro empty vector retrovirus or pBabe puro STAT1 α (WT) retrovirus to generate CV1524 pBabe LCL (empty vector) and CV1524 STAT1 LCL (STAT1 α). These cell lines were cultured with puromycin (1 μ g/ml) and monitored regularly for cell viability by flow cytometry. **(A)** Forward and side scatter dot plot results after 14 days under selection. The R1 gate was used to determine the percentage of viable cells within the population. **(B)** Total lysates were generated from CV1524 LCL, CV1524 pBabe LCL, CV1524 STAT1 LCL and IARC-171 LCL. These lysates were analysed by SDS-PAGE and Western Blotting using antibodies specific to pan-STAT1, pan-STAT2, pan-STAT3, pan-STAT5 and actin. Typically 2x10⁵ cells were applied in each lane of the gel.

4.5 Discussion

This chapter describes the strategy used to generate an *in vitro* model for studying the function of STAT1 in EBV-transformed LCLs. Using two approaches, one using the STAT1-targeting SV5 V-protein and another using a STAT1 deficient LCL (Chapgier et al., 2006), this work has generated two potential cellular models. However, out of the two, the pBabe and V-protein LCLs are possibly the best cell model for the following three reasons. Firstly, the difference in STAT1 protein expression in the V-protein expressing LCLs, compared to their empty vector counterparts (pBabe LCL), is much greater than that between the CV1524 STAT1 LCL and its empty vector counterpart (CV1524 pBabe LCL). Secondly, the effects of the V-protein can be reversed using a chemical proteasome inhibitor like MG132 (Figure 4.7) allowing the phenotype to be rescued. Finally, although the CV1524 pBabe and CV1524 STAT1 LCLs present a cleaner system, the amount of STAT1 expressed in the reconstituted line is not comparable to that seen in a normal EBV-transformed LCL. This is clearly evident when you compare the difference in STAT1 protein expression between the CV1524 STAT1 LCL and the IARC-171 LCL (Figure 4.10B). For these reasons, the V-protein LCL *in vitro* model only was used to investigate the phenotype of reduced STAT1 expression in EBV-transformed LCLs (Chapter 5).

The amount of STAT1 protein expressed in the CV1524 STAT1 LCL was not comparable to that seen in a normal EBV-transformed LCL, such as IARC-171 (Figure 4.10B). The reason for this could be that the endogenous promoter in a normal EBV-transformed LCL (e.g. IARC-171) transcribes more STAT1 mRNA (and subsequently more STAT1 protein) than the 5'-Long Terminal Repeat region (LTR) in the pBabe puro STAT1 α (WT) retrovirus. However, this model does show that you can reconstitute STAT1 into a STAT1-deficient background and could have potential for further research into studying the function of STAT1 in these cells.

In exploiting the effects of the SV5 V-protein in EBV-transformed LCLs, two different methods of viral gene transduction were used: adenoviral and retroviral. At first, an adenoviral based system was chosen for two reasons. Firstly, it permitted transient SV5 V-protein expression in an EBV-transformed LCL. Secondly, a previous member of

the laboratory generated an EBV-transformed LCL, IB4 CAR 8 LCL, which was genetically modified to be more susceptible to adenoviral infection (Richardson et al., 2005) and, therefore, was ideal for infection with a SV5 V-protein-encoding adenovirus. However, although STAT1 protein expression could be reduced in the IB4 CAR 8 LCL, using the SV5 V-protein-encoding adenovirus (Figure 4.3B), this effect was not reproducible in all experiments and was dependent on the percentage of infection at each time. For this reason, a retroviral vector system was then employed.

A retroviral system was chosen to achieve sustained expression of the SV5 V-protein in a selected population of EBV-transformed LCLs. By producing a population of LCLs where 100% of the cells are genetically modified, it was more likely that a phenotype would be seen. On this premise, two SV5 V-protein expressing EBV-transformed LCLs were generated (Figures 4.5 and 4.6) which displayed clear reductions in STAT1 protein expression. However, the use of this technology also had its limitations. The generation of retrovirally transduced EBV-transformed LCLs was time consuming and, in particular, the generation of SV5 V-protein expressing LCLs. Indeed, the time taken to generate pBabe empty vector LCLs (approximately 16 days) was much less compared to the V-protein LCLs (approximately 25-40 days) and may reflect the fact that the pBabe vector is smaller than the pBabe SV5 V-protein vector (5.1kb vs 6.4kb) and would thus affect the titre of the retrovirus. An interesting observation was that the expression of the SV5 V-protein was higher in the IARC-171 V-protein LCL compared to the IB4 V-protein LCL. This may be due to a higher retroviral vector copy number in IARC-171 V-protein LCL compared to IB4 V-protein LCL since transgene expression has been shown to positively correlate with vector copy number (Kusitkova et al., 2003). Although this observation was unexpected, it did not negate the use of both IARC-171 and IB4 V-protein LCLs as *in vitro* models for studying the function of STAT1 in these cells.

The use of the SV5 V-protein provides an alternative way for inhibiting STAT1 activity in EBV-transformed LCLs that does not involve the over-expression of STAT1 β (Le Clorennec et al., 2006) or use of fludarabine (Fagard et al., 2002). Whereas the over-expression of STAT1 β antagonises the actions of STAT1 α , the SV5 V-protein degrades both STAT1 α and STAT1 β which are both found at high levels in EBV-transformed

LCLs. The reduction in STAT1 expression mediated by the SV5 V-protein does not also appear to affect other STATs or even cause STAT compensation. Although marginal effects on STAT2, STAT3 and STAT5 were seen in the Western blot data (Figures 4.5B and 4.6B), this was not representative of all the experiments and in some cases was due to reduced protein loading. In conclusion, it is clear that the pBabe and V-protein LCLs provide a cell model for studying the function of STAT1 in EBV-transformed LCLs. This functional analysis using these cells is described in Chapter 5 of this thesis.

CHAPTER 5

Investigating the function of STAT1 in EBV-transformed B-lymphocytes using V-protein expressing LCLs

5.1 Introduction

In chapter 4 of this thesis an *in vitro* model for studying the function of STAT1 in EBV-transformed LCLs was generated. This model, which utilises the STAT1-specific degradative actions of the SV5 V-protein, enables the comparison of EBV-transformed LCLs with high and low levels of both STAT1 α and STAT1 β and is therefore a good cell model for analysing the functional role of STAT1.

Although the mechanism of STAT1 up-regulation by EBV is well characterised, the role of STAT1 in EBV-transformed LCLs is less established. A couple of studies have identified roles for STAT1 in EBV biology. Firstly, EBV-encoded LMP1 has been shown to induce expression of the glycoprotein Mucin-1 in epithelial cells through STAT1 and STAT3 (Kondo et al., 2007). Mucin-1 has been shown to have a role in tumour invasion and metastasis. Also, LMP1 induced STAT1, in conjunction with NF- κ B and p53, is believed to have role in sensitising EBV-transformed B-lymphocytes to CD95-mediated apoptosis (Le Clorennec et al., 2006). However, this study has really identified only one role for STAT1 in EBV-transformed B-lymphocytes (Le Clorennec et al., 2006) and it seems likely that more roles for STAT1 exist. Since STAT1 expression in B-lymphocytes is induced by LMP1 (Richardson et al., 2003, Zhang et al., 2004), it is possible that it may also regulate a number of LMP1 target genes. The objective of this chapter is to use the *in vitro* model generated in chapter 4 of this thesis in order to investigate what function(s) STAT1 has in EBV-transformed B-lymphocytes. The use of the SV5 V-protein, transduced into an EBV-transformed LCL, presents a novel system

for this investigation and this chapter describes the effect reduced STAT1 protein expression has in these cells.

5.2 STAT1 does not regulate the expression of bcl-2 and mcl-1 in EBV-transformed LCLs

In order to assay the impact of the V-protein in both EBV-transformed LCLs, the expression of a range of LMP1 regulated proteins was compared between pBabe (i.e high STAT1) and V-protein LCLs (i.e low STAT1). LMP1 up-regulates an array of host cellular genes during B-cell transformation including the proto-oncogene bcl-2 (Henderson et al., 1991) and its family homologue mcl-1 (Wang et al., 1996). These anti-apoptotic proteins are up-regulated by LMP1 in order to convey a survival advantage to transformed B-lymphocytes. Mcl-1 expression has been shown to be up-regulated by constitutively serine phosphorylated STAT1 in Wilms' tumour (Timofeeva et al., 2006). Considering that constitutive serine phosphorylation of STAT1 is a feature of EBV-transformed LCLs (Zhang et al., 2004, Chapter 3 of this thesis), it is quite possible that LMP1 induced STAT1 could regulate mcl-1 and/or bcl-2 expression in EBV-transformed LCLs. To investigate this, total lysates were generated from IARC-171 pBabe LCL, IARC-171 V-protein LCL, IB4 pBabe LCL and IB4 V-protein LCL. These lysates were analysed by SDS-PAGE and Western Blotting using antibodies specific to bcl-2 and mcl-1. Actin was also used as a loading control. Figure 5.1 demonstrates that the protein expression of bcl-2 and mcl-1 is not different between the pBabe and V-protein LCLs in both sets of cell lines. This indicates that a reduction in STAT1 protein expression, manifested by the SV5 V-protein, does not affect the expression of these two anti-apoptotic proteins. This suggests STAT1 may not be involved in regulating cell survival proteins in EBV-transformed LCLs.

5.3 Cell surface MHC and CD54 expression is down-regulated in V-protein expressing LCLs

The immune control achieved by circulating cytotoxic T-lymphocytes (CTLs) on EBV-transformed B-cells is dependent on the recognition of antigenic peptides presented by MHC class I molecules. In addition, CD54 (ICAM-1) is an adhesion molecule involved in the interaction between CTLs and target cells (Rammensee & Mies, 1997).

In order to investigate the effect of STAT1 on MHC class I and CD54 (ICAM-1) expression, total cell lysates were generated from IARC-171 pBabe, IARC-171 V-protein, IB4 pBabe and IB4 V-protein LCL cell lines. These lysates were analysed by SDS-PAGE and Western Blotting using antibodies specific to Bcl-2, Mcl-1 and actin. Typically 2×10^5 cells were applied in each lane of the gel.

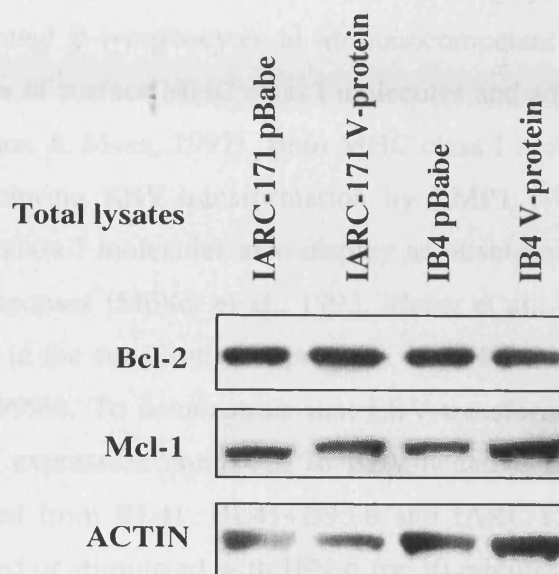


Figure 5.1 Bcl-2 and Mcl-1 protein expression is not regulated by STAT1 in EBV-transformed LCLs

Total cell lysates were generated from IARC-171 pBabe, IARC-171 V-protein, IB4 pBabe and IB4 V-protein LCL cell lines. These lysates were analysed by SDS-PAGE and Western Blotting using antibodies specific to Bcl-2, Mcl-1 and actin. Typically 2×10^5 cells were applied in each lane of the gel.

Since STAT1 is also up-regulated by LCLs (Chen et al., 2003; Zhang et al., 2004), a cell line was generated where STAT1 was over-expressed in EBV-transformed LCLs. This cell line was generated by transfecting the IARC-171 pBabe LCL cell line with a STAT1 expression vector. The cell line was then analysed by flow cytometry. Figure 5.2 shows the results of a representative experiment. The histograms clearly show down-regulation of MHC class I cell surface expression in the V-protein LCL compared to the pBabe LCL. This effect is more dramatic in the IARC-171 cell line compared to the IB4 cell line and is not the result of over-expression of the SV40 V-protein in the V-protein LCL as shown in Figure 5.3 and 5.4 or in Chapter 4 of this thesis. The cell lines were made from the

5.3 Cell surface MHC and CD54 expression is down-regulated in V-protein expressing LCLs

The immune control exhibited by circulating cytotoxic T-lymphocytes (CTLs) on EBV-transformed B-lymphocytes in immunocompetent hosts is crucially dependent on the high levels of surface MHC class I molecules and adhesion molecules found on these cells (Rickinson & Moss, 1997). Both MHC class I molecules and CD54 (ICAM-1) are up-regulated during EBV-transformation by LMP1 (Wang et al., 1988, Rowe et al., 1995). MHC class I molecules also display an absolute requirement for STAT1 in IFN-stimulated responses (Müller et al., 1993, Meraz et al., 1996). To add to this, STAT1 is also involved in the constitutive expression of MHC class I molecules in T-lymphocytes (Lee et al., 1999b). To demonstrate that EBV-transformed LCLs express high levels of MHC class I expression compared to EBV-negative B-lymphocytes, total cell lysates were generated from BL41, BL41+B95.8 and IARC-171 LCLs. These cell lines were either untreated or stimulated with IFN- α for 30 minutes. The lysates were then analysed by SDS-PAGE and Western blotting using antibodies specific to MHC class I, LMP1 and actin. Figure 5.2 shows that the levels of MHC class I protein are higher in the BL41+B95.8 cell line compared to its EBV-negative counterpart BL41. The levels of MHC class I protein are even higher in the IARC-171 LCL and correlates with the relative expression of the EBV latent gene LMP1. These data clearly demonstrate that EBV up-regulates MHC class I protein expression through LMP1.

Since STAT1 is also up-regulated by LMP1 (Richardson et al., 2003, Zhang et al., 2004), it was investigated whether STAT1 was involved in maintaining the levels of surface MHC class and adhesion molecules observed on EBV-transformed LCLs. Using both sets (IARC-171 and IB4) of pBabe and V-protein LCLs, the cell surface expression of MHC class I molecules was compared on these cells by flow cytometry. Figure 5.3A shows the results from a representative experiment. The histograms clearly show a down-regulation of MHC class I cell surface expression in the V-protein LCL compared to the pBabe LCL. This effect is more dramatic in the IARC-171 cell line compared to the IB4 cell line and reflects the relative expression of the SV5 V-protein in these lines, as shown in Figures 4.5 and 4.6 located in Chapter 4 of this thesis. The collective results from four

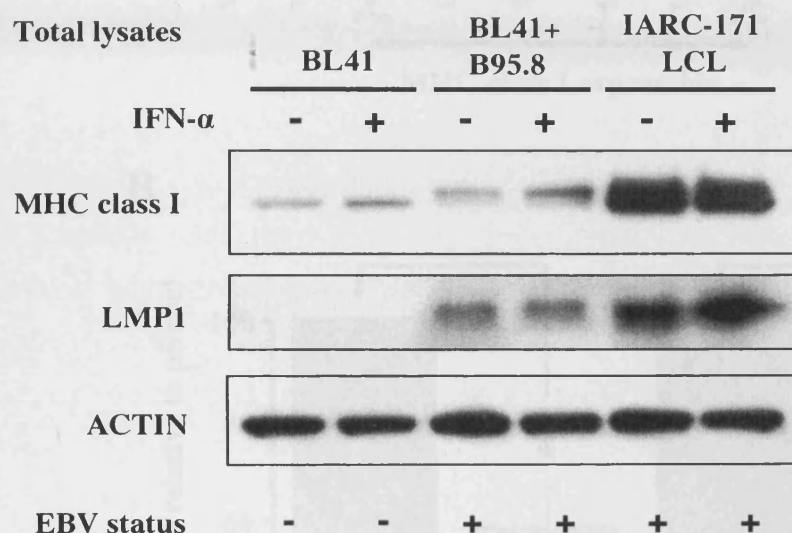
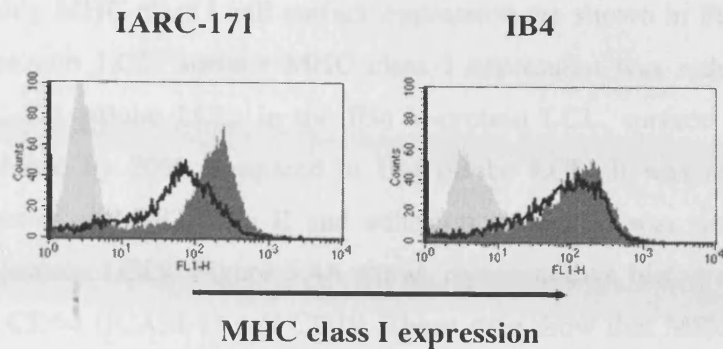


Figure 5.2 EBV up-regulates MHC class I protein expression in B-lymphocytes

Total cell lysates were generated from the BL41, BL41+B95.8 and IARC-171 cell lines. These cells were either untreated or stimulated with IFN- α (1,000 IU) for 30 minutes. These lysates were analysed by SDS-PAGE and Western Blotting using antibodies specific to MHC class I (HC10), LMP1 (CS1-4) and actin. Typically 2×10^5 cells were applied in each lane of the gel. This result is representative of at least three experiments.

A



B

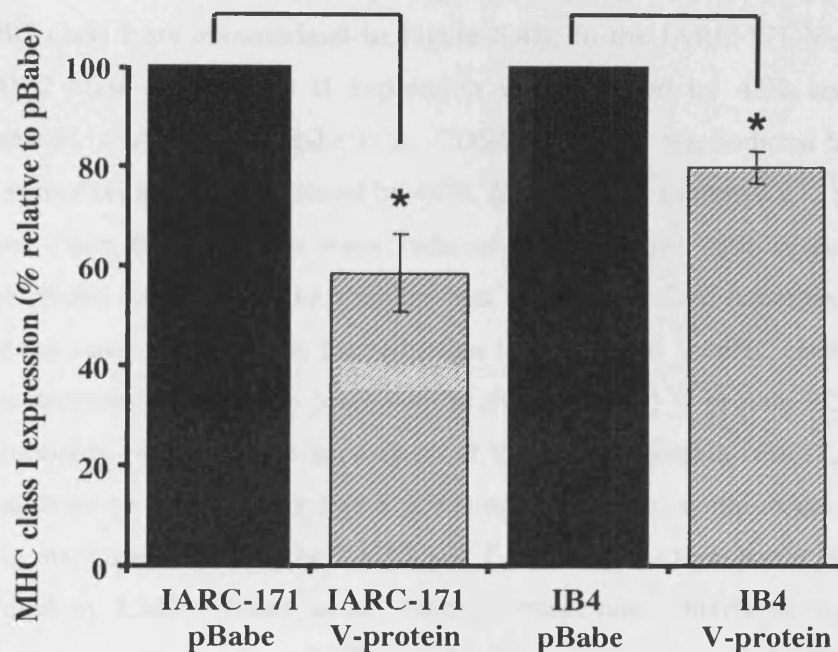


Figure 5.3 MHC class I cell surface expression is down-regulated in EBV-transformed LCLs expressing the simian virus 5 V-protein

Cell surface expression of MHC class I molecules was measured on IARC-171 pBabe, IARC-171V-protein, IB4 pBabe and IB4 V-protein LCLs. Viable cells were stained with MHC class I antibody (W6/32) followed by detection with a FITC-conjugated goat anti-mouse IgG secondary antibody. A representative flow cytometric analysis is shown in (A). Dark grey shading represents cell surface staining on LCLs expressing pBabe empty vector; clear shading with black outline represents cell surface staining on LCLs expressing pBabe simian virus 5 V-protein; light grey shading represents background FITC fluorescence obtained on cells stained with control antibodies. A summary of MHC class I expression on these cell lines is displayed in (B). Each result represents the percentage of MHC class I cell surface expression relative to the pBabe empty vector control from at least four separate experiments. Error bars indicate standard error from the mean. A paired T-test was used ascertain the statistical significance of the results with $*p < 0.05$.

experiments analysing MHC class I cell surface expression are shown in Figure 5.3B. In the IARC-171 V-protein LCL, surface MHC class I expression was reduced by 42% compared to IARC-171 pBabe LCL. In the IB4 V-protein LCL, surface MHC class I expression was reduced by 20% compared to IB4 pBabe LCL. It was next examined whether the expression of MHC class II and adhesion molecules was similarly down-regulated in the V-protein LCLs. Figure 5.4A shows representative histograms for MHC class II molecules, CD54 (ICAM-1) and CD19. These data show that MHC class II and CD54, but not CD19, cell surface expression are also down-regulated in the IARC-171 and IB4 V-protein LCLs compared to their pBabe counterparts.

The collective results from four experiments analysing all three cell surface markers and MHC class I are summarized in Figure 5.4B. In the IARC-171 V-protein LCL, surface MHC class I and class II expression were reduced by 42% and 58% respectively compared to IARC-171 pBabe LCL. CD54 expression was reduced by 51% whereas CD19 expression actually increased by 40%. In the IB4 V-protein LCL, surface MHC class I and class II expression were reduced by 20% and 43% respectively compared to IB4 pBabe LCL. CD54 expression was reduced by 24% whereas CD19 expression stayed the same. Interestingly the reduction in MHC class I, MHC class II and CD54 cell surface expression was more prominent in the IARC-171 V-protein LCL than in the IB4 line, probably reflecting the expression of the SV5 V-protein and STAT1 in these lines. In addition to MHC class I and adhesion molecules, components of the antigen processing machinery, such as the TAP-1 and TAP-2 peptide transporter proteins, are also upregulated by LMP1 (Rowe et al., 1995). Furthermore, interferon-regulated components of the proteasome, such as the low-molecular weight protein-2 (LMP-2), are regulated by both STAT1 and interferon-regulatory factor-1 (IRF1) (Chatterjee-Kishore et al., 2000); and IRF1 has also been implicated in inducing MHC class I expression (Chang et al., 1992, Hobart et al., 1997, Lee et al., 1999b). It was therefore questioned whether the down-regulation of MHC class I cell surface expression on V-protein LCLs might be reflecting an impairment of the antigen processing machinery or a specific reduction in MHC class I heavy chain protein expression. To address this question, the levels of MHC class I, IRF1, TAP1, TAP2 and LMP-2 protein were analysed by SDS-PAGE and Western blotting in IARC-171 pBabe LCL, IARC-171 V-protein LCL, IB4

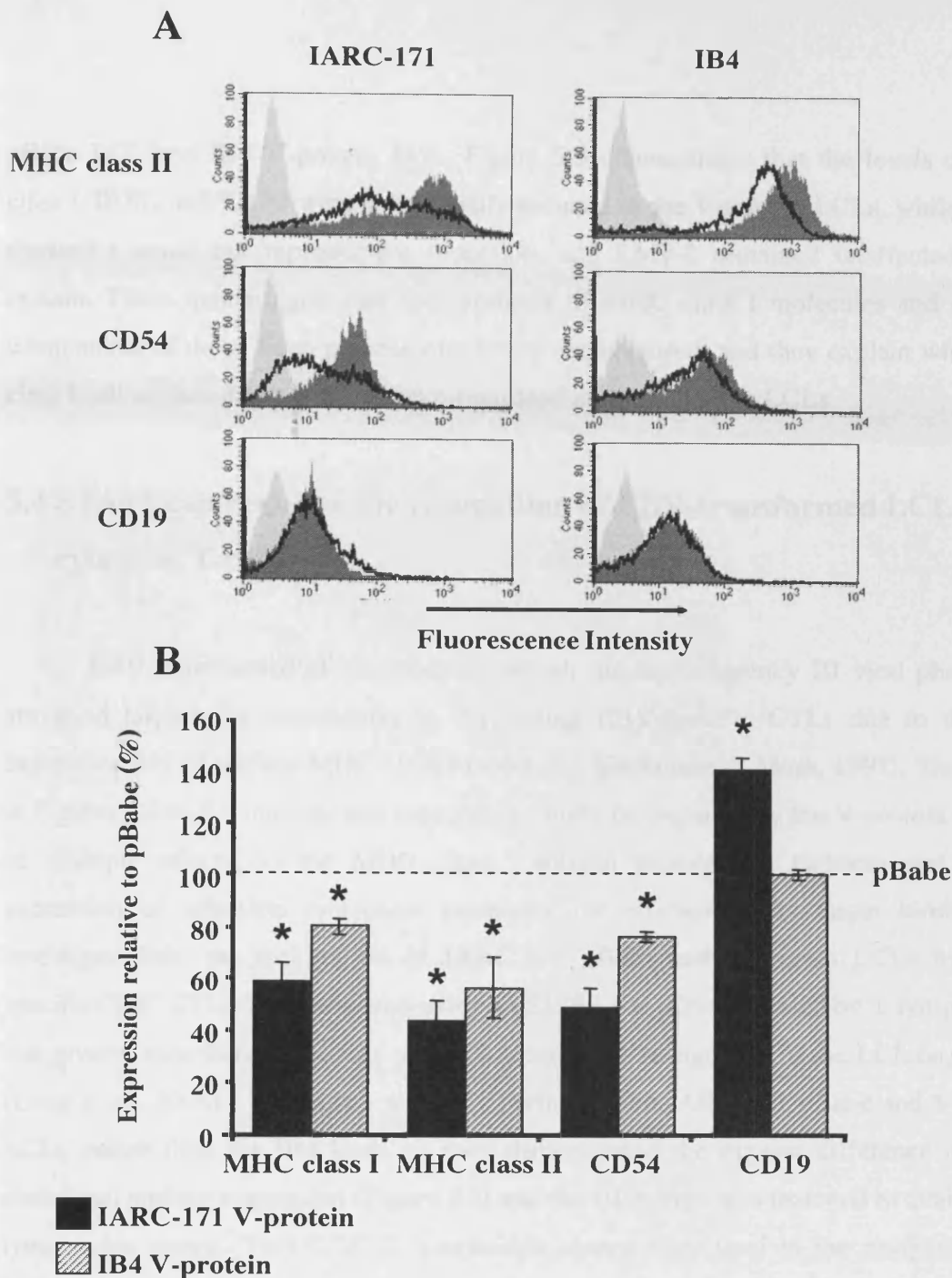


Figure 5.4 MHC class II and CD54 cell surface expression is also down-regulated in EBV-transformed LCLs expressing the simian virus 5 V-protein

Cell surface expression of MHC class II molecules, CD54 and CD19 was measured on IARC-171 pBabe, IARC-171V-protein, IB4 pBabe and IB4 V-protein LCLs. Viable cells were stained with either MHC class II antibody (L243), CD54:FITC antibody or CD19:FITC antibody followed by detection with a FITC-conjugated goat anti-mouse IgG secondary antibody (MHC class II only). A representative flow cytometric analysis is shown in (A). Dark grey shading represents cell surface staining on LCLs expressing pBabe empty vector; clear shading with black outline represents cell surface staining on LCLs expressing pBabe simian virus 5 V-protein; light grey shading represents background FITC fluorescence obtained on cells stained with control antibodies. A summary of MHC class II, CD54 and CD19 expression on these cell lines, along with MHC class I, is displayed in (B). Each result represents the percentage of cell surface expression relative to the pBabe empty vector control from at least three separate experiments. Error bars indicate one standard error from the mean. A paired T-test was used ascertain the statistical significance of the results with * $p < 0.05$. 150

pBabe LCL and IB4 V-protein LCL. Figure 5.5 demonstrates that the levels of MHC class I, IRF1, and TAP2 were substantially reduced in the V-protein LCLs, while TAP-1 showed a small but reproducible reduction, and LMP-2 remained unaffected by V-protein. These data suggest that the synthesis of MHC class I molecules and of other components of the antigen process machinery are impaired, and they explain why MHC class I cell surface expression is down-regulated in the V-protein LCLs.

5.4 STAT1 can regulate the recognition of EBV-transformed LCLs by cytotoxic T-cells

EBV-transformed B-lymphocytes, which display a Latency III viral phenotype, are good targets for recognition by circulating EBV-specific CTLs due to the high expression of cell surface MHC class I molecules (Rickinson & Moss, 1997). The results in Figures 5.3 to 5.5 indicate that recognition might be impaired by the V-protein through its multiple effects on the MHC class I antigen presentation pathway and on the expression of adhesion molecules necessary for efficient CTL: target binding. To investigate this, the recognition of IARC-171 pBabe and V-protein LCLs by EBV-specific CD8⁺ CTLs was compared using an ELISA for IFN- γ released by T-lymphocytes that gives a quantitative measure of the T-lymphocyte recognition of the LCL target cells (Long et al., 2005). This assay was only performed on IARC-171 pBabe and V-protein LCLs, rather than the IB4 lines, as they demonstrated the biggest difference in MHC class I cell surface expression (Figure 5.3) and the HLA-type was matched to available T-lymphocyte clones. Two CD8⁺ T-lymphocyte clones were used in the assays: HPV, a HLA-B35 restricted T-lymphocyte recognising the EBNA1 407-417 HPV epitope (Blake et al., 1997) and AVF, a HLA-A11 restricted T-lymphocyte clone recognising the EBNA3B 399-408 AVF epitope (Gavioli et al., 1993). The T-lymphocytes were kept at the same number for each assay and the LCLs were seeded at different numbers to give a range of effector: target ratios. Culture supernatants were harvested from co-cultures of the LCL targets and T-lymphocyte clones after 18 hours at 37°C, and were then assayed for IFN- γ release. Figures 5.6B and 5.7B show results from a representative experiment

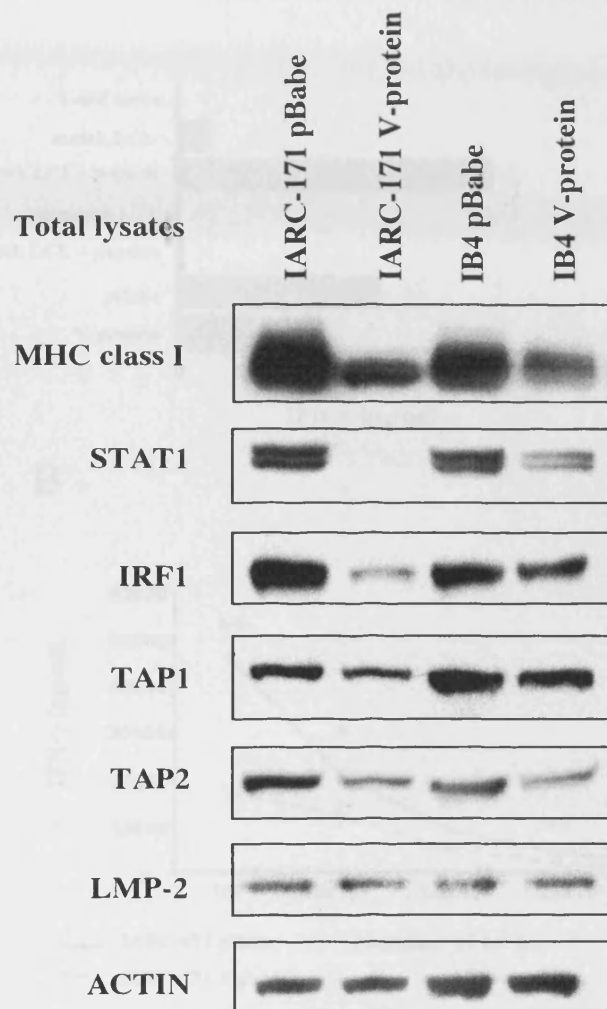


Figure 5.5 The MHC class I antigen processing machinery is impaired in EBV-transformed LCLs expressing simian virus 5 V-protein

Total cell lysates were generated IARC-171 pBabe LCL, IARC-171 V-protein LCL, IB4 pBabe LCL and IB4 V-protein LCL. These lysates were analysed by SDS-PAGE and Western Blotting using antibodies specific to MHC class I (HC10), STAT1, IRF-1, TAP1, TAP2, LMP-2 and actin. Typically 2×10^5 cells were applied in each lane of the gel.

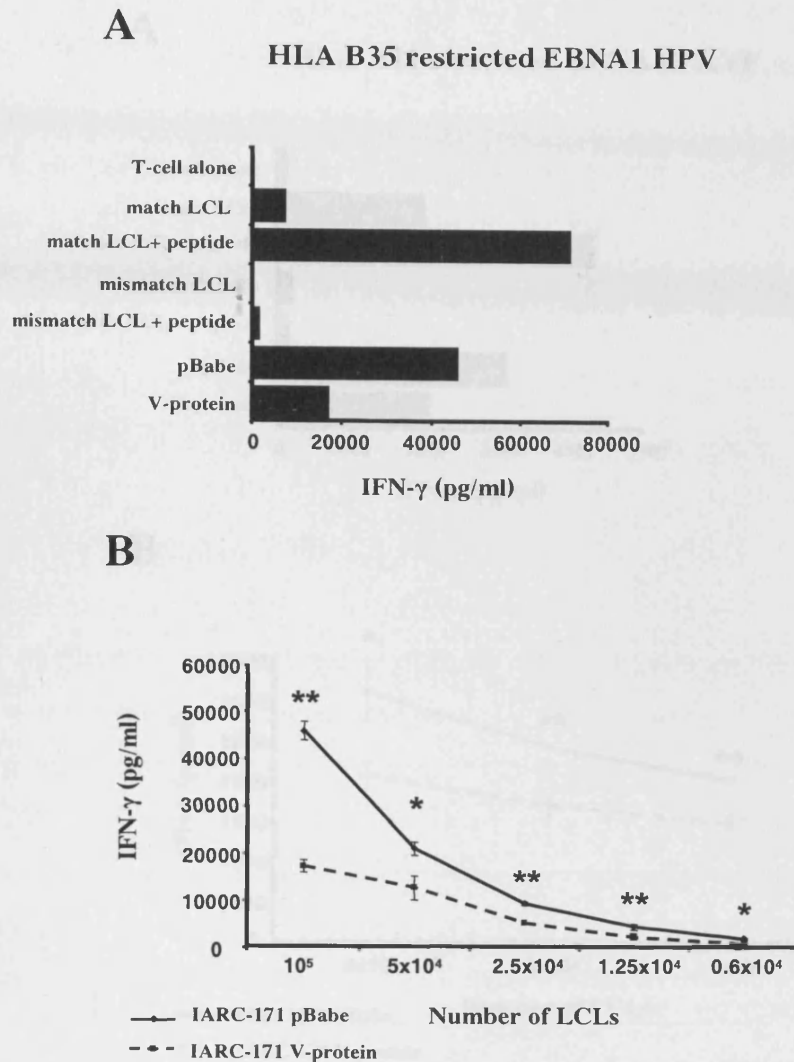


Figure 5.6 V-protein expressing LCLs are less recognized by the HLA restricted B35 EBNA1 HPV EBV-specific CD8⁺ CTL

A known number of HLA-B35 restricted CD8⁺ T-cell clones raised against the EBNA1 407-417 HPV epitope (500 T-cells per well) were incubated at 37°C with either 10⁵, 5x10⁴, 2.5x10⁴, 1.25x10⁴ or 0.6x10⁴ IARC-171 pBabe or V-protein LCLs in a V-bottom 96-well plate. 18 hours post-incubation, supernatant medium was harvested and assayed for IFN-γ release by ELISA. Data generated from a number of controls plus IARC-171 pBabe and V-protein LCLs (10⁵ LCLs) are shown in (A). The data generated from all the IARC-171 pBabe and V-protein LCLs is summarised in the graph in (B). Error bars indicate standard deviation. A paired T-test was used ascertain the statistical significance of the results with *p<0.05 and **p<0.01

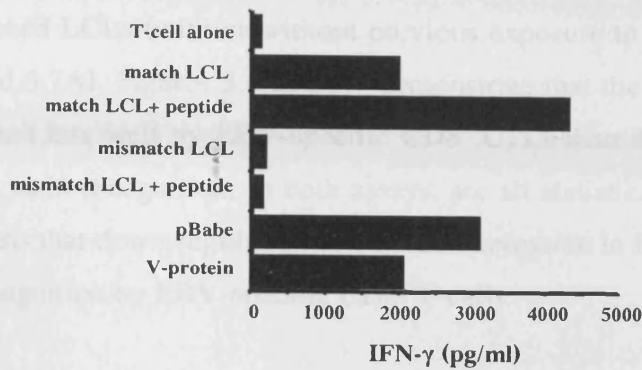
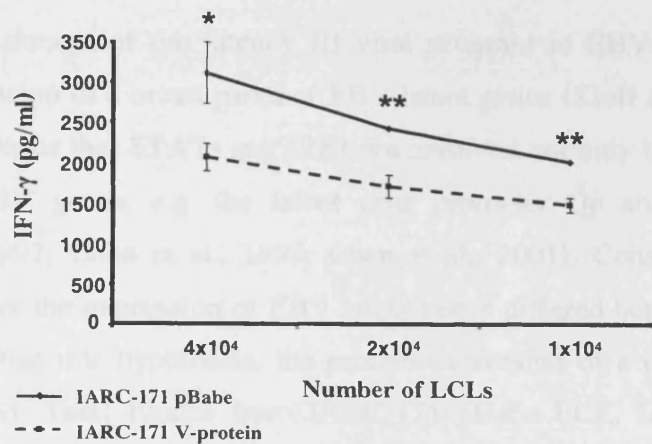
A**HLA A11 restricted EBNA3B AVF****B**

Figure 5.7 V-protein expressing LCLs are less recognized by the HLA restricted A11 EBNA3B AVF EBV-specific CD8⁺ CTL

A known number of HLA-A11 restricted CD8⁺ T-cell clones raised against the EBNA3B 398-408 AVF epitope (200 T-cells per well) were incubated at 37°C with either 4x10⁴, 2x10⁴, or 1x10⁴ IARC-171 pBabe or V-protein LCLs in a V-bottom 96-well plate. 18 hours post-incubation, supernatant medium was harvested and assayed for IFN-γ release by ELISA. Data generated from a number of controls plus IARC-171 pBabe and V-protein LCLs (4x10⁴ LCLs) are shown in (A). The data generated from all the IARC-171 pBabe and V-protein LCLs is summarised in the graph in (B). Error bars indicate standard deviation. A paired T-test was used ascertain the statistical significance of the results with *p<0.05 and **p<0.01

for each of the two T-lymphocyte clones. In each case a number of controls were performed to verify the specificity of the effector T-lymphocytes. These included T-lymphocyte clones incubated alone and T-lymphocytes incubated with HLA matched or mismatched LCLs (with or without previous exposure to 5 μ M epitope peptide) (Figures 5.6A and 5.7A). Figures 5.6 and 5.7 demonstrate that the IARC-171 V-protein LCL was recognized less well by EBV-specific CD8⁺ CTLs than the IARC-171 pBabe LCL. The differences in recognition, in both assays, are all statistically significant and confirm the hypothesis that down-regulation of STAT1 expression in EBV-transformed LCLs impairs their recognition by EBV-specific CD8⁺ T-cells.

5.5 STAT1 represses LMP2A expression

The establishment of the latency III viral program in EBV-transformed LCLs, involves the expression of a broad panel of EBV latent genes (Kieff & Rickinson, 2007). There is some evidence that STATs and IRF1 are involved not only in regulating cellular genes but also EBV genes, e.g. the latent gene promoter Qp and LMP1 promoters (Schaefer et al., 1997, Chen et al., 1999, Chen et al., 2001). Considering this, it was investigated whether the expression of EBV latent genes differed between pBabe and V-protein LCLs. To test this hypothesis, the protein expression of a panel of EBV latent genes was analysed. Total lysates from IARC-171 pBabe LCL, IARC-171 V-protein LCL, IB4 pBabe LCL and IB4 V-protein LCL, were analysed by SDS-PAGE and Western Blotting using either antibodies or human serum specific to pan-STAT1, LMP1, LMP2A, EBNA1, EBNA2, EBNA3A, EBNA3B and EBNA3C. Actin was again used as a loading control. Figure 5.8 demonstrates that the protein expression of LMP1 and most of the EBNAs does not alter between pBabe and V-protein LCLs. There is a marginal reduction in EBNA3A expression in IARC-171 V-protein LCL compared to its pBabe counterpart but this is not representative of all experiments. IB4 LCL, which has been previously reported to be EBNA3B negative (King et al., 1980), displayed a truncated form of EBNA3B. This observation agreed with a recent study (Chen et al., 2005). In contrast to the other latent proteins, expression of LMP2A differed markedly between the two IARC-171 clones, being more highly expressed in the V-protein expressing cells.

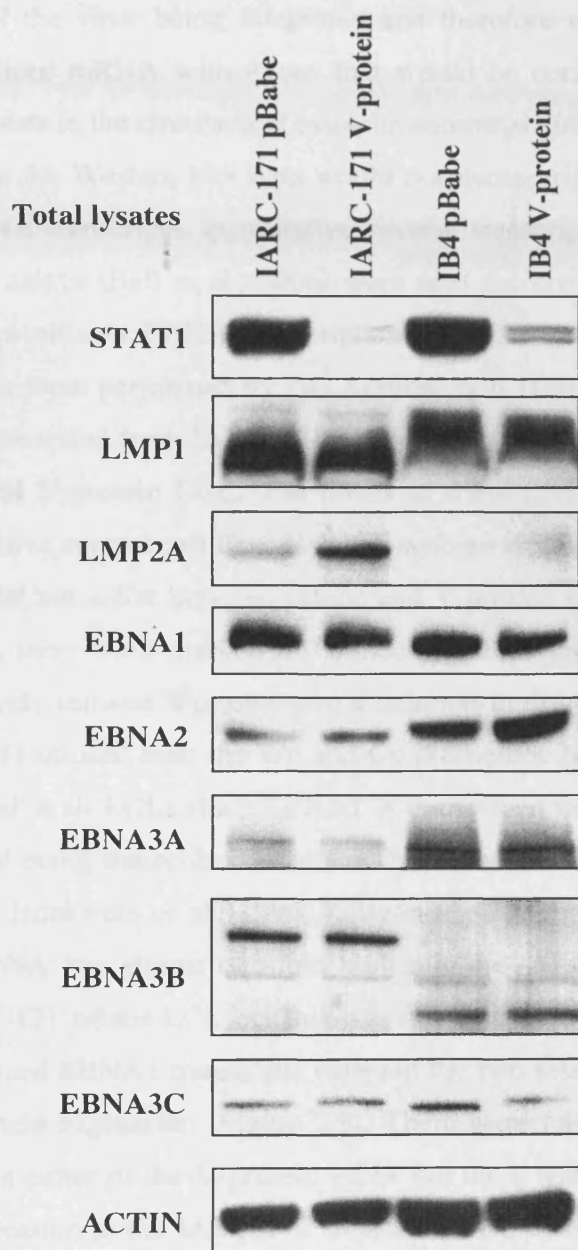


Figure 5.8 STAT1 represses LMP2A expression, measured by Western blotting and antibody detection

Total cell lysates were generated from IARC-171 pBabe LCL, IARC-171 V-protein LCL, IB4 pBabe LCL and IB4 V-protein LCL. These lysates were analysed by SDS-PAGE and Western Blotting using antibodies specific to STAT1, LMP1, LMP2A, EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C and actin. Typically 2×10^5 cells were applied in each lane of the gel.

No LMP2A protein was detected in either of the IB4 cell lines, which is assumed to be the result of the virus being integrated and therefore unable to produce the complete multiple spliced mRNA with exons that would be derived from the other side of the terminal repeats in the circularised extrachromosomal viral DNA (King et al., 1980).

Since the Western blot data would not necessarily detect altered promoter usage for the EBNA transcripts, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays (Bell et al., 2006) were next performed to quantitatively assay Wp-, Cp-, and Qp-initiated EBNA transcripts and LMP1 and LMP2A mRNA (Figure 5.9). These assays were performed by Dr. Andrew Bell (University of Birmingham, UK) on total RNA generated from IARC-171 pBabe LCL, IARC-171 V-protein LCL, IB4 pBabe LCL and IB4 V-protein LCL. The levels of transcription are relative to the seen in a suitable positive control cell line, assigned with an arbitrary value of 1. The Wp- and Cp-transcripts did not differ between pBabe and V-protein clones within each line although, as expected, there were marked differences between the IARC-171 and IB4. Whereas, IB4 exclusively utilised Wp, owing to a deletion in the Cp region (Sample et al., 1986), the IARC-171 utilised both the Wp and Cp promoters. No QUK-spliced EBNA1 mRNA was observed in all LCLs since EBNA1 is transcribed from Wp/Cp promoters in latency III instead of being transcribed from the Qp promoter, as seen in BL cell lines (Schaefer et al., 1995, Nonkwelo et al., 1996, Kelly et al., 2002). The expression of YUK-spliced EBNA1 mRNA was almost two-fold higher in the IARC-171 V-protein LCL compared to the IARC-171 pBabe LCL but this was not replicated in the IB4 lines. The difference in YUK-spliced EBNA1 transcripts between the two sets of lines does not correlate with EBNA1 protein expression (Figure 5.8). There were minimal changes in LMP1 mRNA expression in either of the V-protein LCLs but there was a two-fold increase in LMP2A mRNA expression in the IARC-171 V-protein LCL compared to IARC-171 pBabe LCL which agreed with the protein expression data. Together, these data suggest that the main effect of STAT1 on the latent EBV genome is to repress LMP2A expression.

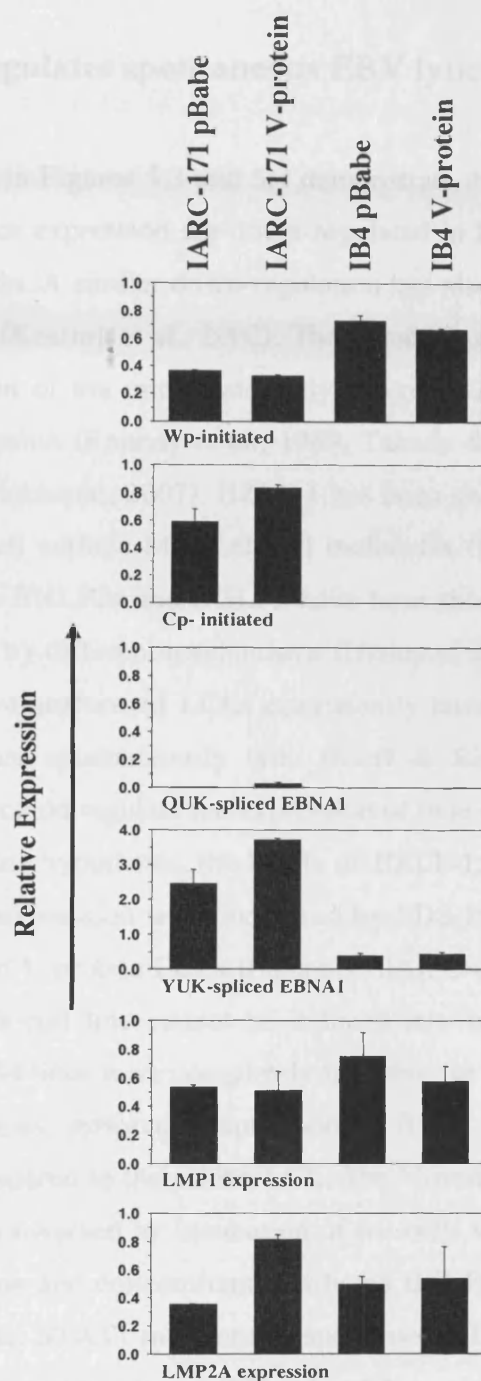


Figure 5.9 STAT1 represses LMP2A expression, measured by quantitative RT-PCR

Quantitative RT-PCR analyses of EBV transcripts in IARC-171 pBabe LCL, IARC-171 V-protein LCL, IB4 pBabe LCL and IB4 V-protein LCL. Assays were used to detect Wp- and Cp-initiated transcripts, QUK- and YUK-spliced EBNA1 mRNA, LMP1 mRNA and LMP2A mRNA. The levels of transcription are relative to the seen in a suitable positive control cell line, assigned with an arbitrary value of 1. Error bars indicate one standard deviation.

Note: This data was produced by Dr. Andrew Bell and is shown with his permission

5.6 STAT1 regulates spontaneous EBV lytic cycle gene expression

The data in Figures 5.3 and 5.4 demonstrate that MHC class I, MHC class II and CD54 cell surface expression are down-regulated in EBV-transformed LCLs expressing the SV5 V-protein. A similar down-regulation has also been described for cells that enter EBV lytic cycle (Keating et al., 2002). The induction of lytic cycle is critically dependent on the expression of the immediate-early antigen BZLF-1 which triggers a cascade of lytic gene expression (Rooney et al., 1989, Takada & Ono, 1989, Flemington & Speck, 1990, Kieff & Rickinson, 2007). BZLF-1 has been shown to impede the ability of LMP1 to up-regulate cell surface MHC class I molecules (Keating et al., 2002) and the early lytic cycle genes BNLF2a and BGLF5 have been shown to reduce surface expression of MHC molecules by different mechanisms (Hislop et al, 2007, Rowe et al., 2007). Since a number of EBV-transformed LCLs consistently have a small population of cells (less than 5%) that are spontaneously lytic (Kieff & Rickinson, 2007), it was postulated whether STAT1 could regulate the expression of lytic cycle genes like BZLF-1.

To test this hypothesis, the levels of BZLF-1, pan-STAT1 and actin (as loading control) protein expression were measured by SDS-PAGE and Western blotting in both sets of pBabe and V-protein LCLs (Figure 5.10A). Owing to the integrated viral genomes in IB4 LCL, this cell line cannot be induced into lytic cycle (King et al., 1980), and therefore both IB4 lines were completely negative for BZLF-1 protein (Figure 5.10A). In the IARC-171 lines, however, expression of BZLF-1 was markedly higher in the V-protein LCL compared to the pBabe LCL. The V-protein phenotype in the IARC-171 V-protein LCL was reversed by incubation of the cells with 20 μ M MG132, which restored STAT1 expression and concomitantly reduced BZLF-1 expression (Figure 5.10B). This result suggests that STAT1 may repress spontaneous BZLF-1 expression. To confirm this result further, the expression of BZLF-1 mRNA, along with Fp-initiated lytic EBNA1 transcripts was measured by qRT-PCR (Figure 5.11). The expression of both BZLF-1 mRNA and Fp-initiated lytic EBNA1 transcripts was found to be 10-fold higher in the IARC V-protein LCL compared to the pBabe LCL as measured by qRT-PCR (Figure 5.11). Although this increase in lytic transcripts was large, the expression of these transcripts is still low since a value of 1 corresponds to an LCL with about 5% of cells

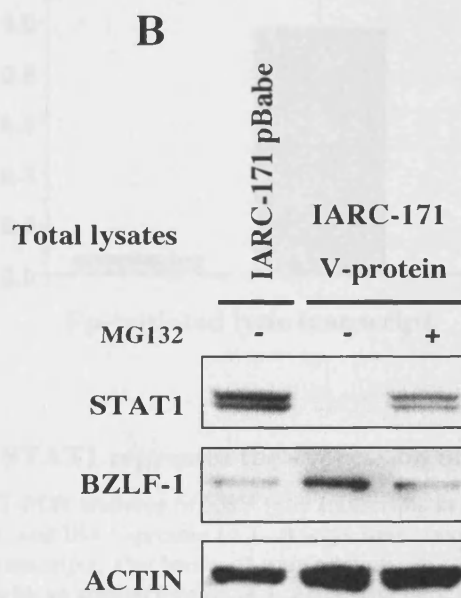
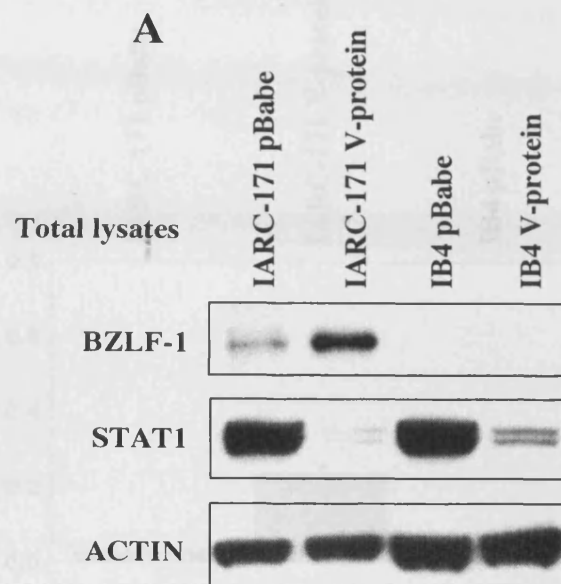


Figure 5.10 STAT1 represses the expression of the lytic cycle gene BZLF-1

(A) Total cell lysates were generated from IARC-171 pBabe LCL, IARC-171 V-protein LCL, IB4 pBabe LCL and IB4 V-protein LCL. These lysates were analysed by SDS-PAGE and Western Blotting using antibodies specific to BZLF-1, pan-STAT1 and actin. Typically 2×10^5 cells were applied in each lane of the gel. (B) Total lysates were generated from IARC-171 V-protein LCL treated with or without $20\mu\text{M}$ MG132 for 1 hour. Total lysates were also generated from IARC-171 pBabe LCL as a control. These lysates were analyzed by SDS-PAGE and Western Blotting using antibodies specific to pan-STAT1, BZLF-1 and actin. Typically 2×10^5 cells were applied in each lane of the gel. This result is representative of at least two experiments.

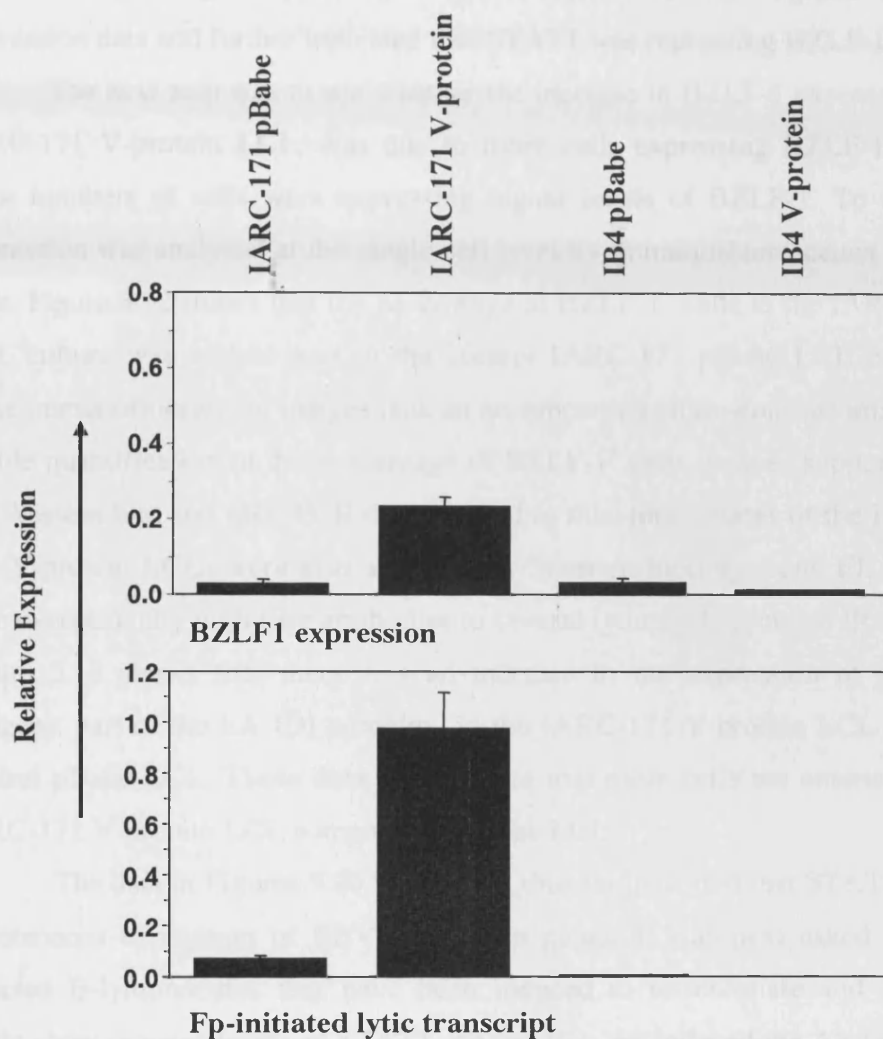


Figure 5.11 STAT1 represses the expression of EBV lytic cycle transcripts

Quantitative RT-PCR analyses of EBV lytic transcripts in IARC-171 pBabe LCL, IARC-171 V-protein LCL, IB4 pBabe LCL and IB4 V-protein LCL. Assays were used to detect BZLF-1 mRNA and Fp-initiated (FQUK-spliced) lytic transcripts. The levels of transcription are relative to the seen in a suitable positive control cell line, assigned with an arbitrary value of 1. Error bars indicate standard deviation.

Note: This data was produced by Dr. Andrew Bell and is shown with his permission

spontaneously in lytic cycle. However, the qRT-PCR data agreed with the protein expression data and further indicated that STAT1 was repressing BZLF-1 expression.

The next step was to see whether the increase in BZLF-1 expression, observed in IARC-171 V-protein LCL, was due to more cells expressing BZLF-1 or whether the same numbers of cells were expressing higher levels of BZLF-1. To do this, BZLF-1 expression was analysed at the single cell level by immunofluorescence staining of fixed cells. Figure 5.12 shows that the percentage of BZLF-1⁺ cells in the IARC-171 V-protein LCL culture was higher than in the control IARC-171 pBabe LCL culture. Although these immunofluorescent images lack an accompanied phase-contrast image which would enable quantification of the percentage of BZLF-1⁺ cells, it does support the findings in the Western blot and qRT-PCR data. To add to this, total lysates of the IARC-171 pBabe and V-protein LCLs were also analysed by Western blotting using EE, a human serum with exceptionally high titre antibodies to several lytic cycle proteins (Rowe et al., 1992). Figure 5.13 shows that there was an increase in the expression of other early lytic antigens, part of the EA (D) complex, in the IARC-171 V-protein LCL compared to the control pBabe LCL. These data demonstrate that more cells are entering lytic cycle, in IARC-171 V-protein LCL compared to pBabe LCL.

The data in Figures 5.10 to 5.13 has thus far indicated that STAT1 can repress the spontaneous expression of EBV lytic cycle genes. It was next asked whether latently infected B-lymphocytes that have been induced to differentiate and enter lytic cycle might show reduced levels of STAT1. To test this, we induced the Akata-derived AKBM reporter line by ligating the B-cell receptor, and isolated a population of cells in lytic cycle (Ressing et al., 2005). Total lysates from the isolated cells and un-induced control AKBM cell lysates were compared by SDS-PAGE and Western blotting using antibodies specific to pan-STAT1, BZLF-1, BGLF-5 (an early lytic cycle protein) and calregulin (loading control). Figure 5.14 shows that successful induction of lytic cycle in AKBM cells was accompanied by a reduction in STAT1 protein levels. Overall, these data are consistent with the hypothesis that STAT1 contributes to the repression of lytic cycle genes in latently infected B cells, and that successful induction of lytic cycle may first require a reduction in STAT1 expression or function.

BZLF-1 immunofluorescence

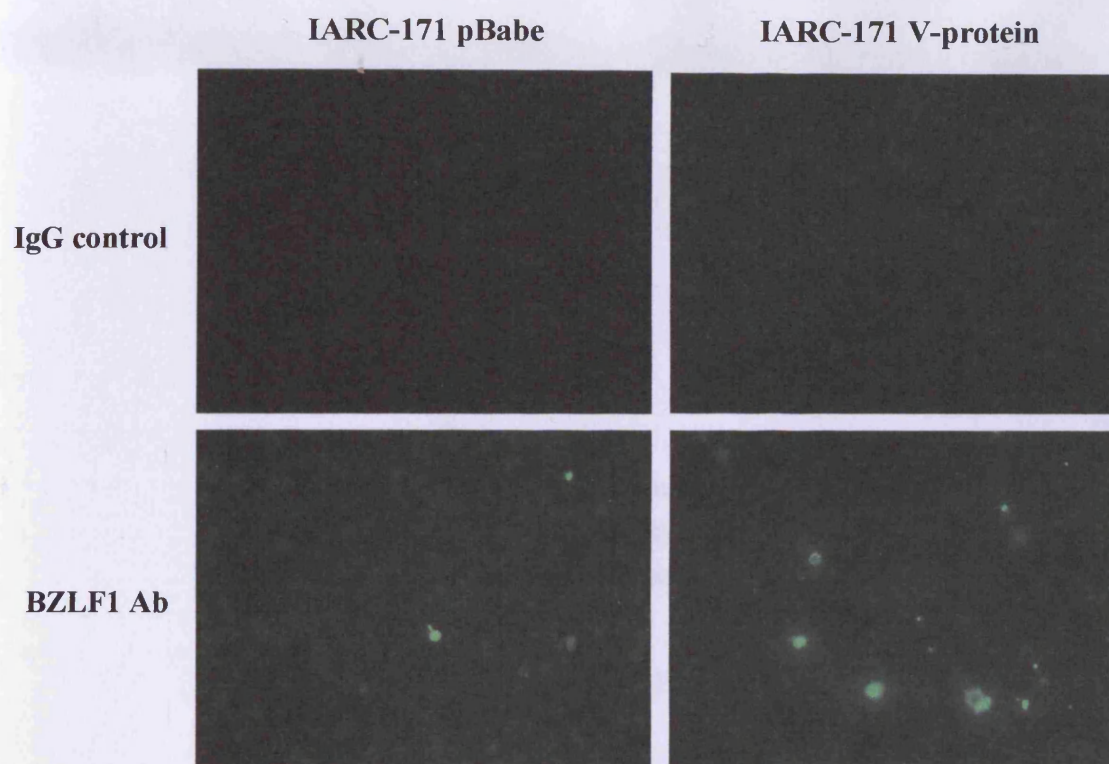


Figure 5.12 More LCLs express BZLF-1 when STAT1 expression is down-regulated

BZLF-1 expression was measured in IARC-171 pBabe LCL and IARC-171 V-protein LCL by indirect immunofluorescence.

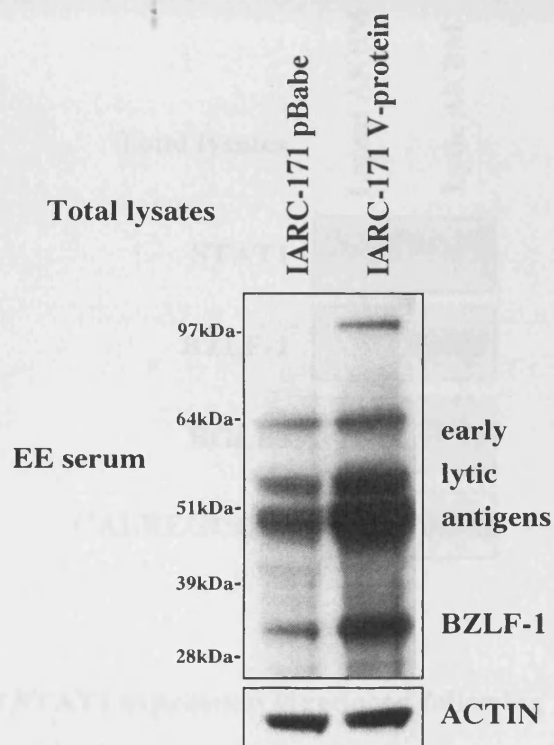


Figure 5.13 STAT1 represses the expression of several early lytic cycle genes

Total lysates were generated from IARC-171 pBabe LCL and IARC-171 V-protein LCL. These lysates were analyzed by SDS-PAGE and Western Blotting using EE human serum, to detect a panel of early and late lytic cycle antigens, and antibody to actin. Typically 2×10^5 cells were applied in each lane of the gel.

5.7 Discussion

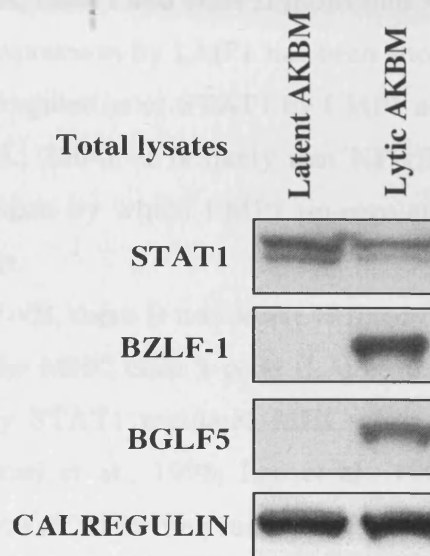


Figure 5.14 STAT1 expression is reduced following induction EBV lytic cycle in AKBM cells

Total lysates were generated from un-induced AKBM and lytically-induced AKBM cells. These lysates were then analysed by SDS-PAGE and Western blotting using antibodies specific to pan-STAT1, BZLF-1, BGLF-5 and calregulin. Typically 2×10^5 cells were loaded in each lane of the gel

Note: This data was produced by Professor Martin Rowe and is shown with his permission.

5.7 Discussion

This chapter provides new evidence of a role for STAT1 in EBV biology. Using a novel strategy to specifically reduce STAT1 protein expression in EBV-transformed LCLs, this chapter has shown that STAT1 contributes to the high levels of cell surface expression of MHC class I and class II molecules found on these cells. The up-regulation of MHC class I expression by LMP1 has been shown to be NF- κ B-dependent (Pai et al., 2002). As the up-regulation of STAT1 by LMP1 also involves NF- κ B (Richardson et al., 2003, Zhang et al., 2004), it is likely that NF- κ B mediated induction of STAT1 is an important mechanism by which LMP1 up-regulates MHC class I expression during B-cell transformation.

Unlike NF- κ B, there is no clearly defined binding site for STAT1 homodimers in the promoter of the MHC class I gene (Lee et al., 1999b). However, a model has been proposed whereby STAT1 regulates MHC class I expression through its downstream target IRF1 (Agresti et al., 1998, Lee et al., 1999b). IRF1 can bind to an interferon consensus sequence (ICS) in the promoter of the MHC class I gene and, in turn, regulate its expression (Agresti et al., 1998, Chang et al., 1992, Lee et al., 1999b). Our data is consistent with the above model as LCLs with down-regulated STAT1 expression display reduced levels of IRF1 expression (Figure 5.5). To add to this, it appears that other members of the MHC class I antigen processing machinery are also regulated by STAT1. The expression of TAP1 and TAP2, but not the proteasome subunit LMP-2, is reduced following a down-regulation of STAT1 protein expression in LCLs (Figure 5.5). This result is consistent with data showing that TAP1 and TAP2, but not LMP-2, are up-regulated in type III latency (Rowe et al., 1995).

This chapter has shown that STAT1 can also regulate the cell surface expression of the adhesion molecule CD54 (or ICAM-1) on EBV-transformed LCLs. ICAM-1 is a known STAT1 target gene (Jahnke & Johnson, 1994, Naik et al., 1997, Ohmori et al., 1997, Tessitore et al., 1998) and its expression is up-regulated by LMP1 during B-cell transformation (Wang et al., 1988). Regulation of ICAM-1 in EBV-transformed B-lymphocytes has been shown to primarily involve NF- κ B (Mehl et al., 2001) but it is likely to require the synergy of both STAT1 and NF- κ B. This is evident since STAT and

NF- κ B binding sites have been located in the promoter of the ICAM-1 gene (Ohmori et al., 1997) and NF- κ B alone is unable to induce ICAM-1 protein expression (Mehl et al., 2001). Therefore, it seems that the induction of ICAM-1 expression by LMP1 requires both STAT1 and NF- κ B.

The effect of STAT1 on antigen presentation and adhesion molecules, like CD54, in EBV-transformed LCLs correlates with their recognition by EBV-specific cytotoxic T-cells. The ability of T-lymphocytes to recognise proliferating, EBV-transformed B-lymphocytes with the latency III programme of gene expression is crucial to the establishment of a host-virus equilibrium and a life-long management of EBV infection, as evidenced by the increased incidence of PTLD in immunosuppressed individuals (Tanner & Alfieri, 2001). This role is compatible with the model proposed by Le Clorennec *et al.*, who showed that induction of CD95 expression by EBV, through STAT1 in conjunction with NF- κ B and p53, plays a key role in the immune control of EBV-transformed B-cells *in vivo* (Le Clorennec et al., 2006). Together, these results provide strong evidence that EBV uses STAT1 to establish the host-virus equilibrium seen in immunocompetent hosts.

Whilst a reduction in STAT1 expression correlated with a ten-fold increase in lytic transcripts, it should be noted that the actual percentage of cells entering lytic cycle remained very low. Therefore, although lytic cycle is associated with an inhibition of cell surface MHC and adhesion molecule expression (Keating et al., 2002) this alone cannot explain the global effect on cell surface phenotype within cultures of LCLs with reduced STAT1 expression.

While the majority of EBV latent genes are not regulated by STAT1, it was found that LMP2A expression was increased in LCLs displaying down-regulated STAT1 expression (Figures 5.8 and 5.9). Interestingly, it has been reported that LMP2A can induce entry into lytic cycle in EBV-infected B-lymphocytes in the absence of lytically-inducing stimuli such as anti-IgG (Schaadt et al., 2005). Therefore, the up-regulation of LMP2A may be a contributory mechanism to the increased activation of lytic cycle in LCLs where STAT1 expression is reduced (Figures 5.10 to 5.13). It is plausible that STAT1 may form a repressive complex in the EBV promoters that encode LMP2A and lytic cycle gene transcription. Intriguingly, it has been shown in EBV-associated

malignancies that the Qp promoter and two LMP1 promoters contain putative STAT binding sites (Chen et al., 1999, Chen et al., 2001). The Qp promoter, in particular, is positively regulated by STATs such as STAT1 in type I and II latency (Chen et al., 1999) but is suppressed in type III latency by interferon-regulatory factors, such as IRF-2 (Zhang & Pagano, 1999). Considering this, it is possible that STAT binding sites could exist in the promoters encoding LMP2A and lytic cycle gene transcripts. Overall, it seems that STAT1 may help maintain a strict latency III phenotype in transformed B-lymphocytes and contributes to the balance of the host-virus equilibrium since EBV-infected cells in lytic cycle are able to evade the host immunosurveillance.

In conclusion, this chapter proposes a model where by EBV induces STAT1 expression in transformed B-lymphocytes in order help maintain viral latency in these cells and their recognition by the host EBV-specific immunosurveillance.

CHAPTER 6

Final Discussion

Constitutive expression and activation of STAT1 is a feature of EBV-associated malignancies such as PTL and NPC (Weber-Nordt et al., 1996, Chen et al., 2001). Although the mechanism for how EBV induces STAT1 up-regulation during B-lymphocyte transformation has been investigated (Richardson et al., 2003, Zhang et al., 2004), little is known about why STAT1 is targeted by the virus. The purpose of this thesis was to investigate how and why EBV modulates STAT1 activity in transformed B-lymphocytes. The three main conclusions of this thesis are as follows. Firstly, LMP1-induced STAT1 is capable of binding DNA in the absence of detectable tyrosine phosphorylation and thus is different to that seen in interferon-stimulated B-lymphocytes. Secondly, STAT1 is constitutively serine phosphorylated, downstream of PI3K and MEK, in EBV-transformed LCLs and this modification appears to repress the DNA binding of interferon-stimulated STAT1. Finally, EBV induces STAT1 expression in transformed B-lymphocytes in order to help maintain viral latency in these cells and their recognition by the host EBV-specific immunosurveillance. These three findings are integrated in Figure 6.1 and are discussed further below in the global context of how and why EBV uses STAT1 for its own ends.

In Chapter 3 of this thesis, it was shown in EBV-transformed LCLs that LMP1-induced STAT1 was found to bind DNA in the absence of detectable tyrosine phosphorylation (Figures 3.5, 3.6 & 3.7). In addition to this, it was shown that STAT1 is only tyrosine phosphorylated in EBV-transformed LCLs in response to extracellular stimulation, such as IFN- α (Figures 3.4 & 3.5) which agrees with previously published studies (Dupuis et al. 2001, Zhang et al., 2004). The identification of this distinct form of DNA-bound STAT1 in EBV-transformed B-lymphocytes adds to reports accumulating in the literature that describe gene expression regulated by non-tyrosine phosphorylated STAT1 (Kumar et al., 1997, Chatterjee-Kishore et al., 2000, Unlu et al., 2007) and also

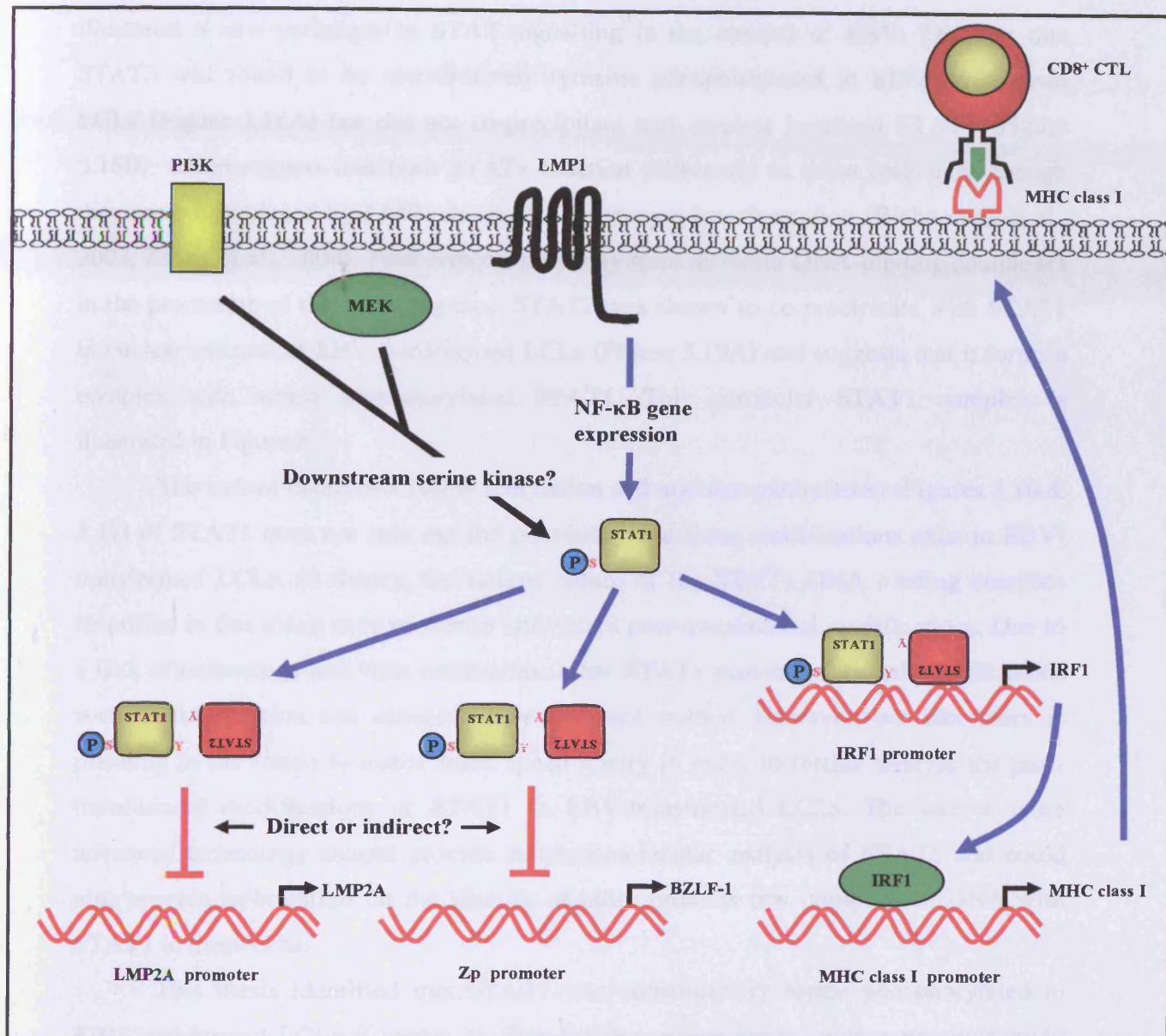


Figure 6.1 Schematic illustrating the main conclusions of this thesis

LMP1-induced STAT1 is constitutively serine phosphorylated downstream of PI3K and MEK and is capable of binding DNA in the absence of tyrosine phosphorylation. Also, EBV induces MHC class I cell surface expression in a mechanism involving both STAT1 and IRF1. To add to this, the expression of the EBV latent gene LMP2A and the lytic cycle gene BZLF-1 is repressed by STAT1 in transformed B-lymphocytes. This repressive effect may involve a direct or indirect interaction with the LMP2A and BZLF-1 promoters and may function to help maintain viral latency in transformed B-lymphocytes.

illustrates a new paradigm in STAT signalling in the context of EBV. The fact that STAT3 was found to be constitutively tyrosine phosphorylated in EBV-transformed LCLs (Figure 3.18A) but did not co-precipitate with nuclear localised STAT1 (Figure 3.18B) would suggest that both STATs function differently in these cells even though they are both induced by LMP1 during B-lymphocyte transformation (Richardson et al., 2003, Zhang et al., 2004). Furthermore, they may form different DNA-binding complexes in the promoters of their target genes. STAT2 was shown to co-precipitate with STAT1 in nuclear extracts of EBV-transformed LCLs (Figure 3.19A) and suggests that it forms a complex with serine phosphorylated STAT1. This particular STAT1 complex is illustrated in Figure 6.1

The lack of detectable lysine acetylation and arginine methylation (Figures 3.16 & 3.17) of STAT1 does not rule out the possibility that these modifications exist in EBV-transformed LCLs. In theory, the unique nature of the STAT1-DNA binding complex identified in this thesis may be due to alternative post-translational modifications. Due to a lack of technology and time constraints, other STAT1 post-translational modifications such as sumoylation and ubiquitination were not studied. However, our laboratory is planning in the future to utilise mass spectrometry in order to further analyse the post-translational modifications of STAT1 in EBV-transformed LCLs. The use of more advanced technology should provide detailed molecular analysis of STAT1 and could also provide information on the identity of other proteins that complex on DNA with STAT1 in these cells.

This thesis identified that STAT1 was constitutively serine phosphorylated in EBV-transformed LCLs (Chapter 3). This finding, which agreed with a previous study (Zhang et al., 2004), provides further evidence that constitutive STAT1 serine phosphorylation is a feature of malignancy since it is also observed in CLL (Frank et al., 1997) and Wilms' tumour (Timofeeva et al., 2006). As it has been shown that serine phosphorylated STAT1 can promote cell survival through the up-regulation of two known pro-survival genes, *mcl-1* and *HSP-27* (Timofeeva et al., 2006), it is possible that EBV may serine phosphorylate STAT1 for some purpose. In Chapter 5 of this thesis, STAT1 was found to be involved in maintaining viral latency in transformed B-lymphocytes and their recognition by the host EBV-specific immunosurveillance. This

function could potentially hinge on STAT1 being constitutively serine phosphorylated. Therefore, through the use of either combined PI3K/MEK inhibition, to reduce STAT1 serine phosphorylation, or by introducing a STAT1 serine phosphorylation mutant into the STAT1 deficient CV1524 LCL, the contribution of serine phosphorylation to this function could be elucidated.

The *in vitro* LCL model generated in this study (Chapter 4) represents the first documented application of the SV5 V-protein in EBV-transformed LCLs. By harnessing the STAT1-degradative actions of the SV5 V-protein, this model presents a unique way of reducing STAT1 protein expression that is not limited by the resistance of EBV-transformed LCLs to standard gene transfer approaches. Indeed, a similar system could be utilised to reduce the protein expression of other STATs in EBV-transformed LCLs since the type II human parainfluenza virus V-protein targets STAT2 for proteasomal degradation (Parisien et al., 2001, Andrejeva et al., 2002) and the mumps virus V-protein similarly targets STAT3 (Ulane et al., 2003).

The use of the SV5 V-protein has enabled this thesis to identify a function for STAT1 in EBV-transformed LCLs. STAT1 appears to regulate immunorecognition of transformed B-lymphocytes by EBV-specific cytotoxic T-lymphocytes and is involved in maintaining the latency III viral program expressed in these cells (Chapter 5). This role for STAT1 in regulating the immunorecognition of EBV-transformed B-lymphocytes is compatible with the model proposed by Le Clorennec et al., who showed that the induction of CD95 expression by EBV, through STAT1 in conjunction with NF- κ B and p53, plays a key role in the immune control of EBV-transformed B-cells *in vivo* (Le Clorennec et al., 2006). To add to this, this thesis proposes a mechanism by which EBV induces MHC class I cell surface expression in transformed B-lymphocytes involving both STAT1 and IRF1 (Figures 5.3 & 5.5). This is illustrated in Figure 6.1 and shows that STAT1 contributes to the maintenance of high levels of MHC class I cell surface expression. This observation could reflect a tumour promoting role for STAT1 in EBV-associated malignancies since STAT1 has been previously shown to promote leukaemia development by sustaining high levels of MHC class I expression *in vivo* (Kovacic et al., 2006). In the context of malignancies like EBV-associated HL, which can manifest in immunocompetent hosts, it is possible that EBV induces STAT1 expression to promote

the development of HRS cells. This is supported by evidence which showed that EBV over-expresses STAT1 and STAT3 in HRS cells and appears use both STATs to contribute to disease progression and tumour survival (Garcia et al., 2003).

In Chapter 5 of this thesis, it was demonstrated that STAT1 repressed the expression of the EBV latent gene LMP2A (Figures 5.8 & 5.9) and several EBV lytic cycle antigens including BZLF-1 (Figures 5.10, 5.11, 5.12 & 5.13) in EBV-transformed LCLs. Considering this, it is plausible that STAT1 may form a repressive complex on the EBV promoters that encode LMP2A and lytic cycle gene transcription. This concept is illustrated in Figure 6.1. Intriguingly, the EBV Qp promoter is positively regulated by STATs such as STAT1 in type I and II latency (Chen et al., 1999) but is suppressed in type III latency by interferon-regulatory factors, such as IRF2 (Zhang & Pagano, 1999). It is possible, therefore, that STAT1 binding sites could exist in the promoters encoding LMP2A and lytic cycle gene transcripts. The DNA sequences of these promoters could be analysed for potential STAT1 binding sites, and use of techniques such as EMSA and chromatin immunoprecipitation could clarify whether STAT1 binds to these promoters. However, the repressive effect of STAT1 on these promoters could be indirect. Having proposed that the induction of MHC class I expression in transformed B-lymphocytes involves IRF1 (Chapter 5), it is possible that STAT1 may act through IRF1, or another transcription factor, in repressing the expression of LMP2A and lytic cycle antigens like BZLF-1. If this is the case, it would highlight how EBV utilises a host signalling pathway to help maintain a latency III infection.

It is possible that STAT1 has other functions in transformed B-lymphocytes and future work, using the SV5 V-protein expressing LCLs, could be focused on identifying them. As mentioned before, *in vivo* and *in vitro* studies have shown that STAT1 is both pro-proliferative and anti-apoptotic (Bromberg et al., 1996, Durbin et al., 1996, Meraz et al., 1996, Kumar et al., 1997). Since EBV promotes cell survival and growth in transformed B-lymphocytes, it is quite possible that STAT1 could regulate these processes. Although this thesis showed that the expression of two LMP1-regulated, anti-apoptotic proteins; bcl-2 and mcl-1, were not affected by reduced STAT1 protein expression (Figure 5.1), this doesn't necessarily rule out the possibility that STAT1 can regulate cell survival in these cells. The fact that EBV can transform B-lymphocytes from

a STAT1-deficient individual *in vivo* (Chapgier et al., 2006) and these cells can survive in culture *in vitro* suggests that STAT1 is not crucial for B-lymphocyte transformation or for cell survival. However, it is possible that STAT1 contributes indirectly to cell survival and this is supported by evidence indicating that STAT1 may have a role in sensitising EBV-transformed B-lymphocytes to CD95-mediated apoptosis (Le Clorennec et al., 2006). One drawback of the V-protein LCL model for studying cell survival is the fact that the cells are selected by their resistance to puromycin. It is possible that selection in this way generates cells in which cell survival is not required. A solution to this would be to generate EBV-transformed LCLs with conditional/inducible SV5 V-protein expression. A system similar to that used in the DG75 tTA LMP1 cell line (Chapter 3) where removal of tetracycline induces LMP1 expression would be ideal. This would be very useful for studying the effects of reduced STAT1 protein expression on LCL survival and proliferation

The therapeutic implications of targeting STAT1 in EBV-associated malignancies, such as HL, are dependent on understanding the function(s) of STAT1 in virally infected cells. In the context of this thesis, it would appear that STAT1 contributes to the establishment of the host-virus equilibrium in immunocompetent hosts by regulating surface MHC expression on transformed B-lymphocytes and subsequently their recognition by host T-lymphocytes. However, although this role for STAT1 appears to be detrimental to EBV, it could promote the onset of malignancies like HL which manifest in immunocompetent hosts. If so, the use of STAT1 targeting therapies could have clinical significance. In addition, the STAT proteins have also been previously indicated to be attractive anti-cancer targets since STATs, like STAT3, have been labelled as oncogenes (Darnell, 2002).

Therapies that target STAT1 could include a STAT1 pharmacological inhibitor or virally delivered STAT1 small interfering RNA (siRNA) or short hairpin RNA (shRNA). Although a STAT1 inhibitor is not commercially available, it is plausible that it could be synthesised. The *in vitro* use of lentiviral STAT1 siRNA or STAT1 shRNA in other cell types has been described (Tassiulas et al., 2004, Ho & Ivashkiv, 2006). However, although the use of lentiviral siRNA and shRNA constructs *in vivo* has been attempted in mouse disease models (Godfrey et al., 2005, Raoul et al., 2005), there are technical

disadvantages. These include the lack of specificity of transduced cell type, especially after systemic administration of the viral vector (Oliveira et al., 2006). Therefore, more development of this technology may permit the use of viral STAT1 siRNA or shRNA vectors for the treatment of malignancies such as HL in the future.

Another potential strategy for inhibiting STAT1 activity, especially in the context of PTLN, would be to target the constitutive serine phosphorylation of STAT1. This thesis demonstrated that this post-translational modification was sensitive to combined use of PI3K and MEK inhibitors (Figure 3.13). Therefore, combined inhibition of the PI3K and MEK pathways could be used to inhibit STAT1 activity in PTLN lesions. It is possible, though, that a kinase downstream of these pathways is responsible for catalysing this modification. Such kinases could include p90 ribosomal S6 kinase which is activated by both ERK and phosphoinositide dependent kinase 1 (PDK1) (Lazar et al., 1995, Dalby et al., 1998) which are downstream of MEK and PI3K respectively. This kinase has also been implicated in catalysing STAT1 serine phosphorylation in another cell model (Zykova et al., 2005). Therefore, targeting p90 RSK2 could provide a more specific way of inhibiting the constitutive serine phosphorylation of STAT1 in EBV-transformed B-lymphocytes.

In summary, the work presented in this thesis shows that STAT1 is involved in maintaining the latency III viral program observed in transformed B-lymphocytes and regulating their immunorecognition by EBV-specific T-lymphocytes. This thesis has extended our knowledge on why EBV targets STAT1 during B-lymphocyte transformation. It is clear that STAT1 may be functionally important to EBV and, as such, may contribute to the pathogenesis of EBV-associated malignancies. As a result, this may ultimately lead to therapeutic intervention of STAT1 itself or molecular targets of STAT1.

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Appendix I

List of Suppliers

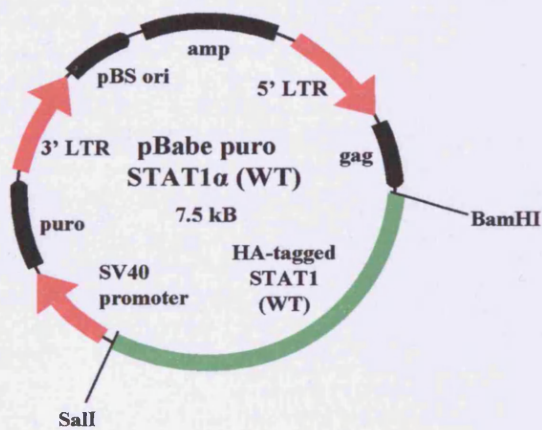
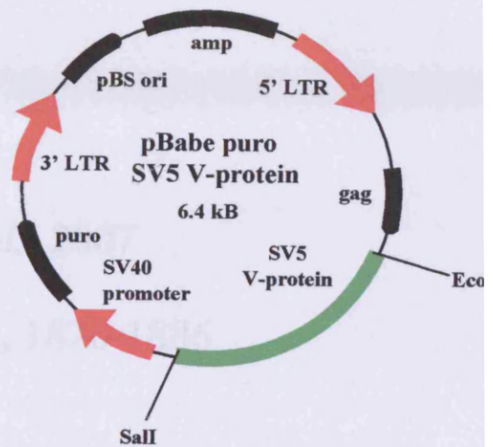
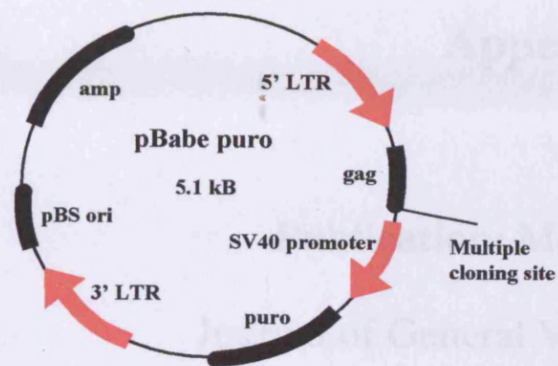
LIST OF SUPPLIERS

Alpha Laboratories	Alpha Laboratories, Eastleigh, Hants, UK
Amersham Biosciences	Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK.
AbCam	AbCam plc, Cambridge, UK
Basic Chemical Company	Basic Chemical Company, High Wycombe, Bucks, UK.
Beckman	Beckman Instruments Ltd., High Wycombe, Bucks, UK.
Biorad	Biorad, Hemel Hempstead, Watford, Herts, UK.
BDH	VWR International Ltd., Poole, Dorset, UK
BD Pharmingen	BD, Franklin Lakes, New Jersey, USA
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, USA.
Dako Cytomation	Dako UK Ltd., Ely, Cambs, UK
Exalpha Biologicals	Exalpha Biologicals Inc., Maynard, Massachusetts, USA.
Fisher	Fisher Scientific Ltd., Loughborough, Leics, UK.
Fluostar	BMG Labtech GmbH, Offenburg, Germany
Gibco BRL	Invitrogen Ltd., Inchinnan Bus. Park, Paisley, UK.
Hamamatsu	Hamamatsu Photonics UK Ltd., Welwyn Garden City, UK.
Heatsystems	Heatsystems-Ultrasonics Inc., Farmingdale, New York, US.
Heraeus	Heraeus Holding GmbH, Hanau, Germany.
Invitrogen	Invitrogen Ltd., Inchinnan Bus. Park, Paisley, UK.
Jencons	Jencons-PLS, Leighton Buzzard, UK.
Kodak	Kodak (IBI Ltd.), Cambridge, UK.
Leica	Leica Microsystems GmbH, Wetzlar, Germany
Lonza	Lonza Group Ltd, Basel, Switzerland
Macherey-Nagel	Abgene Ltd., Epsom, UK
Merck Biosciences	Merck Ltd., Poole, Dorset, UK.
Molecular Probes	Invitrogen Ltd., Inchinnan Bus. Park, Paisley, UK
MWG Biotech	MWG-Biotech AG., Ebersburg, Germany

Novagen	Merck Biosciences Ltd., Beeston, Nottingham, UK.
Oxoid	Oxoid Ltd., Basingtoke, Hampshire, UK.
Promega	Promega Life Sciences, Southampton, UK.
Santa Cruz Biotechnology	Autogen Bioclear UK Ltd., Calne, Wiltshire, UK.
Serotec	AbD Serotec, Langford Bus. Park, Oxford, UK
Sigma	Sigma, Poole, Dorset, UK.
Sorvall	Kendro Lab, Products Ltd., Bishops Stortford, Herts, UK.
Stuart Scientific	Barloworld Scientific, Stone, Staffs, UK
Takara Biomedicals	Takara Bio Inc., Otsu, Shiga, Japan
Tropix	Applied BioSystems, Foster City, California, USA
Upstate Biotechnology	Millipore, Chandlers Ford, Hants, UK
Whatman	Whatman International Ltd., Maidstone, Kent, UK.

Appendix II

Plasmid maps of retroviral DNA used in study



Retroviral vectors used in this study

The maps of pBabe puro, pBabe puro SV5 V-protein and pBabe puro STAT1 α (WT) are shown above

Appendix III

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Epstein–Barr virus induces a distinct form of DNA-bound STAT1 compared with that found in interferon-stimulated B lymphocytes

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Since 'constitutive activation' of STAT1 was first described in Epstein–Barr virus (EBV)-immortalized lymphoblastoid cell lines (LCLs), there has been controversy regarding the molecular identity of the STAT1 DNA-binding complex found in these cells. The post-translational modifications of STAT1 in LCLs have been analysed and an LMP1-induced STAT1 DNA-binding complex, different from that generated by alpha interferon (IFN) stimulation and not involving tyrosine phosphorylation, is demonstrated. STAT1 is serine-phosphorylated downstream of PI3K and MEK in LCLs and this modification restricts IFN-stimulated STAT1–DNA binding. These data suggest that EBV induces a distinct form of DNA-bound STAT1 in virus-infected cells.

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INTRODUCTION

Epstein–Barr virus (EBV) is a lymphotropic γ -herpesvirus that infects >90% of the world's adult population. Although the effects are asymptomatic in most cases, due to the presence of circulating cytotoxic T lymphocytes (Rickinson & Moss, 1997), EBV infection is also associated with the onset of malignancies such as Burkitt's lymphoma, Hodgkin's lymphoma and post-transplant lymphoproliferative disease (PTLD) (Rickinson & Kieff, 1996).

One of the main characteristics of EBV is its ability to establish viral latency. Three types of latency have been described, each of which displays a different subset of EBV latent genes. In type I latency, which is seen in EBV-associated Burkitt's lymphoma, only one viral protein, EBNA-1, is expressed. In type II latency, which is seen in Hodgkin's lymphoma and nasopharyngeal carcinoma, EBNA-1 and three viral latent membrane proteins (LMPs), LMP1, LMP2A and LMP2B, are expressed. In type III latency, which is seen in PTLD, nine viral proteins are expressed (EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, EBNA-LP, LMP1, LMP2A and LMP2B) (Rickinson & Kieff, 1996).

EBV is capable of transforming and immortalizing primary B lymphocytes into lymphoblastoid cell lines (LCLs). LCLs display type III latency and thus act as a cellular model for PTLD (Rickinson & Kieff, 1996). Among the nine viral proteins that are expressed in type III latency, LMP1 is essential to the immortalization process (Kaye *et al.*, 1993; Kilger *et al.*, 1998). LMP1 mimics a constitutively activated CD40 receptor (Gires *et al.*, 1997) and contains two

specialized C-terminal domains (CTAR1 and CTAR2) that trigger cellular signalling (Eliopoulos & Young, 2001). Signal transducer and activator of transcription 1 (STAT1) expression has been shown to be induced by LMP1 and is known to be mediated by the co-operation of these two C-terminal domains (Richardson *et al.*, 2003; Zhang *et al.*, 2004).

STAT1 is a transcription factor that was originally discovered as a target of interferon (IFN) activation. It belongs to a family of latent transcription factors that become activated in response to extracellular ligands such as the cytokine IFN- α . Activation involves a phosphorylation cascade that enables STATs to dimerize, translocate to the nucleus and activate gene transcription at gamma-activated sequence (GAS) and IFN-stimulated response elements located in the promoters of IFN-responsive genes (Darnell *et al.*, 1994). The key post-translational modification involved is tyrosine phosphorylation at residue 701, although other modifications that can regulate STAT activity exist. These include serine phosphorylation at residue 727, which has been shown to be important for STAT1 transactivation (Decker & Kovarik, 2000), arginine methylation (Mowen *et al.*, 2001) and lysine acetylation (Kramer *et al.*, 2006).

Constitutive activation of STAT1 is observed in many cancers, including acute myeloid leukaemia and EBV-associated malignancies (Fagard *et al.*, 2002; Nepomuceno *et al.*, 2002; Rickinson & Kieff, 1996; Weber-Nordt *et al.*, 1996). However, in EBV-immortalized LCLs, the post-translational modifications of the STAT1 DNA-binding complex remain controversial. Some studies have shown

that STAT1 is not constitutively tyrosine-phosphorylated in LCLs, but is capable of being tyrosine-phosphorylated in response to IFN- α (Dupuis *et al.*, 2001; Zhang *et al.*, 2004). However, other studies disagree with this and have reported that STAT1 is constitutively tyrosine-phosphorylated in LCLs, and a mechanism has even been described involving an indirect autocrine loop of IFN secretion (Fagard *et al.*, 2002; Najjar *et al.*, 2005; Nepomuceno *et al.*, 2002). As STATs are capable of regulating the expression of apoptotic and cell-cycle proteins such as Bcl-X_L and cyclin D1 (Bowman *et al.*, 2000), the role of STAT1 in the progression of EBV-associated malignancy may be vital. This is highlighted by recent evidence suggesting that STAT1 acts as a tumour promoter rather than a tumour suppressor in the development of leukaemia (Kovacic *et al.*, 2006). Therefore, the regulation of STAT1 by specific post-translational modifications may provide a key insight into its activity in EBV-associated malignancy.

This study was initiated to investigate the post-translational modifications of STAT1 in EBV-immortalized LCLs. We have shown that STAT1 is only tyrosine-phosphorylated following stimulation with IFN- α , but is capable of binding DNA. This effect is driven specifically by the onco-gene LMP1. STAT1 was found to be constitutively serine-phosphorylated, but lacked detectable lysine acetylation. This modification is regulated by two distinct pathways, PI3K and MEK, and appears to repress the DNA binding of STAT1 following IFN- α stimulation.

METHODS

Cell lines and culture. Kit 225 is a human interleukin-2 (IL-2)-dependent leukaemic T-cell line (Hori *et al.*, 1987). KEM LCL is a patient-derived EBV-immortalized LCL (Rowe *et al.*, 1995). EB LCL is an EBV-immortalized LCL that was derived in house. BL41 is a patient-derived EBV-negative Burkitt's lymphoma cell line. BL41+B95.8 is the same line that has been infected *in vitro* with the B95.8 strain of EBV. IARC-171 is an EBV-immortalized LCL obtained from the same patient as BL41 (Rowe *et al.*, 1986). DG75 is an EBV-negative Burkitt's lymphoma cell line (Ben-Bassat *et al.*, 1977). DG75 tTA LMP1 is a stable transfectant of the DG75 cell line capable of tetracycline-regulated LMP1 expression (Floettmann *et al.*, 1996). All cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics (200 U penicillin ml⁻¹ and 200 µg streptomycin ml⁻¹) and were maintained at 37 °C in a 5% CO₂ humidified incubator. Tetracycline (1 µg ml⁻¹) was used to silence tetracycline-regulated LMP1 expression in the DG75 tTA LMP1 cell line. The Kit 225 T-cell line was cultured in medium supplemented with 20 ng IL-2 ml⁻¹ (Chiron).

Generation of nuclear extracts. Nuclear extracts were prepared by using a method described previously (Brennan & O'Neill, 1996). Following application of any stimuli or inhibitors, 1 × 10⁷ cells were first washed in 1 ml hypotonic buffer [10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl] and centrifuged at 13 000 r.p.m. for 1 min (Heraeus Biofuge). Cells were then lysed in 100 µl hypotonic buffer supplemented with 1 mM PMSF, 1 : 100 dilutions of phosphatase inhibitor cocktails I and II (Sigma; P-2850 and P-5726, respectively) and 0.1% Nonidet P40 detergent and placed on ice for 5 min. Following centrifugation (13 000 r.p.m., 5 min, 4 °C; Heraeus Biofuge), the supernatant was retrieved, representing a cytosolic

extract. Nuclear extracts were prepared by incubating the remaining pellet for 15 min in 50 µl high-salt buffer [20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl₂, 25% glycerol] supplemented with 1 mM PMSF and 1 : 100 dilutions of phosphatase inhibitor cocktails I and II immediately prior to use. Following centrifugation (13 000 r.p.m., 5 min, 4 °C; Heraeus Biofuge), the supernatant was collected, representing the nuclear extract. For extracts prepared specifically for application in an electrophoretic mobility-shift assay (EMSA), 50 µl storage buffer [10 mM HEPES (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 20% glycerol] was also added to the nuclear extract. All extracts were then stored at -20 °C.

Generation of total cell lysates, SDS-PAGE and Western blotting. Cells were counted on a haemocytometer and resuspended in 50 µl 1 × PBS per 10⁶ cells. An equal volume of 2 × gel sample buffer (GSB) [0.1 M Tris/HCl (pH 6.8), 0.2 M dithiothreitol (DTT), 4% SDS, 20% glycerol, 0.1% bromophenol blue] was then added. Cells were sonicated by using a W0385 sonicator (Heatsystems-Ultrasonics Inc.) and, following sonication, samples were heated at 100 °C for 5 min. Proteins were then separated by SDS-PAGE and transferred onto PVDF membranes (Amersham Biosciences) for immunoblotting using a previously described alkaline phosphatase chemiluminescent detection protocol (Rowe & Jones, 2001). Antibodies to phospho-STAT1 (Y701) (sc-7988-R), phospho-STAT1 (S727) (sc-16570-R), pan-STAT1 (sc-346) and phospho-ERK1/2 (Y204) (sc-7383) ERK1/2 (sc-93) were from Santa Cruz Biotechnology and were used at a concentration of 0.2 µg ml⁻¹. Antibodies to phospho-S6 (S235/236) (#2211) and pan-S6 (#2212) were from Cell Signaling Technology and were used at a 1 : 1000 dilution of the stock supplied. The antibody to acetyl-lysine (#06-933) was from Upstate Technology and was used as a 1 : 1000 dilution of the stock supplied. The antibody to actin (A-2066) was from Sigma and was used as a 1 : 1000 dilution of the stock supplied. The primary antibody to LMP1 (CS1-4) has been described previously (Rowe *et al.*, 1987) and was used as a 1 : 1000 dilution.

DNA-affinity precipitation. Nuclear extracts were generated as described above and diluted to 1 ml with salt-free buffer [50 mM Tris/HCl (pH 8), 0.25 mM EDTA, 10 mM NaF, 10% glycerol, 0.5 mM PMSF, 10 µl phosphatase inhibitor cocktails I and II ml⁻¹, 5 mM DTT, 1 mM NaVO₄]. By using an adapted version of the method of Beadling *et al.* (1996), streptavidin-conjugated agarose beads (30 µl of 1 : 1 slurry) and biotinylated double-stranded GRR oligonucleotide (1 µg) were added to the nuclear extract, which was rotated for 1 h at 4 °C. The mixture was centrifuged at 6000 r.p.m. for 5 min (Heraeus Biofuge) and the supernatant was removed. The beads were then washed three times in a wash buffer [50 mM Tris/HCl (pH 8), 0.25 mM EDTA, 10 mM NaF, 10% glycerol, 0.5 mM PMSF, 40 mM NaCl, 5 mM DTT] and the proteins were eluted from the beads by the addition of 2 × GSB. Eluted proteins were then separated by SDS-PAGE, transferred onto PVDF membranes and analysed by using specific antibodies. The GRR oligonucleotide (GTATTTCAGAGAAAGGAAC) corresponds to a STAT consensus sequence derived from the FcγR1-GAS.

EMSA. Nuclear extracts of 2 × 10⁷ cells were generated and quantified by using the Bradford method. Using a previously described method (Brennan & O'Neill, 1996), 10 µg nuclear extract was incubated with either 2 ng ³²P-radiolabelled GRR oligonucleotide, mutant GRR (mGRR) (GTATGTCCCAGAGAGGAAC) or SIE oligonucleotide (GTGCATTTCAGTAAATCTTGTCTACA) (sc-2535; Santa Cruz Biotechnology), generated by T4 polynucleotide labelling. For super-shift assays, the nuclear extracts were pre-incubated for 30 min with 2 µg antibody to STAT1 from Santa Cruz Biotechnology (sc-592 X) and BD Transduction Laboratories (610119). For cold competitor assays, the nuclear extracts were pre-incubated for 30 min with 100 ng cold GRR oligonucleotide. The binding reaction was

performed in binding buffer [10 mM Tris/HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, 4 % glycerol, 0.1 mg BSA ml⁻¹, 5 mM DTT] with the addition of 2 ng poly(dIdC) (Amersham Biosciences) for 30 min at room temperature prior to separation of protein–DNA complexes by using a native 4 % polyacrylamide gel.

Immunoprecipitation. Nuclear extracts were generated as described above and diluted to 1 ml with salt-free buffer [50 mM Tris/HCl (pH 8.0), 0.25 mM EDTA, 10 mM NaF, 10 % glycerol, 0.5 mM PMSF, 10 µl phosphatase inhibitor cocktails I and II ml⁻¹, 5 mM DTT, 1 mM NaVO₄]. Forty microlitres (1 : 1 slurry) of pre-washed STAT1 antibody (sc-346; Santa Cruz Biotechnology)- or ATF-3 antibody (sc-188; Santa Cruz Biotechnology)-bound Sepharose–protein G beads was added to the nuclear extract and rotated at 4 °C for 120 min. The antibody-conjugated beads were collected by centrifugation (6000 r.p.m., 3–4 s, 4 °C; Heraeus Biofuge) and washed two times in cold lysis buffer [50 mM HEPES (pH 7.9), 2 mM EDTA, 250 mM NaCl, 0.1 % Nonidet P40 detergent] and two times in cold PBS. Antibody-bound proteins were eluted by the addition of 25 µl PBS and 25 µl 2 × GSB and heating at 100 °C for 5 min.

Plasmids. The GRR (5)-luc reporter construct, containing five copies of the STAT consensus sequence derived from the FcγR1-GAS upstream of a firefly luciferase gene, has been described previously (Beadling *et al.*, 1996; Richardson *et al.*, 2003). The phRL-SV40 reporter contains a synthetic *Renilla* luciferase gene downstream of a T7 promoter and was obtained from Promega (E-6261).

Transient transfection and luciferase reporter assay. Cells (1 × 10⁷) were transiently transfected by electroporation using a Bio-Rad Gene Pulser II electroporator at 270 V and 950 µF at room temperature in 500 µl RPMI 1640 medium. Following transfection, cells were seeded in 3.5 ml fresh RPMI 1640 medium and incubated for 20 h at 37 °C in a 5 % CO₂ humidified incubator. To assay luciferase activity from the reporter constructs, cells were washed in chilled PBS and lysed in 100 µl 1 × passive lysis buffer provided within the Promega Dual Luciferase reporter assay kit (E-1910). Lysates were clarified by centrifugation (13 000 r.p.m., 1 min; Heraeus Biofuge) and 50 µl of supernatant was then assayed for firefly and *Renilla* luciferase levels in a 96-well plate by using reagents supplied with the kit. Light release was integrated for 10 s and measured by using a FLUOstar OPTIMA luminometer.

Inducing expression of LMP1 in the DG75 tTA LMP1 cell line. The stable transfectant DG75 tTA LMP1 was maintained under drug selection with 1 µg tetracycline ml⁻¹ until required. Prior to an experiment, cells were washed five times in RPMI 1640 medium and were recultured in the presence or absence of 1 µg tetracycline ml⁻¹ for a period of 72 or 96 h. Total cell lysates and nuclear extracts were generated as described above.

RESULTS

STAT1 is tyrosine-phosphorylated following IFN-α stimulation, but is capable of binding DNA

We have previously demonstrated DNA binding of STAT1 in EBV-immortalized LCLs (Richardson *et al.*, 2003), but have not determined the post-translational modifications of the increased levels of STAT1 found in these cells. Given the conflicting literature, we first analysed STAT1 tyrosine phosphorylation in a range of LCLs that were either untreated or stimulated with IFN-α for 30 min. Total cell lysates were analysed by SDS-PAGE and Western blotting

using specific antibodies. Fig. 1(a) illustrates the absence of detectable STAT1 tyrosine phosphorylation in two LCLs, KEM and EB, a result also seen in three other LCLs including IARC-171 (not shown). Only after stimulation with IFN-α was STAT1 tyrosine-phosphorylated, as has been reported previously (Dupuis *et al.*, 2001; Zhang *et al.*, 2004).

Transcriptional regulation by STAT1 is dependent on its ability to bind DNA in the nucleus. We investigated STAT1 DNA binding in a set of B-cell lines differing in their EBV gene-expression profile. These lines include: BL41, an EBV-negative Burkitt's lymphoma; BL41 + B95.8, the same line that has been infected *in vitro* with the B95.8 strain of EBV; and IARC-171, an EBV-immortalized LCL (Rowe *et al.*, 1986). STAT1 DNA binding was measured in untreated or IFN-α-stimulated cells by using DNA-affinity precipitation. DNA-bound proteins were analysed by SDS-PAGE and Western blotting using specific antibodies to tyrosine-phosphorylated and pan-STAT1 (Fig. 1b). STAT1 DNA binding was observed in the BL41 and BL41 + B95.8 cell lines only after IFN-α stimulation, whereas in the IARC-171 LCL, it was seen in both untreated and IFN-α-stimulated cells.

To confirm this constitutive binding, we investigated STAT1 DNA binding in the same cell lines by EMSA. Cells were either untreated or stimulated with IFN-α, and 10 µg nuclear extract was incubated with 2 ng ³²P-radiolabelled GRR probe or SIE oligonucleotide probe, representing a consensus sequence for the binding of Sis-inducible factor (Sadowski *et al.*, 1993). Protein–DNA complexes were then separated by using a native 4 % polyacrylamide gel and visualized by autoradiography (Fig. 1c). Protein–DNA complexes are seen on both probes in the IFN-α-stimulated BL41 and BL41 + B95.8 cell lines. In the IARC-171 LCL, these complexes are seen in both untreated and IFN-α-stimulated cells, an observation that is consistent with the data in Fig. 1(b). This suggested that, in LCLs, STAT1 can bind DNA in the absence of detectable tyrosine phosphorylation. This observation was consistent with previously published data that demonstrated LMP1-induced STAT1 DNA binding (Richardson *et al.*, 2003).

STAT1 forms a distinct DNA-binding complex

To elucidate the identity of the protein–DNA complexes in IARC-171 LCLs that can be observed in Fig. 1(c), a STAT1 antibody (sc-592 X) was pre-incubated with nuclear extracts of both untreated and IFN-α-stimulated IARC-171 LCL cells before ³²P-radiolabelled GRR or SIE probe was added (Fig. 2a). Although no reduction in electrophoretic mobility was observed following pre-incubation with the STAT1 antibody, a reduction in the intensity of the protein–DNA complexes was seen. This indicated that the antibody was preventing a full protein–DNA interaction and thus identified STAT1 as a component of the DNA-bound protein with both probes. The specificity of this complex was ascertained through incubation of a

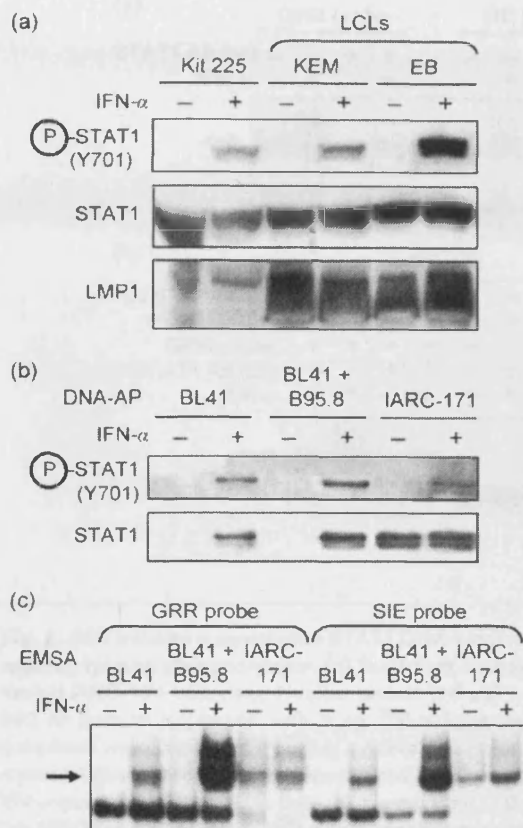


Fig. 1. STAT1 is tyrosine-phosphorylated in LCLs after IFN- α stimulation, but can bind DNA in the absence of stimulation. (a) Total cell lysates were generated from three cell lines: Kit 225, KEM LCL and EB LCL. These cell lines were either unstimulated or incubated with IFN- α (1000 IU) for 30 min. These lysates were analysed by SDS-PAGE and Western blotting using antibodies specific to phospho-STAT1 (Y701), pan-STAT1 and LMP1. The Kit 225 T-cell line was used as a positive control for the presence of tyrosine-phosphorylated STAT1 following IFN- α stimulation, and LMP1 detection was used as a positive marker for EBV. (b) STAT1 DNA binding was measured in the BL41, BL41+B95.8 and IARC-171 cell lines by using a DNA-affinity precipitation assay. These cell lines were either unstimulated or incubated with IFN- α (1000 IU) for 30 min. DNA-bound proteins were analysed by SDS-PAGE and Western blotting using antibodies specific to phospho-STAT1 (Y701) and pan-STAT1. Typically, 1×10^7 cell equivalents were applied to each lane of the gel. These results are representative of four separate experiments. (c) STAT1 DNA binding was measured in the BL41, BL41+B95.8 and IARC-171 cell lines by using an EMSA. These cell lines were either unstimulated or incubated with IFN- α (1000 IU) for 30 min. Nuclear extract (10 μ g) was then incubated with 2 ng 32 P-radiolabelled GRR oligonucleotide or SIE oligonucleotide probe. Protein-DNA complexes were separated by using a native 4% polyacrylamide gel and visualized by autoradiography. Only protein-DNA complexes are shown, as free probe has been removed from the figure. The arrow indicates a specific protein-DNA complex. The results shown are representative of three separate experiments.

mutant 32 P-radiolabelled GRR probe and through competitive binding with an excess of cold GRR competitor probe (Fig. 2b). In order to confirm fully that the protein-DNA complex contained STAT1, untreated and IFN- α -stimulated IARC-171 LCL cells were pre-incubated with a different STAT1 antibody (610199; BD Transduction Laboratories) before 32 P-radiolabelled GRR probe was added (Fig. 2c). The supershift seen with the STAT1 antibody reiterated its presence in the DNA-binding complex.

We investigated STAT transcriptional activity in untreated IARC-171 LCL cells by transiently transfecting them with a STAT luciferase reporter. Cells were transfected with either 20 μ g empty vector-luc reporter or increasing amounts (5, 10 or 20 μ g) of GRR (5)-luc reporter. Fig. 2(d) shows that STAT transcriptional activity increased, at levels higher than the empty-vector control, with rising amounts of the STAT reporter.

From these data, we conclude that EBV-immortalized LCLs contain a constitutive STAT1 DNA-binding complex, unphosphorylated at tyrosine 701, that can probably stimulate transcriptional activation.

LMP1 induces STAT1 protein expression, nuclear translocation and DNA binding without triggering tyrosine phosphorylation

LMP1 has been shown to be responsible for inducing STAT1 expression and transcriptional activity in EBV-infected B lymphocytes (Richardson *et al.*, 2003; Zhang *et al.*, 2004). Given that we observed constitutive STAT1 DNA binding in the absence of tyrosine phosphorylation, we investigated whether this effect was LMP1-specific. In order to study the impact of LMP1 in a cellular context, we sought use of a stable transfectant of an EBV-negative Burkitt's lymphoma cell line in which LMP1 expression is controlled by a tetracycline-regulated LMP1 expression vector (Floettmann *et al.*, 1996). This cell line, DG75 tTA LMP1, allows LMP1 to be induced in an EBV-negative B-cell system through removal of tetracycline. To show successful induction of LMP1, total cell lysates were generated of cells cultured in the presence or absence of tetracycline for 72 or 96 h. The lysates were analysed by SDS-PAGE and Western blotting using specific antibodies to LMP1. Fig. 3(a) shows the presence of LMP1 in the cells that were cultured in the absence of tetracycline for 72 or 96 h, but not in those cultured with tetracycline. IARC-171 was used as a positive control for LMP1 expression and actin was used as a loading control. To see whether LMP1 induces STAT1 expression, nuclear translocation and tyrosine phosphorylation, nuclear extracts were generated of DG75 tTA LMP1 cells cultured in the presence or absence of tetracycline for 72 or 96 h. These cells were also either stimulated with IFN- α or left untreated. Nuclear extract (10 μ g) was then analysed by SDS-PAGE and Western blotting using specific antibodies. Fig. 3(b) shows that LMP1 induces STAT1 nuclear expression, but does not trigger tyrosine phosphorylation. Only

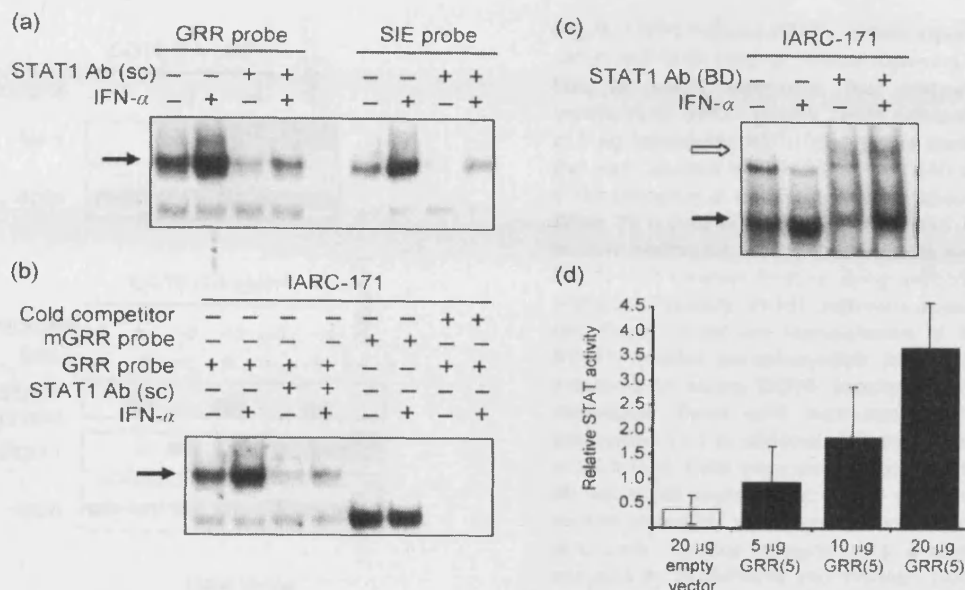


Fig. 2. EBV induces a constitutive STAT1 DNA-binding complex that is capable of stimulating transcriptional activation without requiring tyrosine phosphorylation. (a) Supershift analysis of protein–DNA complexes was measured in unstimulated and IFN- α -treated IARC-171 LCL cells. Nuclear protein (10 μ g) was pre-incubated for 30 min with 2 μ g STAT1 supershift antibody (sc-592 X) prior to incubation with 2 ng 32 P-radiolabelled GRR oligonucleotide or SIE oligonucleotide probe. Protein–DNA complexes were separated by using a native 4% polyacrylamide gel and visualized by autoradiography. The results shown are representative of two separate experiments. (b) The specificity of STAT1–DNA complexes was measured in unstimulated and IFN- α -treated IARC-171 LCL cells. Nuclear protein (10 μ g) was pre-incubated for 30 min with 2 μ g STAT1 supershift antibody (sc-592 X) or 100 ng cold GRR oligonucleotide prior to incubation with 2 ng 32 P-radiolabelled GRR oligonucleotide or mGRR oligonucleotide probe. Protein–DNA complexes were then separated by using a native 4% polyacrylamide gel and were visualized by autoradiography. (c) Supershift of protein–DNA complexes was measured in unstimulated and IFN- α -treated IARC-171 LCL cells. Nuclear protein (10 μ g) was pre-incubated for 30 min with 2 μ g STAT1 supershift antibody (BD #610119) prior to incubation with 2 ng 32 P-radiolabelled GRR oligonucleotide. Protein–DNA complexes were separated by using a native 4% polyacrylamide gel and visualized by autoradiography. Arrows indicate specific protein–DNA and supershifted protein–DNA complexes. The results shown are representative of two separate experiments. (d) STAT transcriptional activation was measured in IARC-171 LCL cells by using a STAT reporter assay. Cells (1×10^7) were transfected with 20 μ g empty vector-luc reporter, 5 μ g GRR (5)-luc reporter, 10 μ g GRR (5)-luc reporter or 20 μ g GRR (5)-luc reporter. One microgram of pRL-SV40 reporter was also co-transfected and luciferase activity was assayed 20 h post-transfection. Relative luciferase activity was calculated as a ratio of firefly over *Renilla* luciferase. The results are mean values representative of at least three experiments. Error bars indicate 1 SEM.

after IFN stimulation was tyrosine phosphorylation observed. This shows that LMP1 elevates STAT1 expression in LCLs, but does not induce tyrosine phosphorylation. The impact of LMP1 on STAT1 DNA binding in LCLs was investigated by using an EMSA (Fig. 3c). DG75 tTA LMP1 cells were cultured in the presence or absence of tetracycline for 72 or 96 h and were either incubated with IFN- α or left unstimulated. Nuclear extract (10 μ g) was incubated with 2 ng 32 P-radiolabelled GRR probe, and any protein–DNA complexes formed were then separated by using a native 4% polyacrylamide gel and visualized by autoradiography. Fig. 3(c) displays the emergence of a constitutive STAT1 DNA-binding complex in DG75 tTA LMP1 cells in the absence of tetracycline after 96 h. This observation was seen in unstimulated cells and the levels of DNA binding were comparable to those seen in the IARC-171 LCL. From

these data, we conclude that LMP1 alone is sufficient for inducing a constitutive STAT1 DNA-binding complex that is unphosphorylated at tyrosine Y701.

STAT1 is constitutively serine-phosphorylated in LCLs, but lacks detectable lysine acetylation

STAT1 may also be serine-phosphorylated at the C-terminal residue 727, and this type of modification is proposed to be important for full transactivation potential (Decker & Kovarik, 2000). We compared serine phosphorylation of STAT1, following STAT1 immunoprecipitation, in the IARC-171 LCL and an EBV-negative Burkitt's lymphoma line, DG75. Nuclear extracts were generated from untreated or IFN- α -stimulated cells and incubated with 1 μ g STAT1 antibody pre-coupled to Sepharose–protein G.

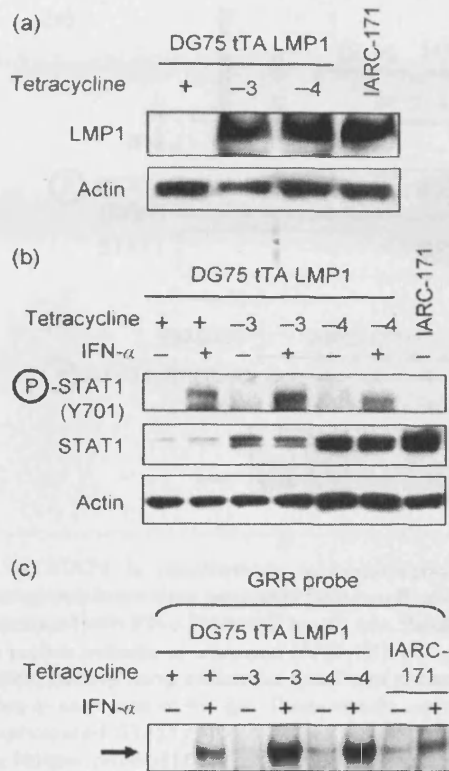


Fig. 3. LMP1 induces STAT1 protein expression, nuclear translocation and DNA binding without triggering tyrosine phosphorylation. (a) LMP1 expression was measured in stable DG75 transfectants (with inducible LMP1 expression) following removal of $1 \mu\text{g}$ tetracycline ml^{-1} . Total lysates were generated from cells that were washed five times in RPMI 1640 medium and recultured in the presence of tetracycline (+) or absence of tetracycline for either 72 h (-3) or 96 h (-4). IARC-171 LCLs were used as a positive control for LMP1. These lysates were analysed by SDS-PAGE and Western blotting using antibodies specific to LMP1 and actin. Typically, 2×10^5 cells were applied to each lane of the gel. These results are representative of three experiments. (b) STAT1 tyrosine phosphorylation and nuclear expression were measured in stable DG75 transfectants with inducible LMP1 expression. These cells were recultured in the presence of tetracycline (+) or absence of tetracycline for either 72 h (-3) or 96 h (-4). Cells were also incubated with IFN- α (1000 IU) for 30 min or left unstimulated. STAT1 tyrosine phosphorylation and nuclear expression were also measured in unstimulated IARC-171 LCL cells. Nuclear extracts were generated and were then analysed by SDS-PAGE and Western blotting using antibodies specific to phospho-STAT1 (Y701), pan-STAT1 and actin. Nuclear extract ($10 \mu\text{g}$) was applied to each lane of the gel. These results are representative of three experiments. (c) STAT1 DNA binding was measured in stable DG75 transfectants with inducible LMP1 expression by using an EMSA. These cells were recultured in the presence of tetracycline (+) or absence of tetracycline for either 72 h (-3) or 96 h (-4). STAT1 DNA binding was also measured in IARC-171 LCL cells. Cells were incubated with IFN- α for 30 min or left unstimulated. Nuclear extract ($10 \mu\text{g}$) was incubated with 2 ng ^{32}P -radiolabelled GRR oligonucleotide probe. Protein-DNA complexes were separated by using a native 4% polyacrylamide gel and visualized by autoradiography. Only the protein-DNA complexes are shown, as free probe has been removed from the figure. The arrow indicates a specific protein-DNA complex. The results shown are representative of three separate experiments.

Immunoprecipitated proteins were analysed by SDS-PAGE and Western blotting using specific antibodies to serine-phosphorylated and pan-STAT1. Fig. 4(a) shows that STAT1 is constitutively serine-phosphorylated in both lines, although at a much higher level in the IARC-171 LCL, reflecting the higher levels of STAT1 in this line.

Given that STAT1 can bind DNA in the absence of tyrosine phosphorylation, we investigated whether DNA-bound STAT1 is serine-phosphorylated by using an EMSA. Nuclear extracts were generated from untreated and IFN- α -stimulated IARC-171 LCL cells and were pre-incubated with $2 \mu\text{g}$ phospho-STAT1 (S727) antibody before ^{32}P -radiolabelled GRR probe was added (Fig. 4b). The reduction in the intensity of the protein-DNA complex indicated that DNA-bound STAT1 in untreated and IFN- α -stimulated IARC-171 LCL cells is serine-phosphorylated.

Both tyrosine and serine phosphorylation are key regulatory modifications of STAT1, but other post-translational modifications of STAT1 have been characterized. Arginine methylation of STAT1 at the N-terminal residue 31 has been documented and is believed to enhance the DNA-binding activity of STAT1 (Mowen *et al.*, 2001), although other studies have disputed these claims (Komyod *et al.*, 2005; Meissner *et al.*, 2004). Lysine acetylation of STAT1 at residues 410 and 413 has recently been demonstrated and is believed to regulate the activity of NF- κB (Kramer *et al.*, 2006). To investigate whether STAT1 is acetylated in LCLs, we immunoprecipitated STAT1 from

the nuclei of IARC-171 LCL cells that were untreated, stimulated with IFN- α and/or incubated with trichostatin A, a specific histone deacetylase inhibitor that has previously been shown to enhance STAT3 acetylation (Wang *et al.*, 2005; Yuan *et al.*, 2005). Immunoprecipitates were analysed by SDS-PAGE and Western blotting using an antibody specific for acetylated lysine residues. The results showed that STAT1 lysine acetylation cannot be detected in the immunoprecipitates of nuclear extracts of IARC-171 LCL (Fig. 4c), although the acetylation of an unidentified protein was observed in standard nuclear extracts. STAT1 acetylation was also not detected in immunoprecipitates of cytosolic extracts of this LCL (data not shown).

STAT1 is serine-phosphorylated downstream of PI3K and MEK and seems to restrict IFN-stimulated STAT1 DNA binding

The observation of constitutive STAT1 serine phosphorylation in our LCL model led us to the question of which

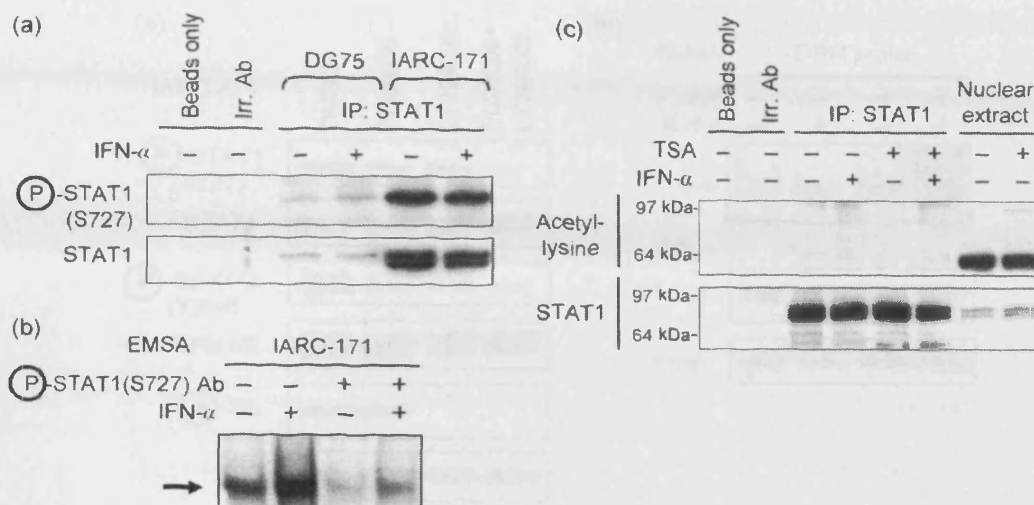


Fig. 4. STAT1 is constitutively serine-phosphorylated in LCLs, but lacks detectable lysine acetylation. (a) STAT1 immunoprecipitates were generated from two B-cell lines, DG75 and IARC-171 LCL. These cell lines were either unstimulated or incubated with IFN- α (1000 IU) for 30 min. Beads only and irrelevant antibody (Irr. Ab; ATF-3) controls were also incubated with nuclear extracts of untreated IARC-171 LCL cells. STAT1 immunoprecipitates were then analysed by SDS-PAGE and Western blotting using antibodies specific to phospho-STAT1 (S727) and pan-STAT1. Typically, 5×10^6 cell equivalents were loaded in each lane of the gel. These results are representative of three separate experiments. (b) The presence of serine-phosphorylated STAT1 in DNA-bound protein complexes was measured in unstimulated and IFN- α -treated IARC-171 LCL cells. Nuclear protein (10 μ g) was pre-incubated for 30 min with 2 μ g phospho-STAT1 (S727) antibody prior to incubation with 2 ng 32 P-radiolabelled GRR oligonucleotide. Protein-DNA complexes were separated by using a native 4% polyacrylamide gel and visualized by autoradiography. Arrows indicate specific protein-DNA and supershifted protein-DNA complexes. The results shown are representative of two separate experiments. (c) STAT1 immunoprecipitates were generated from IARC-171 LCL cells that were unstimulated, incubated with IFN- α (1000 IU) for 30 min and/or treated with trichostatin A (TSA) (2 μ M) for 24 h. STAT1 immunoprecipitates were then analysed by SDS-PAGE and Western blotting using antibodies specific to acetyl-lysine and pan-STAT1. Typically, 5×10^6 cell equivalents were loaded in lanes 1–6 of the gel, and 2.5×10^5 cell equivalents of nuclear extracts from unstimulated and TSA-treated IARC-171 LCL cells were loaded in lanes 7 and 8 as controls. These results are representative of four separate experiments.

serine kinase(s) regulates this process and whether it can be inhibited. Many kinases have been implicated in other cell models, including p38 mitogen-activated protein kinase (p38 MAPK) (Goh *et al.*, 1999; Zykova *et al.*, 2005), extracellular signal-regulated kinase (ERK) (Zykova *et al.*, 2005) and phosphatidylinositol 3-kinase (PI3K) (Nguyen *et al.*, 2001; Rahimi *et al.*, 2005; Zykova *et al.*, 2005). We used a selection of serine kinase inhibitors (all supplied by Calbiochem) to investigate their effect on IARC-171 LCLs: PD98059, a MEK1/2 inhibitor; staurosporine, a broad-spectrum serine/threonine kinase inhibitor; and LY294002, a PI3K inhibitor. We found that staurosporine inhibited STAT1 serine phosphorylation, but also produced dramatic cytotoxicity to the cells (data not shown). However, PD98059 and LY294002 both inhibited STAT1 serine phosphorylation without causing dramatic cell death. We also found that, in combination, they further reduced the levels of STAT1 serine phosphorylation compared with that seen with the two inhibitors incubated alone (Fig. 5a). As LY294002 has been shown to inhibit the phosphorylation of S6 ribosomal protein (Breslin *et al.*, 2005), phospho-S6

detection was used to demonstrate the actions of LY294002. Phospho-ERK detection was used to demonstrate the actions of PD98059. Thus, serine phosphorylation of STAT1 in our LCL model is sensitive to inhibition of both PI3K and MEK, suggesting that EBV stimulates serine phosphorylation through two distinct pathways.

Having shown earlier that DNA-bound STAT1 in EBV-immortalized LCLs was serine-phosphorylated, but not tyrosine-phosphorylated, we investigated whether inhibiting STAT1 serine phosphorylation in EBV-immortalized LCLs affected its DNA-binding capabilities. STAT1 DNA binding was analysed in IARC-171 LCLs that were either untreated or incubated with a combination of PD98059 and LY294002 for 24 h, and/or stimulated with IFN- α for 30 min, by EMSA (Fig. 5b). The results show that inhibition of serine phosphorylation did not abrogate STAT1 DNA binding in untreated or IFN- α -stimulated IARC-171 LCL cells. In fact, surprisingly, inhibition of serine phosphorylation actually increased the amount of STAT1 DNA binding in IFN- α -treated cells. Interestingly, IFN- α treatment

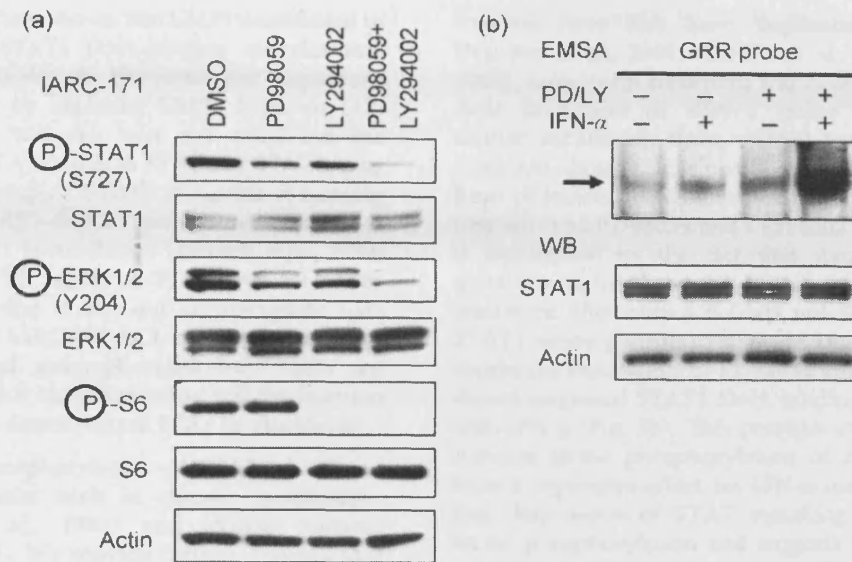


Fig. 5. STAT1 is serine-phosphorylated downstream of PI3K and MEK and seems to restrict IFN-stimulated STAT1 DNA binding. (a) Total cell lysates were generated from IARC-171 LCL cells incubated for 24 h with different combinations of PD98059 and LY294002. These combinations were: PD98059 (50 μ M) alone; LY294002 (20 μ M) alone; and PD98059 (50 μ M)+LY294002 (20 μ M). Total cell lysates were incubated with DMSO for 24 h as a control. These lysates were then analysed by SDS-PAGE and Western blotting using antibodies specific to phospho-STAT1 (S727), pan-STAT1, phospho-ERK1/2 (Y204), pan-ERK1/2, phospho-S6, pan-S6 and actin. Typically, 5×10^5 cells were applied to each lane of the gel. These results are representative of four experiments. (b) The effect of serine phosphorylation on STAT1 DNA binding was measured in IARC-171 LCL cells by using an EMSA. These cells were unstimulated, treated with a combination of PD98059 (50 μ M) and LY294002 (20 μ M) for 24 h and/or incubated with IFN- α (1000 IU) for 30 min. Nuclear extract (10 μ g) was then incubated with 2 ng 32 P-radiolabelled GRR oligonucleotide probe. Protein-DNA complexes were separated by using a native 4% polyacrylamide gel and visualized by autoradiography. Nuclear extract (10 μ g) was also analysed by SDS-PAGE and Western blotting using antibodies specific to STAT1 and actin. This demonstrates that the nuclear levels of STAT1 were equal in each sample analysed. The results shown are representative of five separate experiments.

did not increase the amount of STAT1 found in nuclear extracts. This effect is attributed to the high levels of STAT1 that already exist in the nucleus of an LCL and shows that IFN- α does not stimulate further STAT1 nuclear translocation.

DISCUSSION

This study provides new evidence that STAT1 is capable of binding DNA in the absence of detectable tyrosine phosphorylation in EBV-immortalized LCLs. This form of constitutive STAT1 activation illustrates a new paradigm in STAT signalling in the context of EBV and adds to reports accumulating in the literature that describe gene expression regulated by unphosphorylated STATs. STAT1 lacking tyrosine phosphorylation has been shown to induce the constitutive expression of genes in other cells, including low-molecular-mass protein 2 (Chatterjee-Kishore *et al.*, 2000) and the caspase 1–3 genes (Kumar *et al.*, 1997). Also, a role for STAT1 in the constitutive expression of major histocompatibility complex (MHC) class I antigens has

been demonstrated in T lymphocytes (Lee *et al.*, 1999). Thus, STAT1 can drive gene transcription without requiring tyrosine phosphorylation to form classical homodimers. Unphosphorylated STAT3 has also been shown to activate oncogene expression through a mechanism distinct from that used by classical STAT3 dimers (Yang *et al.*, 2005). In the context of EBV-immortalized LCLs, MHC class I antigens are known to be elevated by LMP1 (Rowe *et al.*, 1995). Thus, LMP1-induced STAT1 may play a role in the regulation of genes such as MHC class I.

Although we have characterized a STAT1 DNA-binding complex lacking tyrosine phosphorylation in EBV-immortalized LCLs, this complex does not seem to exist in untreated BL41 + B95.8 cells (Fig. 1b, c). This observation conflicts with previously published data that show DNA-bound STAT1 in untreated BL41 + B95.8 cells (Richardson *et al.*, 2003). However, this contrast reflects the variable expression of LMP1 in these cells, as clones high in LMP1 display DNA-bound STAT1, whereas those low in LMP1 lack detectable STAT1 DNA binding. The role of LMP1 in this STAT1 DNA-binding complex is evident from the data

displayed in Fig. 3. We have shown that LMP1 is sufficient to induce a constitutive STAT1 DNA-binding complex that lacks tyrosine phosphorylation. However, our hypothesis could be strengthened by depleting LMP1 from an LCL by RNA interference. We also have not ruled out the possibility that other STATs, such as STAT2 or STAT3, may comprise part of this complex. STAT1 is capable of forming STAT1–STAT2 and STAT1–STAT3 heterodimers, as well as forming classical STAT1 homodimers (Darnell *et al.*, 1994; Zhong *et al.*, 1994). With regard to STAT3, we found, by immunoprecipitation, that it did not co-precipitate with STAT1 in the nuclei of IARC-171 LCL cells, even though it was both tyrosine- and serine-phosphorylated (data not shown). Only with further characterization will the function of this complex in EBV-immortalized LCLs be elucidated.

Constitutive serine phosphorylation of STAT1 has been observed in malignancies such as chronic lymphocytic leukemia (Frank *et al.*, 1997) and Wilms' tumour (Timofeeva *et al.*, 2006). We provide further evidence that STAT1 is constitutively serine-phosphorylated in EBV-infected cells, agreeing with reports in the literature (Zhang *et al.*, 2004). Our data suggest that this phenomenon is not EBV-specific, as constitutive serine phosphorylation was also observed in the EBV-negative Burkitt's lymphoma cell line DG75 (Fig. 4a). However, constitutive STAT1 DNA binding was seen in IARC-171 LCL cells, but not in DG75 cells, even though both cell lines exhibit serine-phosphorylated STAT1. This would suggest that DNA-bound serine-phosphorylated STAT1 is a feature of EBV-immortalized LCLs. Also, as constitutive serine phosphorylation is absent in normal peripheral blood B lymphocytes (Frank *et al.*, 1997), this suggests that constitutive serine phosphorylation of STAT1 may be a feature of B-cell malignancy in general. Recent evidence has shown that serine-phosphorylated STAT1 promotes cell survival through the up-regulation of two known pro-survival genes, MCL-1 and HSP-27 (Timofeeva *et al.*, 2006), indicating why malignant B cells may accumulate this molecular change.

Lysine acetylation of STAT1 has been demonstrated recently (Kramer *et al.*, 2006) and could regulate its transcriptional abilities, as lysine acetylation of STAT3 has been shown to be vital for its DNA-binding and transcriptional capacity (Wang *et al.*, 2005; Yuan *et al.*, 2005). Our evidence suggests that STAT1 is not lysine-acetylated in EBV-immortalized LCLs (Fig. 4c). This observation would suggest that this modification is not necessary for STAT1 function in LCLs, although we do not rule out the possibility that we cannot detect it with the technology at our disposal. More specific antibodies for lysine-acetylated STAT1 may provide a different answer but, at present, do not exist commercially.

Many serine kinases have been implicated in catalysing STAT1 serine phosphorylation in various cell systems. We have shown that the constitutive serine phosphorylation of STAT1 in LCLs is abrogated following long-term treatment with inhibitors of PI3K and MEK (Fig. 5a). Both of these

enzymes have also been implicated by other studies (Nguyen *et al.*, 2001; Rahimi *et al.*, 2005; Zykova *et al.*, 2005). Long-term treatment was necessary to ensure sufficient inhibition of STAT1 serine phosphorylation, as shorter incubation times yielded very little or no effect (data not shown). It is possible that this may reflect some form of indirect mechanism or perhaps just a slow inhibitory effect by PD98059 and LY294002 in combination. This is highlighted by the fact that staurosporine, a broad-spectrum serine/threonine kinase inhibitor, caused rapid inhibition after only 1 h (data not shown). By inhibiting STAT1 serine phosphorylation in LCLs through use of the combined incubation of PD98059 and LY294002, we have shown increased STAT1 DNA binding in LCLs stimulated with IFN- α (Fig. 5b). This provides evidence that the constitutive serine phosphorylation of STAT1 in LCLs may have a repressive effect on IFN- α -induced STAT1 signalling. Repression of STAT signalling has been linked to serine phosphorylation and suggests that its role is more complex than thought previously (Bowman *et al.*, 2000). STAT3 serine phosphorylation has been shown to prevent tyrosine phosphorylation and DNA binding through either a direct influence upon or an indirect negative interaction with upstream tyrosine kinases (Chung *et al.*, 1997; Jain *et al.*, 1998; Sengupta *et al.*, 1998). Our data agree with these findings, in that serine phosphorylation seems to repress STAT1 DNA binding in IFN- α -stimulated LCLs. This effect could explain why no constitutive STAT1 tyrosine phosphorylation was seen in our LCLs (Fig. 1a). These observations provide new data supporting a repressive role of serine phosphorylation on STAT1 rather than an enhancing role, and may indicate some form of reprogramming in IFN signalling by EBV.

In summary, this study builds on previous reports by being the most complete survey of post-translational modifications of STAT1 in EBV-immortalized LCLs. Our work illustrates three key advances in our knowledge. Firstly, we have shown that LMP1-induced STAT1 lacks tyrosine phosphorylation and lysine acetylation, but is capable of binding DNA. Secondly, we have also demonstrated, for the first time in EBV-immortalized LCLs, that the serine phosphorylation of STAT1 is regulated by two distinct pathways, PI3K and MEK. Thirdly, and most surprisingly, this modification appears to repress the DNA binding of IFN-stimulated STAT1. This indicates that STAT1 may be subject to some form of viral reprogramming by EBV during cellular transformation.

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Appendix IV

List of publications

LIST OF PUBLICATIONS

- White, P.C., Shore, A.M., Clement, M., McLaren, J., Soeiro, I., Lam, E.W-F. & Brennan, P. (2006) Regulation of cyclin D2 and the cyclin D2 promoter by protein kinase A and CREB in lymphocytes. *Oncogene* **15**, 2170-80
- McLaren, J.E., Rowe, M. & Brennan, P. (2007) Epstein-Barr virus induces a distinct form of DNA-bound STAT1 compared to that found in interferon stimulated B-lymphocytes. *J. Gen Virol* **88**, 1876-1886
- McLaren, J.E., Zuo, J., Grimstead, J., Poghosyan, Z., Bell, A.I., Rowe, M., & Brennan, P. (2007) STAT1 contributes to the maintenance of the latency III viral program observed in Epstein-Barr virus transformed B-lymphocytes, and their recognition by CD8⁺ T-cells. *Manuscript in preparation.*



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I also acknowledge the Leukaemia Research Appeal for Wales who funded this PhD project.

Finally, I would like to thank my parents for all their encouragement. I also want to especially thank Bec for all her support and care throughout these last three years and more notably having to suffer my endless scientific ramblings to her at home which I know for a fact she never ever truly understood.

SUMMARY

Constitutive expression and activation of Signal Transducer and Activator of Transcription 1 (STAT1) is a feature of Epstein-Barr virus (EBV)-associated malignancies such as post-transplant lymphoproliferative disease and nasopharyngeal carcinoma. Although the mechanism for how EBV induces STAT1 up-regulation during B-lymphocyte transformation is well established, little is known about why STAT1 is targeted by the virus. This thesis sought to investigate how and why EBV modulates STAT1 activity in transformed B-lymphocytes.

Following analysis of the post-translational modifications of STAT1 in EBV-transformed lymphoblastoid cell lines (LCLs), this thesis demonstrated a LMP1-induced STAT1-DNA binding complex, different from that generated by interferon-alpha stimulation, which does not involve tyrosine phosphorylation. To add to this, STAT1 was also found to be constitutively serine phosphorylated, downstream of PI3K and MEK, in EBV-transformed LCLs and this modification restricts interferon-stimulated STAT1 DNA binding. This evidence suggested that qualitative differences exist between EBV-induced STAT1 and that seen in interferon-stimulated B-lymphocytes.

In order to study the function of STAT1 in EBV-transformed LCLs, an *in vitro* model was generated by introducing the simian virus 5 (SV5) V-protein by retroviral gene transfer. The SV5 V-protein is a virally evolved STAT1 inhibitor that specifically targets STAT1 for proteasomal degradation. Using this cell model, this thesis has demonstrated that MHC class I and class II molecules are down-regulated at the cell surface following a reduction in STAT1 protein expression. With regards to MHC class I, the impairment of the antigen processing machinery renders the cells to be less recognised by the host EBV-specific immunosurveillance. In addition, down-regulation of STAT1 increases the expression of the EBV latent gene LMP2A and several EBV lytic cycle antigens including BZLF-1, resulting in a higher proportion of cells entering lytic cycle. These results suggest that STAT1 is involved in maintaining the latency III viral program observed in transformed B-lymphocytes and regulating immunorecognition by EBV-specific T-lymphocytes.

In conclusion, the work presented in this thesis has extended our knowledge on why EBV targets STAT1 during B-lymphocyte transformation. It is clear that STAT1 may be functionally important to EBV and, as such, may contribute to the pathogenesis of EBV-associated malignancies.

APPENDIX 1:
Specimen Layout for Thesis Summary and Declaration/Statements page
to be included in a Thesis

DECLARATION

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

Signed (candidate) Date 21/12/07

STATEMENT 1

This thesis is being submitted in partial fulfillment of the requirements for the degree of
PhD (insert MCh, MD, MPhil, PhD etc, as appropriate)

Signed (candidate) Date 21/12/07

STATEMENT 2

This thesis is the result of my own independent work/investigation, except where otherwise stated.

Other sources are acknowledged by explicit references.

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I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

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