

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository: <https://orca.cardiff.ac.uk/id/eprint/111376/>

This is the author's version of a work that was submitted to / accepted for publication.

Citation for final published version:

Zhang, Le, Zhou, Momei, Stanton, Richard , Kamil, Jeremy and Ryckman, Brent J. 2018. Expression levels of glycoprotein O (gO) vary between strains of human cytomegalovirus, influencing the assembly of gH/gL complexes and virion infectivity. *Journal of Virology* 92 (15) , e00606-18. 10.1128/JVI.00606-18

Publishers page: <http://dx.doi.org/10.1128/JVI.00606-18>

Please note:

Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher's version if you wish to cite this paper.

This version is being made available in accordance with publisher policies. See <http://orca.cf.ac.uk/policies.html> for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.



**Expression levels of glycoprotein O (gO) vary between strains of human cytomegalovirus, influencing the assembly of gH/gL complexes and virion infectivity.**

**Le Zhang<sup>1,3,4</sup>, Momei Zhou<sup>5</sup>, Richard Stanton<sup>6</sup>, Jeremy Kamil<sup>7</sup>, and Brent J. Ryckman<sup>1,2,3,4</sup> \***

<sup>1</sup>Division of Biological Sciences, <sup>2</sup>Cellular, Molecular and Microbial Biology Program, <sup>3</sup>Biochemistry and Biophysics Program, <sup>4</sup>Center for Biomolecular Structure and Dynamics, University of Montana, Missoula, Montana, U.S.A.;

<sup>5</sup>Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA, USA;

<sup>6</sup>Division of Infection and Immunity, School of Medicine, Cardiff University, Cardiff, United Kingdom; MRC-University of Glasgow Centre for Virus Research, Glasgow, United Kingdom;

<sup>7</sup>Department of Microbiology and Immunology and Center for Molecular and Tumor Virology, Louisiana State University Health Sciences Center, Shreveport, Louisiana, USA;

**Running title:** Variation in gO expression among HCMV strains

**\*Corresponding author:** Dr. Brent J. Ryckman  
Division of Biological Sciences  
Interdisciplinary Science Building Rm. 215  
The University of Montana  
Missoula, MT 59812  
Tel: 406-243-6948  
Fax: 406-243-4304  
Email: [brent.ryckman@mso.umt.edu](mailto:brent.ryckman@mso.umt.edu)

45 **ABSTRACT**

46 Tropism of human cytomegalovirus (HCMV) is influenced by the envelope glycoprotein  
47 complexes gH/gL/gO and gH/gL/UL128-131. During virion assembly, gO and the UL128-131  
48 proteins compete for binding to gH/gL in the ER. This assembly process clearly differs among  
49 strains since Merlin (ME) virions contain abundant gH/gL/UL128-131 and little gH/gL/gO,  
50 whereas TR contains much higher levels of total gH/gL, mostly in the form of gH/gL/gO, but  
51 much less gH/gL/UL128-131 than ME. Remaining questions include 1) what are the  
52 mechanisms behind these assembly differences, and 2) do differences reflect *in vitro* culture  
53 adaptations or natural genetic variations? Since the UL74(gO) ORF differs by 25% of amino  
54 acids between TR and ME, we analyzed recombinant viruses in which the UL74(gO) ORF was  
55 swapped. TR virions were >40-fold more infectious than ME. Transcriptional repression of  
56 UL128-131 enhanced infectivity of ME to the level of TR, despite still far lower levels of  
57 gH/gL/gO. Swapping the UL74(gO) ORF had no effect on either TR or ME. A quantitative  
58 immunoprecipitation approach revealed that gH/gL expression was within 4-fold between TR  
59 and ME, but gO expression was 20-fold less by ME, and suggested differences in mRNA  
60 transcription, translation or rapid ER-associated degradation of gO. Trans-complementation of  
61 gO expression during ME replication gave 6-fold enhancement of infectivity beyond the 40-fold  
62 effect of UL128-131 repression alone. Overall, strain variations in assembly of gH/gL  
63 complexes result from differences in expression of gO and UL128-131, and selective  
64 advantages for reduced UL128-131 expression during fibroblast propagation are much stronger  
65 than for higher gO expression.

66 **IMPORTANCE**

67 Specific genetic differences between independently isolated HCMV strains may result from  
68 purifying selection on *de novo* mutations arising during propagation in culture, or random  
69 sampling among the diversity of genotypes present in clinical specimens. Results presented  
70 indicate that while reduced UL128-131 expression may confer a powerful selective advantage

during cell-free propagation of HCMV in fibroblast cultures, selective pressures for increased gO expression are much weaker. Thus, variation in gO expression among independent strains may represent natural genotype variability present *in vivo*. This may have important implications for virus-host interactions such as immune recognition, and underscores the value of studying molecular mechanisms of replication using multiple HCMV strains.

## INTRODUCTION

Human cytomegalovirus (HCMV) is widely spread throughout the world, found in approximately 60% of adults in developed countries and 100% in developing countries (reviewed in (1–3) (4)). Immunocompromised individuals such as those infected with HIV patients, or transplant recipients under antirejection treatments can suffer HCMV related pathologies including gastroenteritis, encephalitis, retinitis, and vasculopathies, which can accelerate allograft rejection. HCMV infection can also be acquired in utero and this is a significant cause of congenital neurological impairments and sensorineural hearing loss. The transmission of HCMV is mainly through body liquid, such as urine and saliva (5). Once infection is established, HCMV can spread throughout the body, infecting many of the major somatic cell types including, fibroblasts smooth muscle cells, epithelial and endothelial cells, neurons, and leukocytes such as monocytes-macrophages, and dendritic cells (6–9). HCMV does not replicate efficiently in transformed cells (10, 11), thus most studies of the mechanisms governing HCMV tropism have involved dermal fibroblasts, and retinal pigment epithelial cells and umbilical cord endothelial cells, all of which can be easily cultured as normal, non-transformed cells.

Much focus has been on the gH/gL complexes, which as for other herpesviruses, likely engage cell receptors and promote infection by contributing to the gB-mediated membrane fusion event or through activating cell signaling pathways (reviewed in (12, 13) (14)). During virus assembly, the HCMV UL128-131 proteins and gO compete for binding to gH/gL to form

the pentameric complex gH/gL/UL128-131, or the trimeric complex gH/gL/gO. Structural studies involving purified soluble complexes showed that gO and UL128 can each make a disulfide bond with cysteine 144 of gL, and this was suggested to be the basis of the competitive assembly of the complexes (15). However, Stegmann 2017 demonstrated that a mutant gO lacking the cysteine implicated in the disulfide bond with gL formed an intact, and functional gH/gL/gO (16). This suggests that gO can engage in extensive non-covalent interactions with gH/gL. The gH/gL/UL128-131 complex is dispensable for infection of cultured fibroblasts and neuronal cells, but required for infection of epithelial endothelial cells and monocyte-macrophages (17) (18) (19) (20) (21). In contrast, gH/gL/gO is critical for infection of all cell types (22) (23) (24) (25). Both complexes likely interact with cell receptors. gH/gL/gO can bind platelet-derived growth factor receptor-alpha (PDGFR $\alpha$ ) through the gO subunit, and this interaction is critical for infection of fibroblasts (26–28). Epithelial and endothelial cells do not express PDGFR $\alpha$ , but blocking of gH/gL/gO either with neutralizing antibodies or with soluble PDGFR $\alpha$  can inhibit infection of these cells, suggesting the existence of other gH/gL/gO receptors (26, 27). Receptors for gH/gL/UL128-131 might include epidermal growth factor receptor (EGFR; also known as ErbB1), and  $\beta$ 1 or  $\beta$ 3 integrins, and these interactions may induce signaling cascades, critical for infection of selected cell types such as epithelial and endothelial cells and monocyte-macrophages (26, 29).

We recently reported that the amounts of gH/gL/gO, and gH/gL/UL128-131 in the virion envelope can differ dramatically among commonly studied strains of HCMV, and that this can affect the infectivity of the virions (25, 30). The salient results of these studies were; 1) ME virions contained gH/gL mostly in the form of gH/gL/UL128-131, whereas TR and TB virions had mostly gH/gL/gO, 2) in terms of “total gH/gL”, the amount of gH/gL/gO in TR and TB virions was more than the gH/gL/UL128-131 in ME virions, 3) the infectivity of all three strains on both fibroblasts and epithelial cells correlated with the amount of gH/gL/gO, and 4) when the

121 expression of UL128-131 was suppressed in ME, virions contained dramatically less  
122 gH/gL/UL128-131, but only slightly more gH/gL/gO. This latter point was especially curious  
123 since the model that gO and the UL128-131 proteins compete for binding to gH/gL would predict  
124 that the fraction of gH/gL normally bound by UL128-131 would, in their absence, be instead  
125 bound by gO. This discrepancy could be explained by differences in the stoichiometric  
126 expression of gH/gL, gO, and UL128-131 between strains. An alternative hypothesis was  
127 suggested by the fact that there are at least eight alleles of the UL74 gene that encodes gO  
128 (31). Isoforms of gO can vary between 10-30% of amino acids, and this could affect competition  
129 with UL128-131 for binding to gH/gL. Both of these non-mutually exclusive hypotheses were  
130 addressed in the experiments reported here.

## 131 RESULTS

132 **Strains of HCMV display different patterns of glycoprotein expression and**  
133 **trafficking to virion assembly compartments.** The dramatic differences in the composition  
134 of gH/gL complexes in TR and ME virions described in Zhou 2013/2015 (25, 30) suggested  
135 corresponding differences in glycoprotein expression, and/or trafficking of glycoproteins to virion  
136 assembly compartments (AC). To address these possibilities, cells were infected for 2 days  
137 (Fig 1A) or 5 days (Fig 1B) with TR or ME, and steady state amounts of viral proteins were  
138 compared by immunoblot. At 2 dpi, immediate-early (IE)-1/2 levels were similar for both TR and  
139 ME, consistent with an equal multiplicity of infection. At 5 dpi, the levels of the virion structural  
140 proteins MCP, gB, gH, and gL were also very similar between the two strains. In contrast, ME  
141 infected cells contained dramatically more of the UL128-131 proteins than TR. The UL148  
142 protein was also included in these analyses because it was recently described as an ER  
143 chaperone protein that influences the ratio of gH/gL complexes (32). In TR-infected cells, an  
144 anti-UL148 antibody detected a prominent 35 kDa protein species, consistent with the previous  
145 description of the UL148 protein (32). This 35-kDa species was not detected in ME-infected



146 cells. Instead, ME-infected cells contained two species that were less abundant, and of faster  
147 and slower electrophoretic motilities than the single UL148 species detected in TR-infected  
148 cells. The basis of the apparent size difference was not characterized, but could reflect  
149 differences in translational start/stop codon usage, splicing of the UL148 mRNA, or  
150 posttranslational modifications of the UL148 protein between strains. Overall, the pattern of  
151 expression of the UL128-131, and UL148 proteins correlated well with the previously described  
152 pentamer-rich nature of ME virions and the trimer-rich nature of TR virions (25, 30). Note that  
153 the expression of gO was not addressed in these analyses because the gO amino acid  
154 sequence differences between strains affects antibody recognition and precluded direct  
155 comparison (30).

156        Trafficking of gH/gL from the ER to TGN-derived assembly compartments was assessed  
157 by treating the 5 dpi infected-cell extracts with either endoglycosidase H (endo H) or PNGaseF,  
158 and then analyzing gH and gL by immunoblot (Fig 2). The majority of gH and gL in TR-infected  
159 cells was endo H resistant, consistent with efficient transport from the ER to *trans*-Golgi-derived  
160 ACs. In contrast, most of the gH and gL in ME-infected cells was sensitive to endo H digestion.  
161 In HFFFtet cells, which repress transcription from the UL128-131 locus (30) (33), there was  
162 even less endo H resistant gH and gL. This suggested that the bulk of gH/gL trafficked to ACs  
163 in ME-infected nHDF, which allow UL128-131 expression, represented gH/gL/UL128-131 and is  
164 consistent with the previous observations that, 1) the bulk of gH/gL in the ME virion is pentamer,  
165 and 2) the loss of gH/gL in the form of pentamer in ME-T virions due to the repression of the  
166 UL128-131 proteins is apparently not fully compensated by the formation of complexes with gO  
167 (25, 30).

168        **Differences in the amino acid sequence of gO between TR and ME do not affect**  
169 **the infectivity of cell free virus.** The predicted amino acid sequence of gO differs by 25%  
170 between TR and ME. This sequence divergence precluded direct comparison of gO expression

171 levels because antibodies do not cross-react (30). Furthermore, these sequence differences  
172 could potentially affect the ability of the distinct gO isoforms to compete with the UL128-131  
173 proteins for binding to gH/gL (thus influencing the amounts of gH/gL complexes in the mature  
174 virion envelope), or the function(s) of gO during entry, such as binding PDGFR $\alpha$  or other  
175 receptors. To address these possibilities, BAC recombineering methods were used to replace  
176 the gO ORF (UL74) of TR with the analogous sequences from ME, and visa versa to generate  
177 recombinant viruses denoted TR\_MEgO and ME\_TRgO.

178         Zhou 2015 demonstrated a positive correlation between the infectivity of HCMV virions  
179 and the amounts of gH/gL/gO in the virion envelope (25). To assess the effects of gO  
180 sequences on infectivity, cell free virus stocks of parental wild type and heterologous gO  
181 recombinants were analyzed by qPCR to determine the number of virions, and infectivity was  
182 determined by plaque assay. No difference in particles/PFU was observed between TR and the  
183 corresponding recombinant, TR\_MEgO (Fig 3A), or between ME and the corresponding  
184 recombinant ME\_TRgO (Fig 3B). When the ME-based HCMV were grown in HFFFtet cells,  
185 which repress UL128-131 expression, the resultant virions, ME-T and ME-T\_TRgO, were  
186 dramatically more infectious, as shown before (25) (33), but consistently there were no  
187 differences due to the isoform of gO expressed (Fig. 3B). In parallel analyses, the amounts of  
188 gH/gL complexes were analyzed by non-reducing immunoblot probing for gL to detect intact,  
189 disulfide linked gH/gL/gO, and disulfide-linked gH/gL/UL128 (note that UL130 and UL131 are  
190 not disulfide-linked to the intact pentamer complex and are thus separated by SDS-PAGE) (Fig  
191 4). Consistent with our previous reports (25, 30), TR virions contained much greater amounts  
192 of total gH/gL, mostly in the form of gH/gL/gO, whereas ME virions contained less gH/gL, mostly  
193 as gH/gL/UL128-131. Repression of the UL128-131 proteins (ME-T) drastically reduced the  
194 amount of gH/gL/UL128-131, and increased the amount of gH/gL/gO. However, note that the  
195 amount of gH/gL/gO in ME-T virions was still less than the gH/gL/UL128-131 in ME virions,



196 indicating that the repression of UL128-131 was not fully compensated by gO. In no case did  
197 expression of the heterologous gO isoform detectably influence the amounts of gH/gL  
198 complexes in HCMV virions. Together these results suggest that the amino acid sequence  
199 differences between TR and ME gO do not influence gH/gL complex assembly, or the function  
200 of gO in entry into fibroblasts.

201 **ME expresses less gO during replication than TR.** The heterologous gO  
202 recombinants allowed comparison of gO expression level between TR and ME. In the first  
203 analyses cells infected with parental or the heterologous gO recombinants were analyzed by  
204 reducing immunoblot using TR and ME specific anti-gO antibodies (30) (Fig 5). TR-specific gO  
205 antibodies detected two bands in TR-infected cells, a prominent species migrating just above  
206 the 100kDa marker, and a minor, more diffuse species migrating at approximately 130-140kDa.  
207 The ME-specific antibodies detected similarly migrating bands in TR\_MEgO infected cells,  
208 however their relative abundance appeared more equal. No similar bands were detected in  
209 cells infected with ME or ME\_TRgO analyzed with either gO antiserum. The failure to detect  
210 either isoform of gO in cells infected with ME-based HCMV suggested that protein expression  
211 from the UL74 locus of ME was lower than in TR.

212 To directly compare differences in glycoprotein expression between TR and ME, infected  
213 cells were labeled with [35S]-methionine/cysteine for 15 min, then analyzed by  
214 immunoprecipitation with anti-peptide antibodies specific for gH, gL, or gO, followed by SDS-  
215 PAGE and band density analysis (Fig 6, Tables 1 and 2). Two approaches were taken to allow  
216 for direct quantitative comparisons of labeled-proteins between extracts. First, cell extracts  
217 were denatured and reduced with SDS/DDT prior to immunoprecipitation to allow maximum  
218 epitope access by the anti-peptide antibodies. Second, for each analysis, multiple  
219 immunoprecipitation reactions were performed in parallel with increasing amounts of protein  
220 extract input to insure that antibodies were not limiting. In these experiments, expression of gH  
221 was nearly identical between TR and ME, gL expression was approximately 4-fold higher for TR

222 than for ME, but gO expression was strikingly 27-fold higher for TR than for ME (Fig. 6A, Table  
223 1). To address the possibility that the MEgO-specific antibodies were simply less efficient at  
224 capturing MEgO from ME extracts, similar experiments were performed with the TR-ME  
225 heterologous gO recombinants (Fig 6B, Table 2). Again, gH and gL were similar between  
226 TR\_MEgO and ME\_TRgO, but gO levels were approximately 20-fold lower higher for the TR-  
227 based virus. To address the hypothesis that differences in gO expression between TR and ME  
228 reflect differences in protein turnover, the [35]S-methionine/cysteine label was chased for up to  
229 6 hours (Fig 7). The pattern of gH detection over the chase time was very similar in both TR  
230 and ME samples. In both cases, labeled gH dropped to 60% after 3 hours and to 30-40% after  
231 6 hours. The pattern of gO detection for both TR and ME was comparable to that of gH.  
232 Together, these results confirmed that ME-infected cells express less gO than TR-infected cells,  
233 and suggested differences in early steps of expression such as mRNA transcription, translation  
234 or rapid ER-associated degradation, which can degrade proteins in the timescale of minutes  
235 (34).

236 **Overexpression of gO during ME replication increased gH/gL/gO assembly and**  
237 **virus infectivity.** To directly test the hypothesis that the low abundance of gH/gL/gO in ME  
238 virions was due not simply to competition from the UL128-131 proteins, but also from low gO  
239 expression, Ad vectors were used to increase gO levels during ME replication. Ad vectors  
240 expressing GFP were used to control for potential effects of the Ad vectors themselves.  
241 Consistent with the above analyses, gO levels were below the limits of immunoblot detection in  
242 ME-infected nHDF or HFFF-tet cells, but gO was readily detected in cells superinfected with  
243 AdMEgO (Fig 8A). The overall expression of gL in ME infected cells was reduced by the  
244 presence of either Ad vector (Fig 8A). In the case of the control AdGFP, the lower intracellular  
245 gL correlated with reduced gH/gL/gO complexes in virions from HFFFtet cells (ME-T) (Fig 8B),  
246 and this in turn correlated with reduced infectivity (i.e., increased particle PFU ratio) (Fig 9). The  
247 “Ad effect” on virion gH/gL levels and infectivity was less apparent in HFF cells (ME), perhaps

248 masked by the overall higher amounts of gH/gL and much lower infectivity of these virions (Fig  
249 8B, and Fig. 9). Controlling for the “Ad effect”, AdMEgO expression in HFFFtet increased the  
250 amounts of gH/gL/gO in ME-T virions compared to the AdGFP, and this resulted in a 6-fold  
251 enhancement of infectivity, beyond the 40-fold enhanced infectivity resulting from repression of  
252 UL128-131 alone (Fig 8B, and Fig. 9). By contrast, AdMEgO expression had little effect on the  
253 virions from HFF cells.

## 254 **DISCUSSION**

255       Recent population genetic studies have demonstrated a greater degree of genetic  
256 diversity of HCMV in clinical specimens than had been previously appreciated (35, 36) (37).  
257 The cell type and propagation methods likely narrow the resultant genotypes by purifying  
258 selection (38, 39). During propagation in cultured fibroblasts, inactivating mutations in the  
259 UL128-131 ORFs are rapidly selected in a BAC clone of ME, and this selective pressure can be  
260 relieved by transcriptional repression of the UL131 promoter, which reduces expression of the  
261 pentameric gH/gL/UL128-131 (33). In contrast, the UL128-131 ORFs are more stable in  
262 BAC clones of strain TR, and TB (39, 40). The UL128-131 ORF of TB contains a single  
263 nucleotide polymorphism relative to ME that reduces splicing of the mRNA encoding the UL128  
264 protein, which may help stabilize the UL128-131 ORFs through reduced expression of  
265 gH/gL/UL128-131 (40). However, TR is identical to ME at this nucleotide position, and a  
266 recombinant ME in which the UL128-131 locus was replaced with the UL128-131 sequences  
267 from TR was as sensitive to selective inactivation of the locus as was wild type ME (40).  
268 Together, these observations suggest that factors beyond expression level of the UL128-131  
269 proteins can influence the selective pressures on the UL128-131 ORFs.

270       The results reported here demonstrated that TR and ME differ in stoichiometry of  
271 expression of gO and UL128-131, and this seems to be a major factor determining the  
272 abundance of gH/gL/gO and gH/gL/UL128-131 in the virion envelope, and the infectivity of cell

273 free virion. Fibroblasts infected with TR or ME were found to be comparable in the steady state  
274 levels of gH/gL, but ME-infected cells contained more UL128-131 than TR infected cells. In ME-  
275 infected cells, most of the gH/gL was an ER-associated form, whereas TR-infected cells  
276 contained a large amount of Golgi-associated gH/gL. This correlated well with the previous  
277 observations that TR contained more total gH/gL than ME virions (25, 30). The amount of  
278 Golgi-associated gH/gL in ME-infected cells was reduced when expression of the UL128-131  
279 proteins was repressed, consistent with the observation that most of the gH/gL in ME virions  
280 was in the form of gH/gL/UL128-131 (25, 30). Comparison of gO expression between strains  
281 was complicated because the amino acid sequence differences between genotypes affected  
282 antibody recognition (30). To circumvent this caveat, recombinant HCMV were engineered in  
283 which the UL74(gO) ORF of TR were replaced with the homologous sequences of ME, and *vice*  
284 *versa*. This approach allowed the analysis of expression of both gO isoforms in both genetic  
285 backgrounds eliminating the possibility that the results were due to differences in antibody-  
286 antigen affinities. Immunoblot and radiolabeling experiments clearly demonstrated that ME-  
287 infected cells contained less gO than TR-infected cells. Overexpression of gO during ME  
288 replication had no effect on levels of gH/gL/gO, or infectivity of the virions unless UL128-131  
289 proteins were also transcriptionally repressed, and even then gH/gL/gO levels and infectivity  
290 were only modestly enhanced. Together these results underscore the competition between gO  
291 and UL128-131 for binding to gH/gL, and suggest other factors may influence the efficiency of  
292 gH/gL/gO assembly.

293       The molecular mechanisms underpinning the discrepancy between TR and ME in  
294 expression UL128-131, and gO remain unclear. As mentioned above, Murrell 2013 described a  
295 SNP in the TB UL128-131 locus that affected mRNA splicing, in part explaining the lower  
296 expression of these proteins in TB (40). However, this splicing effect does not explain the  
297 difference in UL128-131 expression between TR and ME since these strains are conserved at

298 this nucleotide position. For gO, the radiolabeling analyses reported in Figures 6 and 7,  
299 suggest the differences are due to early events in UL74(gO) expression such as transcription,  
300 mRNA processing/stability, translation, or rapid ER-associated degradation occurring in the  
301 timescale of minutes (34). Attempts to analyze UL74(gO) mRNA levels between TR and ME by  
302 quantitative RT-PCR were complicated by the fact that HCMV genomes contain many  
303 overlapping RNAPII transcription units that vary between strains (41, 42). It is interesting that  
304 ME-infected cells contained less UL148 than TR-infected cells. UL148 was first described as an  
305 ER-resident chaperone protein that promotes the assembly of gH/gL/gO (32). The mechanism  
306 may well involve interactions between UL148 and the cellular ER-associated degradation  
307 pathway (C. Nguyen, M. Siddiquey, H.Zhang, J. Kamil; presented at 42<sup>nd</sup> International  
308 Herpesvirus Workshop, 2017 Ghent Belgium).

309 The TR-ME heterologous gO recombinant viruses also allowed analysis of the effects of  
310 gO amino acid sequence differences on assembly of gH/gL complexes and the function of gO in  
311 entry. No differences between TR and TR\_MEgO or between ME and ME\_TRgO were  
312 observed in either the amounts of gH/gL complexes in virions, or cell free infectivity. These  
313 results argue against the notion that the amino acid sequence differences between gO  
314 genotypes affect interactions with gH/gL or the binding of the fibroblast entry receptor,  
315 PDGFR $\alpha$ . Interestingly Kalser 2017 showed that replacing the endogenous gO of TB with the  
316 gO from Towne did not alter replication in cultured fibroblasts, but did enhance replication in  
317 epithelial cell cultures. (43). Thus, it may be that gO sequence variation affects interactions  
318 with receptors other than PDGFR $\alpha$  that mediated infection of epithelial cells.

319 Laib-Sampaio 2016 reported that mutational disruption of UL74(gO) expression in ME  
320 had little effect on replication unless the UL128-131 locus was also disrupted (24). These  
321 authors suggested that spread of ME was mediated principally by gH/gL/UL128-131 in a cell-  
322 associated manner, but when UL128-131 was inactivated, spread could also occur in a cell-free

323 manner mediated by gH/gL/gO. This is in stark contrast to the dramatic phenotype reported for  
324 a gO null TR mutant (22). Our finding that expression of gO by ME is low compared to TR may  
325 provide a partial explanation of these different gO null phenotypes.

326 It remains unclear whether the described difference in gO expression between TR and  
327 ME represents a *bona fide* variation that naturally exists between HCMV genotypes *in vivo*, or  
328 reflects differential selection on *de novo* mutations that occurred during the independent  
329 isolation of these strains from clinical specimens. It seems clear that serial propagation of ME in  
330 cultured fibroblasts selects for *de novo* mutations that reduce or abolish the robust expression of  
331 the UL128-131 proteins (33, 39). The selective pressure that fixes these mutations in the  
332 culture population may be explained by the specific infectivity analyses reported here (Figs. 3  
333 and 9) and in Zhou 2015 (25). In both analyses the specific infectivity of TR was measured at  
334 approximately 100-200 particles/PFU, whereas ME was more 30-50-fold less infectious.  
335 Repression of the UL128-131 proteins enhanced the infectivity of ME ("ME-T") to levels  
336 comparable to TR (approximately 100 particles/PFU). While the infectivity of ME-T and TR  
337 virions was comparable, ME-T virions still contained far less gH/gL/gO than TR (Fig 4, and  
338 ((25)). Ad vector overexpression of gO enhanced infectivity of ME only 6-fold beyond the  
339 enhancement due to UL128-131 repression alone (Fig. 8 and 9). Together, these observations  
340 would seem to suggest that *in vitro* selective pressures for reduced UL128-131 expression are  
341 much more pronounced than any for enhanced gO expression. Thus, it is possible that the  
342 difference in gO expression between HCMV TR and ME derives not from selection on *de novo*  
343 mutations occurring during propagation in culture, but from nonselective, random sampling of  
344 the multitude of different genotypes that likely preexist in clinical specimens (35, 36) (37).  
345 Distinguishing these possibilities will require clear identification of the genomic sequences that  
346 determine gO expression level.

## 347 MATERIALS AND METHODS



348       **Cell lines.** Primary neonatal human dermal fibroblasts (nHDF; Thermo Fisher  
349 Scientific), MRC-5 fibroblasts (American Type Culture Collection; CCL-171), and HFFFtet cells  
350 (which express the tetracycline (Tet) repressor protein provided by Richard Stanton (Cardiff  
351 University, Cardiff, United Kingdom) (33) were grown in Dulbecco's modified Eagle's medium  
352 (DMEM: Thermo Fisher Scientific) supplemented with 6% heat-inactivated fetal bovine serum  
353 (FBS; Rocky Mountain Biologicals, Inc., Missoula, Montana, USA.) and 6% bovine growth  
354 serum (BGS; Rocky Mountain Biologicals, Inc., Missoula, Montana, USA.).

355       **Human cytomegaloviruses.** All HCMV were derived from bacterial artificial  
356 chromosome (BAC) clones. The BAC clone of TR was provided by Jay Nelson (Oregon Health  
357 and Sciences University, Portland, OR, USA) (44). The BAC clone of Merlin (ME) (pAL1393),  
358 which carries tetracycline operator sequences in the transcriptional promoter of UL130 and  
359 UL131 was provided by Richard Stanton (Cardiff University, Cardiff, United Kingdom) (33).  
360 Infectious HCMV was recovered by electroporation of BAC DNA into MRC-5 fibroblasts as  
361 described by Wille et al. (22). Cell-free HCMV stocks were produced by infecting HFF or  
362 HFFFtet at 2 plaque-forming unit (PFU) per cell. At 8-10 days post infection (when cells were  
363 still visually intact), culture supernatants were harvested, and cellular contaminants were  
364 removed by centrifugation at 1,000 X g for 10min, and again at 6,000 X g for 10min. Stocks  
365 were judged cell-free by the lack of calnexin, and actin in western blot analyses, and then stored  
366 at -80°C. Freeze/thaw cycles were avoided. Plaque-forming-units were determined by plating a  
367 series of 10-fold dilutions of each stock on replicate cultures of HFF for 2h at 37° C, and  
368 replacing the inoculum with a DMEM supplemented with 5% FBS, and 0.6% SeaPlaque  
369 agarose (to limit cell free spread). Plaques were counted by light microscopy 3 weeks after  
370 infection.

371       **Heterologous UL74(gO) recombinant HCMV.** A two step BAC recombineering  
372 process was performed as previously described (33). In the first step, endogenous UL74 ORF

373 from start codon to stop codon of both TR and ME was replaced by a selectable marker.  
374 Briefly, overnight cultures of SW102 *E. coli* containing either the BAC clone of TR or ME were  
375 grown at 32° C until OD600=0.55. Recombination genes were induced by incubating at 42° C  
376 for 15 mins. Purified PCR product containing the selectable marker cassette, KanR/LacZ/RpsL  
377 flanked by sequences homologous to 80 base pairs upstream and downstream of the TR or ME  
378 UL74 ORF, was electroporated into the bacteria, cultures were recovered for 1h at 32° C, and  
379 then selected on media containing kanamycin (15ug/ml), IPTG (50uM), X-gal (20ug/ml) and  
380 chloramphenicol (12.5ug/ml). First-step primer sequences were; TR: 5'-

381 CTTGGTGGACTATGCTTAACGCTCTCATTCTCATGGGAGCTTTTTGTATCGTATTACGACAT  
382 TGCTGTTTCCAGAACTCCTGTGACGGAAGATCACTTCG-3'; 5'-  
383 CGACCAGAATCAGCAGTGAGTACACGCAGGCAAGCCAAACCACAAGGCAGACGGACGGT  
384 GCGGGGTCTCCTCCTCTGTCCTGAGGTTCTTATGGCTCTTG-3'; ME: 5'-  
385 CCTGGTGGACTATGCTTAACGCTCTCATTCTGATGGGAGCTTTTTGTATCGTATTACGACAT  
386 TGCTGCTTCCAGAACTCCTGTGACGGAAGATCACTTCG-3'; 5'-  
387 CGACCAGAATCAGCAGTGAGTACACGCAGGCAAGCCAAACCACAAGGCAGACGGACGGT  
388 GCGGGGTCTCCTCCTCTGTACTGAGGTTCTTATGGCTCTTG-3'

389 In the second step, the selectable marker cassette in the TR and ME first-step  
390 intermediate BACs was replaced with the UL74(gO) sequence from the heterologous strain.  
391 Briefly, *E. coli* were prepared for recombination as described for step one above, and  
392 electroporated with purified PCR products containing the UL74 ORF from TR or ME strain  
393 flanked by sequence homologous to 80 base pairs upstream and downstream of the opposite  
394 strain. Transformed *E. coli* were selected for the removal of the KanR/LacZ/RpsL cassette by  
395 growth on media containing streptomycin (1.5mg/ml), IPTG (50uM), X-gal (20ug/ml) and  
396 chloramphenicol (12.5ug/ml). Primers used to generate the second-step PCR produce were,

397 TR                      UL74                      into                      ME:                      5'-  
398 GCCTGGTGGACTATGCTTAACGCTCTCATTCTGATGGGAGCTTTTTGTATCGTATTACGACA

399 TTGCTGCTTCCAGAACTTTACTGCAACCACCACCAAAG-3', and 5'-  
400 CGACCAGAATCAGCAGTGAGTACACGCAGGCAAACCAACCACAAGGCAGACGGACGGT  
401 GCGGGGTCTCCTCCTCTGTAATGGGGAGAAAAGGAGAGATG-3'. ME UL74 into TR: 5'-  
402 GGCTTGGTGGACTATGCTTAACGCTCTCATTCTCATGGGAGCTTTTTGTATCGTATTACGAC  
403 ATTGCTGTTTCCAGAACTTTACTGCGACCACCACCAAA-3', and 5'-  
404 CAGAATCAGCAGTGAGTACACGCAGGCAAGCCAAACCACAAGGCAGACGGACGGTGCGG  
405 GGTCTCCTCCTCTGTCATGGGGAAAAAAGAGATGATAATGG

406 The final heterologous UL74(gO) recombinants were verified by Sanger sequencing  
407 PCR products using the following primers; TRΔMEgO: 5'-  
408 GATGATTTTTACAAGGCACATTGTACATC-3', and 5'-AACTAGGTCGTCTTGAAGC-3',  
409 MEΔTRgO: 5'-CTCACAATGATTTTTACAATGCG-3', and 5'-AACTAGGTCGTCTTGAAGC-3'.

410 **Antibodies.** Rabbit polyclonal anti-peptide antibodies specific for TBgO and MEgO were  
411 described previously (30). Rabbit polyclonal antibodies specific for UL148 were described  
412 (32). Rabbit polyclonal, anti-peptide antibodies against gH, gL, UL130 and UL131 were  
413 provided by David Johnson (Oregon Health and Sciences University, Portland, OR, USA) (45).  
414 Anti-UL128 monoclonal antibodies (mAb) 4B10 were provided by Tom Shenk (Princeton  
415 University, Princeton, NJ, USA) (46). mAbs directly against major capsid protein (MCP) 28-4  
416 and gB 27-156 were provided by Bill Britt (47, 48). mAb (CH160) against CMV immediate early  
417 protein1 and immediate early protein2 (IE1/IE2) was purchased from Abcam (Cambridge, MA,  
418 USA).

419 **Immunoblot.** HCMV-infected cells or cell free virions were solubilized in 2% SDS/  
420 20mM Tris-buffered saline (TBS) (pH 6.8). Insoluble material was cleared by centrifugation at  
421 16,000 × g for 15 min and then extracts were boiled for 10 min. For endoglycosidase H (endo  
422 H) or peptide N-glycosidase F (PNGase F) treatment assay, proteins were extracted in 1%  
423 Triton X-100 (TX100), 0.5% sodium deoxycholate (DOC) in 20 mM Tris (pH 6.8), 100 mM NaCl

424 (TBS-TX/DOC). Extracts were clarified by centrifugation 16,000 × g for 15 min and treated with  
425 with endo H or PNGase F according to manufacturer's instructions (New England BioLabs). For  
426 reducing blots, dithiothreitol (DTT) were added to extracts to a final concentration of 25 mM.  
427 After separation by SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF)  
428 membranes (Whatman) in a buffer containing 10 mM NaHCO<sub>3</sub>, 3 mM Na<sub>2</sub>CO<sub>3</sub> (pH 9.9) plus  
429 10% methanol. Transferred proteins were probed with mAbs or rabbit polyclonal antibodies,  
430 anti-rabbit or anti-mouse secondary antibodies conjugated with horseradish peroxidase (Sigma-  
431 Aldrich), and Pierce ECL-Western Blotting Substrate (ThermoFisher Scientific).  
432 Chemiluminescence was detected using a Bio-Rad ChemiDoc MP imaging system.

433 **Radiolabeling proteins.** Cell cultures were incubated in labeling medium (met/cys-free  
434 DMEM + 2% dialyzed FBS) lacking methionine and cysteine) for 2 h at 37 °C, then [<sup>35</sup>S]  
435 methionine-cysteine was added to 1 mCi/ml (EasyTag Express 35S Protein Labeling Mix;  
436 Perkin Elmer). For chase experiments, label medium was removed and cultures were washed  
437 twice in DMEM + 2% FBS supplemented with a 10-fold excess of nonradioactive methionine  
438 and cysteine, then incubated in this medium for the indicated time.

439 **Immunoprecipitation.** Cell extracts were harvested in TBS-TX/DOC supplemented  
440 with 0.5% bovine serum albumin (BSA) and 1mM phenylmethylsulfonyl fluoride (PMSF),  
441 clarified by centrifugation at 16,000 × g for 15 min, adjusted to 2% SDS, 30 mM DTT and  
442 heated at 75 °C for 15 min. The extracts were then diluted 35-fold with TBS-TX/DOC  
443 supplemented with 0.5% BSA, and 10 mM iodoacetamide, incubated on ice for 15 min and pre-  
444 cleared with protein A-agarose beads (Invitrogen/ThermoFisher Scientific) for at 4 °C for 2 h.  
445 Immunoprecipitation reactions were set up with specific antibodies and protein A-agarose beads  
446 and incubated overnight at 4°C. Protein A-agarose beads were washed 3 times with TBS-  
447 TX/DOC and proteins were eluted with 2% SDS, 30 mM DTT in TBS at RT for 15 min, followed  
448 by 75 °C for 10 min. Eluted proteins were separated by SDS-PAGE and analyzed with a

449 Typhoon FLA-9500 imager (GE Healthcare Life Sciences). Band densities were determined  
450 using ImageJ version 1.48 software.

451 **Quantitative PCR.** Viral genomes were determined as described (25). Briefly, Cell-  
452 free HCMV stocks were treated with DNase I before extraction of viral genomic DNA (PureLink  
453 Viral RNA/DNA minikit; Life Technologies/ThermoFisher Scientific). Primers specific for  
454 sequences within UL83 were used with the MyiQ real-time PCR detection system (Bio-Rad).

455 **Superinfection of HCMV-infected cells with replication-defective adenovirus**  
456 **vectors.** Construction of Ad vectors expressing MEgO or GFP was described (30). At 2 days  
457 post HCMV infection, cells were superinfected with 20 PFU/cell of AdMEgO or AdGFP. 6 days  
458 later, cell-free HCMV was collected from the supernatant culture by centrifugation, and cells  
459 were harvested for immunoblot.

460

461 **ACKNOWLEDGMENTS**

462 We are grateful to Bill Britt, David Johnson, Jay Nelson, and Tom Shenk, for generously  
463 supplying HCMV BAC clones, antibodies, and cell lines as indicated in the Material and  
464 Methods, and members of the Ryckman laboratory for support, and insightful discussions.

465 This work was supported by grant from the National Institutes of Health to B.J.R  
466 (R01AI097274).

467 Experiments were designed by B.J.R., LZ, and M.Z., and performed by L.Z., and M.Z.  
468 Data were analyzed, and manuscript was prepared by B.J.R., L.Z., J.P.K. and, R.J.S.

469

470 **FIGURE LEGENDS**

471 **Figure 1. Comparison of protein expression between TR and ME.** nHDF were infected with  
472 1 PFU/cell of TR or ME. At day 2 (A) or day 5 (B) total cell extracts were separated by reducing  
473 SDS-PAGE and analyzed by immunoblot probing for immediate early (IE)-1/2, major capsid  
474 protein (MCP), gB, gH, gL, UL128, UL130, UL131, or UL148. Arrowheads indicate the positions  
475 of the cleaved 100kDa and 55 kDa fragments of gB.

476

477 **Figure 2. Analysis of ER-to-transGolgi compartment trafficking of glycoproteins in TR or**  
478 **ME infected cells.** Extracts of nHDF infected with TR or ME, or HFFFtet cells infected with ME  
479 were treated with endoglycosidase H (H), PNGaseF (F) or left untreated (U), and then  
480 separated by reducing SDS-PAGE and analyzed by immunoblot probing for gH or gL.  
481 Arrowheads indicate the position of the faster migrating, deglycosylated species.

482

483 **Figure 3. Specific infectivity of parental and TR-ME heterologous gO recombinants.**

484 Extracellular virions of TR, TR\_MEgO, ME, ME\_TRgO, ME-T, or ME-T\_TRgO were analyzed by  
485 quantitative PCR for viral genomes and PFU were determined by plaque assay on nHDF.  
486 Average particle-PFU ratios from at least 4 independent experiments are plotted. Error bars



487 represent standard deviation.

488

489 **Figure 4. Immunoblot analysis of gH/gL complexes in parental and TR-ME heterologous**

490 **gO recombinants.** Extracellular virion extracts of TR, TR\_MEgO, ME, ME\_TRgO, ME-T, or

491 ME-T\_TRgO were separated by reducing (A and B) or non-reducing (C) SDS-PAGE and

492 analyzed by immunoblot probing for major capsid protein (A) or gL (B and C)

493

494 **Figure 5. Immunoblot analysis of gO expression in cells infected with parental and TR-**

495 **ME heterologous gO recombinants.** nHDF were infected with 1 PFU/cell of TR, TR\_MEgO,

496 ME, or ME\_TRgO. At day 5, total cell extracts of infected cells were separated by reducing

497 SDS-PAGE and analyzed by immunoblot probing for TRgO, MEgO, major capsid protein

498 (MCP), or actin.

499

500 **Figure 6. Quantitative comparison of glycoprotein expression in TR and ME infected**

501 **cells.** nHDF were infected with 1 PFU/cell of TR or ME (A), or TR\_MEgO or ME\_TRgO (B). At

502 5 dpi, infected cells were metabolically labeled with [35]-S cysteine/methionine for 15 min and

503 membrane proteins were extracted in 1% Triton X-100. All samples were adjusted to

504 2%SDS/30mM DTT, heated to 75° C for 10 min, cooled to room temperature and then diluted

505 35-fold. Parallel immunoprecipitations were performed in which equal amounts of anti-gH, gL,

506 or gO (TR or ME specific) antibodies were reacted with 3-fold increasing amounts of protein

507 extract as input, and precipitated proteins were analyzed by SDS-PAGE.

508

509 **Figure 7. Analysis of glycoprotein turnover in TR and ME infected cells.** nHDF were

510 infected with 1 PFU/cell of TR or ME. At 5 dpi, infected cells were metabolically labeled with

511 [35]-S cysteine/methionine for 15 minutes and then label was chased for 0, 10, 60, 180, or 360

512 minutes. Membrane proteins were extracted in 1% Triton X-100, adjusted to 2%SDS/30mM

513 DTT, heated to 75° C for 10 min, cooled to room temperature and then diluted 35-fold.  
514 Immunoprecipitation was performed with anti-gH, gO (TR or ME specific) antibodies and  
515 precipitated proteins were analyzed by SDS-PAGE. Band densities were determined relative to  
516 the 0 minute chase time. Results shown are representative of 4 independent experiments.

517

518 **Figure 8. Ad vector overexpression of gO during ME replication.** nHDF or HFFF-tet cells  
519 were infected with ME for 2 days, then superinfected with Ad vectors expressing either GFP or  
520 MEgO for an additional 4 days. Extracts of infected cells (A), or extracellular virions (B) were  
521 separated by reducing (A, and B; top) or non-reducing (B; bottom) SDS-PAGE and analyzed by  
522 immunoblot probing for MEgO, actin, major capsid protein (MCP), or gL as indicated to the right.

523

524 **Figure 9. Specific infectivity of ME virions produced under conditions of gO**  
525 **overexpression.** nHDF or HFFF-tet cells were infected with ME for 2 days, then superinfected  
526 with Ad vectors expressing either GFP or MEgO for an additional 4 days. Extracellular virions  
527 from nHDF (ME) or HFFFtet (ME-T) were analyzed by quantitative PCR for viral genomes, and  
528 PFUs determined by plaque assay on nHDF. Shown are average particle-PFU ratios of virions  
529 produced in 2 independent experiments, each analyzed in triplicate. Error bars represent the  
530 standard deviation. Asterisks (\*) above fold differences indicate  $P < 0.03$  (Student's unpaired  
531 T-test (2-tailed)).

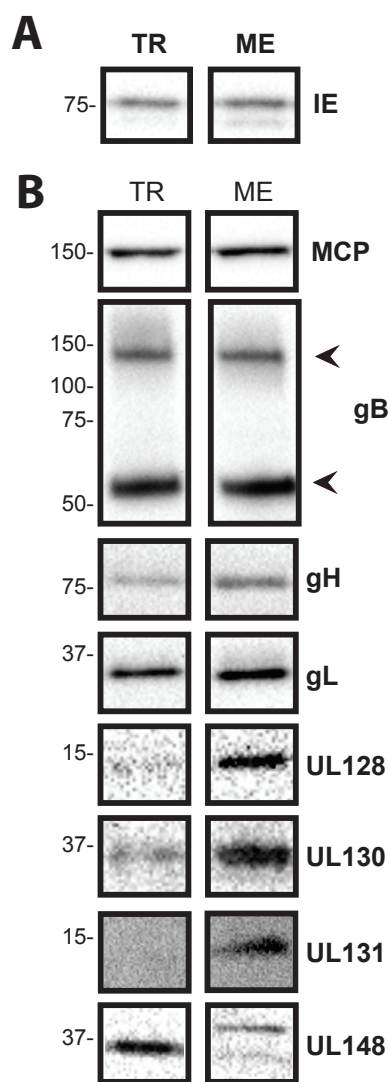
## 532 REFERENCES

- 533  
534 1. **Boppana SB, Fowler KB, Pass RF, Rivera LB, Bradford RD, Lakeman FD, Britt WJ.**  
535 2005. Congenital cytomegalovirus infection: association between virus burden in infancy  
536 and hearing loss. *J Pediatr* **146**:817–823.
- 537 2. **Britt WJ.** 2008. Manifestations of human cytomegalovirus infection: proposed  
538 mechanisms of acute and chronic disease., p. In T.E. S, M.F. S (ed), *Human*  
539 *Cytomegalovirus. Current Topics in Microbiology and Immunology* 325,
- 540 3. **Griffiths P, Baraniak I, Reeves M.** 2015. The pathogenesis of human cytomegalovirus. *J*  
541 *Pathol* **235**:288–297.
- 542 4. **Streblow DN, Orloff SL, Nelson JA.** 2007. Acceleration of allograft failure by  
543 cytomegalovirus. *Curr Opin Immunol* **19**:577–582.
- 544 5. **Cannon MJ, Hyde TB, Schmid DS.** 2011. Review of cytomegalovirus shedding in bodily  
545 fluids and relevance to congenital cytomegalovirus infection. *Rev Med Virol* **21**:240–255.
- 546 6. **Plachter B, Sinzger C, Jahn G.** 1996. Cell types involved in replication and distribution of  
547 human cytomegalovirus. *Adv Virus Res* **46**:195–261.
- 548 7. **Sinzger C, Grefte A, Plachter B, Gouw AS, The TH, Jahn G.** 1995. Fibroblasts,  
549 epithelial cells, endothelial cells and smooth muscle cells are major targets of human  
550 cytomegalovirus infection in lung and gastrointestinal tissues. *J Gen Virol* **76**:741–750.
- 551 8. **Sinzger C, Jahn G.** 1996. Human cytomegalovirus cell tropism and pathogenesis.  
552 *Intervirology* **39**:302–319.
- 553 9. **Sinzger C, Digel M, Jahn G.** 2008. Cytomegalovirus cell tropism. *Curr Top Microbiol*  
554 *Immunol* **325**:63–83.
- 555 10. **Xu S, Schafer X, Munger J.** 2016. Expression of Oncogenic Alleles Induces Multiple  
556 Blocks to Human Cytomegalovirus Infection. *J Virol* **90**:4346–4356.
- 557 11. **Smith JD.** 1986. Human cytomegalovirus: demonstration of permissive epithelial cells and  
558 nonpermissive fibroblastic cells in a survey of human cell lines. *J Virol* **60**:583–588.
- 559 12. **Connolly SA, Jackson JO, Jardetzky TS, Longnecker R.** 2011. Fusing structure and  
560 function: a structural view of the herpesvirus entry machinery. *Nat Rev Microbiol* **9**:369–  
561 381.
- 562 13. **Campadelli-Fiume G, Collins-McMillen D, Gianni T, Yurochko AD.** 2016. Integrins as  
563 Herpesvirus Receptors and Mediators of the Host Signalingosome. *Annu Rev Virol* **3**:215–  
564 236.
- 565 14. **Heldwein EE.** 2016. gH/gL supercomplexes at early stages of herpesvirus entry. *Curr*  
566 *Opin Virol* **18**:1–8.
- 567 15. **Ciferri C, Chandramouli S, Donnarumma D, Nikitin PA, Cianfrocco MA, Gerrein R,**  
568 **Feire AL, Barnett SW, Lilja AE, Rappuoli R, Norais N, Settembre EC, Carfi A.** 2015.  
569 Structural and biochemical studies of HCMV gH/gL/gO and Pentamer reveal mutually  
570 exclusive cell entry complexes. *Proc Natl Acad Sci U S A* **112**:1767–1772.
- 571 16. **Stegmann, Abdellatif, Sampaio L, Walther, Sinzger.** 2017. **Importance of Highly**  
572 **Conserved Peptide Sites of Human Cytomegalovirus gO for Formation of the**  
573 **gH/gL/gO Complex.** *J Virol* **91**
- 574 17. **Hahn G, Revello MG, Patrone M, Percivalle E, Campanini G, Sarasini A, Wagner M,**  
575 **Gallina A, Milanesi G, Koszinowski U, Baldanti F, Gerna G.** 2004. Human  
576 cytomegalovirus UL131-128 genes are indispensable for virus growth in endothelial cells  
577 and virus transfer to leukocytes. *J Virol* **78**:10023–10033.
- 578 18. **Ryckman BJ, Jarvis MA, Drummond DD, Nelson JA, Johnson DC.** 2006. Human  
579 cytomegalovirus entry into epithelial and endothelial cells depends on genes UL128 to  
580 UL150 and occurs by endocytosis and low-pH fusion. *J Virol* **80**:710–722.
- 581 19. **Wang D, Shenk T.** 2005. Human cytomegalovirus UL131 open reading frame is required

- 582 for epithelial cell tropism. *J Virol* **79**:10330–10338.
- 583 20. **Luo MH, Schwartz PH, Fortunato EA.** 2008. Neonatal neural progenitor cells and their  
584 neuronal and glial cell derivatives are fully permissive for human cytomegalovirus  
585 infection. *J Virol* **82**:9994–10007.
- 586 21. **Gerna G, Percivalle E, Lilleri D, Lozza L, Fornara C, Hahn G, Baldanti F, Revello MG.**  
587 2005. Dendritic-cell infection by human cytomegalovirus is restricted to strains carrying  
588 functional UL131-128 genes and mediates efficient viral antigen presentation to CD8+ T  
589 cells. *J Gen Virol* **86**:275–284.
- 590 22. **Wille PT, Knoche AJ, Nelson JA, Jarvis MA, Johnson DC.** 2010. A human  
591 cytomegalovirus gO-null mutant fails to incorporate gH/gL into the virion envelope and is  
592 unable to enter fibroblasts and epithelial and endothelial cells. *J Virol* **84**:2585–2596.
- 593 23. **Jiang XJ, Adler B, Sampaio KL, Digel M, Jahn G, Ettischer N, Stierhof YD, Scrivano**  
594 **L, Koszinowski U, Mach M, Sinzger C.** 2008. UL74 of human cytomegalovirus  
595 contributes to virus release by promoting secondary envelopment of virions. *J Virol*  
596 **82**:2802–2812.
- 597 24. **Laib Sampaio K, Stegmann C, Brizic I, Adler B, Stanton RJ, Sinzger C.** 2016. The  
598 contribution of pUL74 to growth of human cytomegalovirus is masked in the presence of  
599 RL13 and UL128 expression. *J Gen Virol* **97**:1917–1927.
- 600 25. **Zhou M, Lanchy JM, Ryckman BJ.** 2015. Human cytomegalovirus gH/gL/gO promotes  
601 the fusion step of entry into all cell types whereas gH/gL/UL128-131 broadens virus  
602 tropism through a distinct mechanism. *J Virol*
- 603 26. **Kabanova A, Marcandalli J, Zhou T, Bianchi S, Baxa U, Tsybovsky Y, Lilleri D,**  
604 **Silacci-Fregni C, Foglierini M, Fernandez-Rodriguez BM, Druz A, Zhang B, Geiger R,**  
605 **Pagani M, Sallusto F, Kwong PD, Corti D, Lanzavecchia A, Perez L.** 2016. Platelet-  
606 derived growth factor- $\alpha$  receptor is the cellular receptor for human cytomegalovirus  
607 gHgLG $\alpha$  trimer. *Nat Microbiol* **2016**
- 608 27. **Stegmann C, Hochdorfer D, Lieber D, Subramanian N, Stöhr D, Laib Sampaio K,**  
609 **Sinzger C.** 2017. A derivative of platelet-derived growth factor receptor  $\alpha$  binds to the  
610 trimer of human cytomegalovirus and inhibits entry into fibroblasts and endothelial cells.  
611 *PLoS Pathog* **13**:e1006273.
- 612 28. **Wu Y, Prager A, Boos S, Resch M, Brizic I, Mach M, Wildner S, Scrivano L, Adler B.**  
613 2017. Human cytomegalovirus glycoprotein complex gH/gL/gO uses PDGFR- $\alpha$  as a key  
614 for entry. *PLoS Pathog* **13**:e1006281.
- 615 29. **Nogalski MT, Chan GC, Stevenson EV, Collins-McMillen DK, Yurochko AD.** 2013. The  
616 HCMV gH/gL/UL128-131 Complex Triggers the Specific Cellular Activation Required for  
617 Efficient Viral Internalization into Target Monocytes. *PLoS Pathog* **9**:e1003463.
- 618 30. **Zhou M, Yu Q, Wechsler A, Ryckman BJ.** 2013. Comparative analysis of gO isoforms  
619 reveals that strains of human cytomegalovirus differ in the ratio of gH/gL/gO and  
620 gH/gL/UL128-131 in the virion envelope. *J Virol* **87**:9680–9690.
- 621 31. **Rasmussen L, Geissler A, Cowan C, Chase A, Winters M.** 2002. The genes encoding  
622 the gCIII complex of human cytomegalovirus exist in highly diverse combinations in clinical  
623 isolates. *J Virol* **76**:10841–10848.
- 624 32. **Li G, Nguyen CC, Ryckman BJ, Britt WJ, Kamil JP.** 2015. A viral regulator of  
625 glycoprotein complexes contributes to human cytomegalovirus cell tropism. *Proc Natl*  
626 *Acad Sci U S A* **112**:4471–4476.
- 627 33. **Stanton RJ, Baluchova K, Dargan DJ, Cunningham C, Sheehy O, Seirafian S,**  
628 **McSharry BP, Neale ML, Davies JA, Tomasec P, Davison AJ, Wilkinson GW.** 2010.  
629 Reconstruction of the complete human cytomegalovirus genome in a BAC reveals RL13 to  
630 be a potent inhibitor of replication. *J Clin Invest* **120**:3191–3208.
- 631 34. **Wiertz EJ, Jones TR, Sun L, Bogyo M, Geuze HJ, Ploegh HL.** 1996. The human  
632 cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the

- 633 endoplasmic reticulum to the cytosol. *Cell* **84**:769–779.
- 634 35. **Renzette N, Bhattacharjee B, Jensen JD, Gibson L, Kowalik TF.** 2011. Extensive
- 635 genome-wide variability of human cytomegalovirus in congenitally infected infants. *PLoS*
- 636 *Pathog* **7**:e1001344.
- 637 36. **Renzette N, Gibson L, Bhattacharjee B, Fisher D, Schleiss MR, Jensen JD, Kowalik**
- 638 **TF.** 2013. Rapid Intrahost Evolution of Human Cytomegalovirus Is Shaped by
- 639 Demography and Positive Selection. *PLoS Genet* **9**:e1003735.
- 640 37. **Sijmons S, Thys K, Mbong Ngwese M, Van Damme E, Dvorak J, Van Loock M, Li G,**
- 641 **Tachezy R, Busson L, Aerssens J, Van Ranst M, Maes P.** 2015. High-throughput
- 642 analysis of human cytomegalovirus genome diversity highlights the widespread
- 643 occurrence of gene-disrupting mutations and pervasive recombination. *J Virol*
- 644 38. **Dargan DJ, Douglas E, Cunningham C, Jamieson F, Stanton RJ, Baluchova K,**
- 645 **McSharry BP, Tomasec P, Emery VC, Percivalle E, Sarasini A, Gerna G, Wilkinson**
- 646 **GW, Davison AJ.** 2010. Sequential mutations associated with adaptation of human
- 647 cytomegalovirus to growth in cell culture. *J Gen Virol* **91**:1535–1546.
- 648 39. **Murrell I, Wilkie GS, Davison AJ, Statkute E, Fielding CA, Tomasec P, Wilkinson GW,**
- 649 **Stanton RJ.** 2016. Genetic Stability of Bacterial Artificial Chromosome-Derived Human
- 650 Cytomegalovirus during Culture In Vitro. *J Virol* **90**:3929–3943.
- 651 40. **Murrell I, Tomasec P, Wilkie GS, Dargan DJ, Davison AJ, Stanton RJ.** 2013. Impact of
- 652 sequence variation in the UL128 locus on production of human cytomegalovirus in
- 653 fibroblast and epithelial cells. *J Virol* **87**:10489–10500.
- 654 41. **Balázs Z, Tombácz D, Szűcs A, Csabai Z, Megyeri K, Petrov AN, Snyder M,**
- 655 **Boldogkői Z.** 2017. Long-Read Sequencing of Human Cytomegalovirus Transcriptome
- 656 Reveals RNA Isoforms Carrying Distinct Coding Potentials. *Sci Rep* **7**:15989.
- 657 42. **Gatherer D, Seirafian S, Cunningham C, Holton M, Dargan DJ, Baluchova K, Hector**
- 658 **RD, Galbraith J, Herzyk P, Wilkinson GW, Davison AJ.** 2011. High-resolution human
- 659 cytomegalovirus transcriptome. *Proc Natl Acad Sci U S A* **108**:19755–19760.
- 660 43. **Kalser J, Adler B, Mach M, Kropff B, Puchhammer-Stöckl E, Görzer I.** 2017.
- 661 Differences in Growth Properties among Two Human Cytomegalovirus Glycoprotein O
- 662 Genotypes. *Front Microbiol* **8**:1609.
- 663 44. **Murphy E, Yu D, Grimwood J, Schmutz J, Dickson M, Jarvis MA, Hahn G, Nelson JA,**
- 664 **Myers RM, Shenk TE.** 2003. Coding potential of laboratory and clinical strains of human
- 665 cytomegalovirus. *Proc Natl Acad Sci U S A* **100**:14976–14981.
- 666 45. **Ryckman BJ, Rainish BL, Chase MC, Borton JA, Nelson JA, Jarvis MA, Johnson DC.**
- 667 2008. Characterization of the human cytomegalovirus gH/gL/UL128-131 complex that
- 668 mediates entry into epithelial and endothelial cells. *J Virol* **82**:60–70.
- 669 46. **Wang D, Shenk T.** 2005. Human cytomegalovirus virion protein complex required for
- 670 epithelial and endothelial cell tropism. *Proc Natl Acad Sci U S A* **102**:18153–18158.
- 671 47. **Schoppel K, Hassfurth E, Britt W, Ohlin M, Borrebaeck CA, Mach M.** 1996.
- 672 Antibodies specific for the antigenic domain 1 of glycoprotein B (gpUL55) of human
- 673 cytomegalovirus bind to different substructures. *Virology* **216**:133–145.
- 674 48. **Chee M, Rudolph SA, Plachter B, Barrell B, Jahn G.** 1989. Identification of the major
- 675 capsid protein gene of human cytomegalovirus. *J Virol* **63**:1345–1353.
- 676

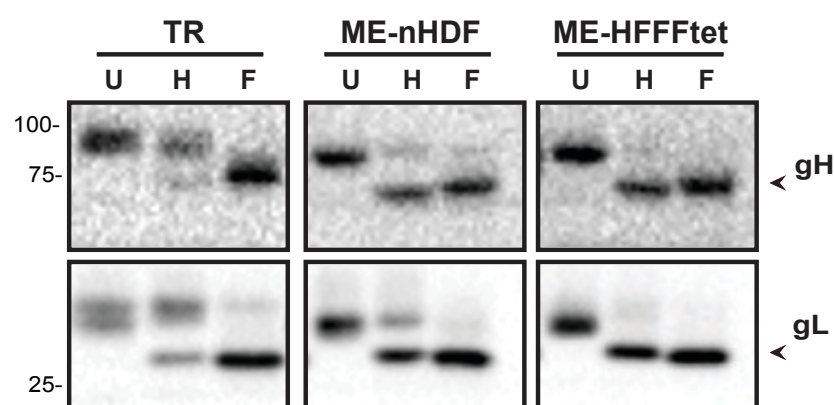
# Zhang et al., Figure 1



**Figure 1. Comparison of protein expression between TR and ME.** nHDF were infected with 1 PFU/cell of TR or ME. At day 2 (A) or day 5 (B) total cell extracts were separated by reducing SDS-PAGE and analyzed by immunoblot probing for immediate early (IE)-72, major capsid protein (MCP), gB, gH, gL, UL128, UL130, UL131, or UL148. Arrowheads indicate the positions of the cleaved 100kDa and 55 kDa fragments of gB.

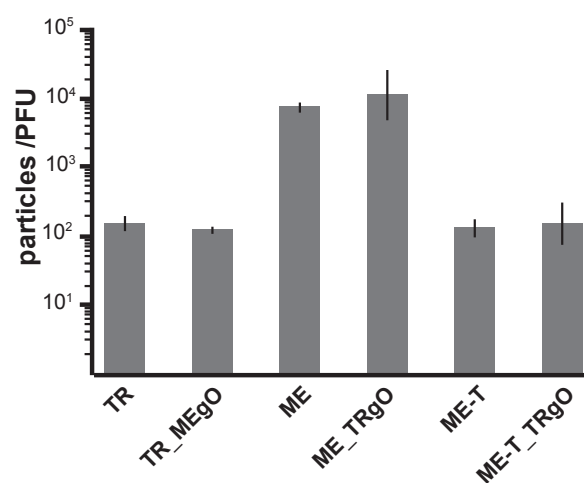


## Zhang et al., Figure 2



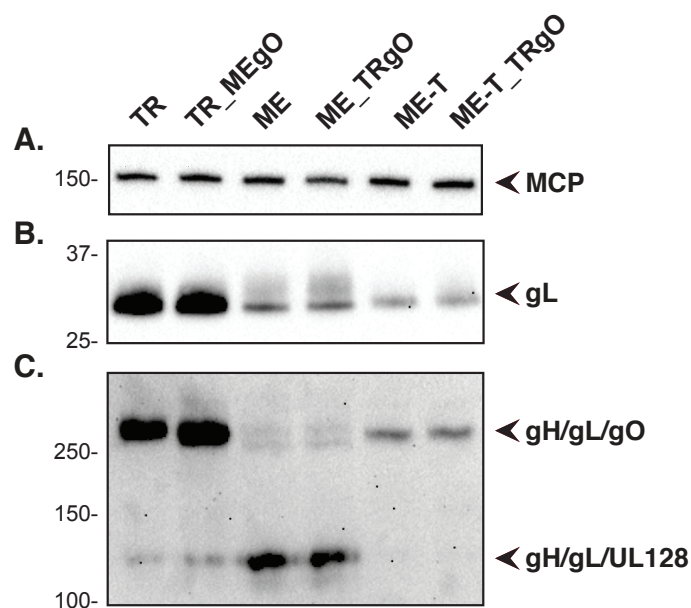
**Figure 2. Analysis of ER-to-*trans*Golgi compartment trafficking of glycoproteins in TR or ME infected cells.** Extracts of nHDF infected with TR or ME, or HFFFtet cells infected with ME were treated with endoglycosidase H (H), PNGaseF (F) or left untreated (U), and then separated by reducing SDS-PAGE and analyzed by immunoblot probing for gH or gL. Arrowheads indicate the position of the faster migrating, deglycosylated species.

## Zhang et al., Figure 3



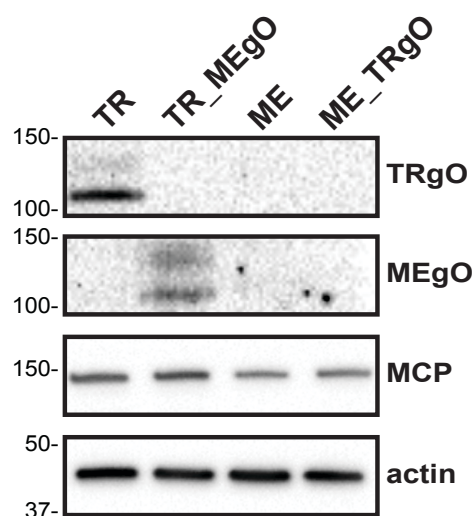
**Figure 3. Specific infectivity of parental and TR-ME heterologous gO recombinants.** Extracellular virions of TR, TR\_MEgO, ME, ME\_TRgO, ME-T, or ME-T\_TRgO were analyzed by quantitative PCR for viral genomes and PFU were determined by plaque assay on nHDF. Average particle-PFU ratios from at least 4 independent experiments are plotted. Error bars represent standard deviation.

## Zhang et al., Figure 4



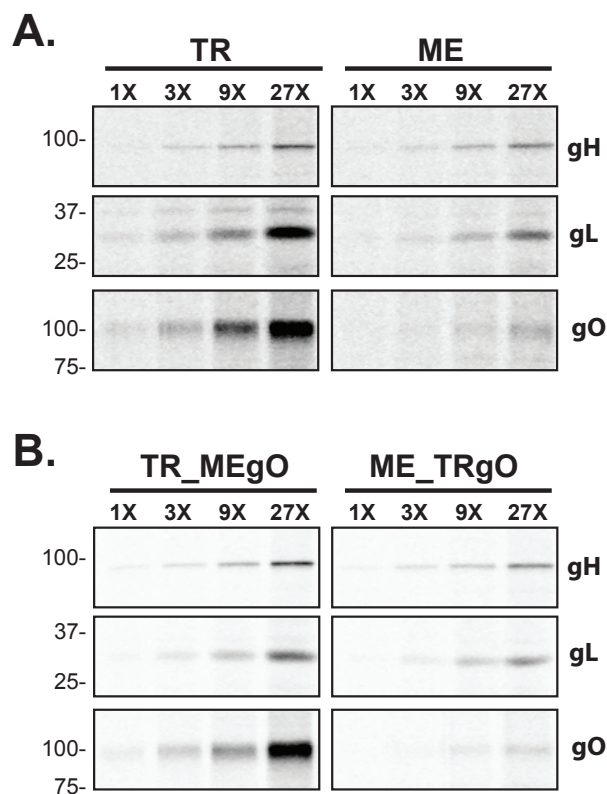
**Figure 4. Immunoblot analysis of gH/gL complexes in parental and TR-ME heterologous gO recombinants.** Extracellular virion extracts of TR, TR\_MEgO, ME, ME\_TRgO, ME-T, or ME-T\_TRgO were separated by reducing (A and B) or non-reducing (C) SDS-PAGE and analyzed by immunoblot probing for major capsid protein (A) or gL (B and C).

## Zhang et al., Figure 5



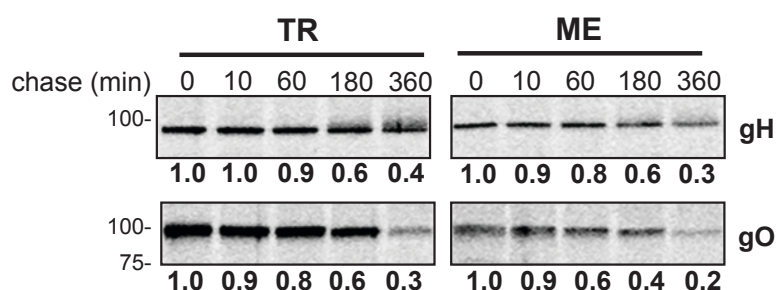
**Figure 5. Immunoblot analysis of gO expression in cells infected with parental and TR-ME heterologous gO recombinants.** nHDF were infected with 1 PFU/cell of TR, TR\_MEgO, ME, or ME\_TRgO. At day 5, total cell extracts of infected cells were separated by reducing SDS-PAGE and analyzed by immunoblot probing for TRgO, MEgO, major capsid protein (MCP), or actin.

## Zhang et al., Figure 6



**Figure 6. Quantitative comparison of glycoprotein expression in TR and ME infected cells.** nHDF were infected with 1 PFU/cell of TR or ME (A), or TR\_MEgO or ME\_TRgO (B). At 5 dpi, infected cells were metabolically labeled with [<sup>35</sup>S]-S cysteine/methionine for 15 min and membrane proteins were extracted in 1% Triton X-100. All samples were adjusted to 2%SDS/30mM DTT, heated to 75° C for 10 min, cooled to room temperature and then diluted 35-fold. Parallel immunoprecipitations were performed in which equal amounts of anti-gH, gL, or gO (TR or ME specific) antibodies were reacted with 3-fold increasing amounts of protein extract as input, and precipitated proteins were analyzed by reducing SDS-PAGE.

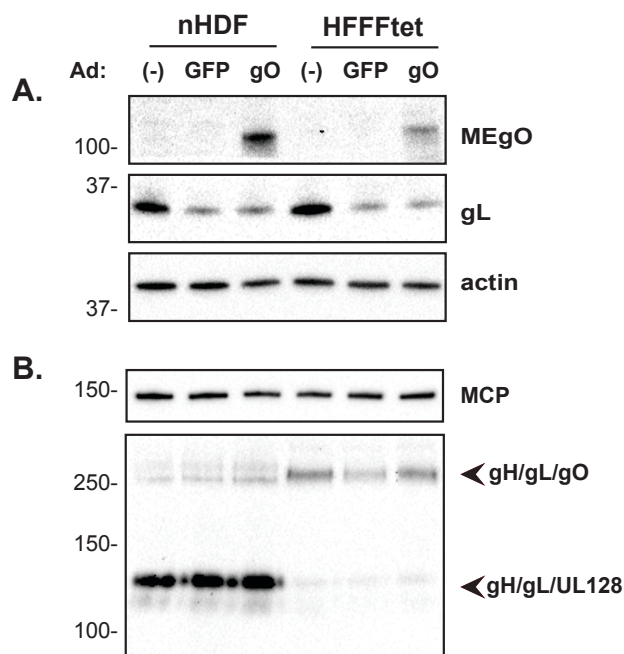
## Figure 7. Zhang et al



**Figure 7. Analysis of glycoprotein turnover in TR and ME infected cells.** nHDF were infected with 1 PFU/cell of TR or ME. At 5 dpi, infected cells were metabolically labeled with [35]-S cysteine/methionine for 15 minutes and then label was chased for 0, 10, 60, 180, or 360 minutes. Membrane proteins were extracted in 1% Triton X-100, adjusted to 2%SDS/30mM DTT, heated to 75° C for 10 min, cooled to room temperature and then diluted 35-fold. Immunoprecipitation was performed with anti-gH, gO (TR or ME specific) antibodies and precipitated proteins were analyzed by SDS-PAGE. Band densities were determined relative to the 0 minute chase time. Results shown are representative of 4 independent experiments.

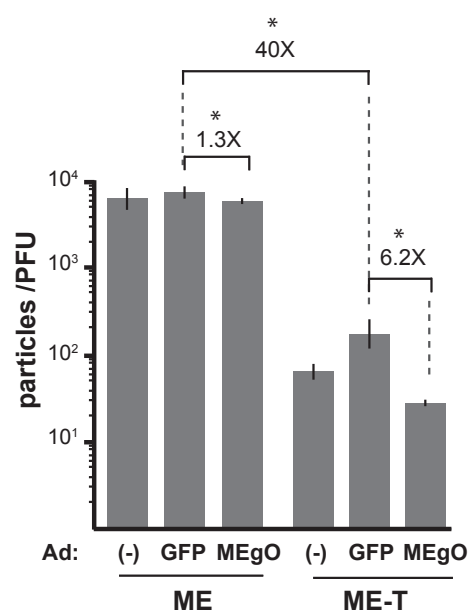


# Zhang et al., Figure 8



**Figure 8. Ad vector overexpression of gO during ME replication.** nHDF or HFFF-tet cells were infected with ME for 2 days, then superinfected with Ad vectors expressing either GFP or MEGO for an additional 4 days. Extracts of infected cells (A), or extracellular virions (B) were separated by reducing (A, and B; top) or non-reducing (B; bottom) SDS-PAGE and analyzed by immunoblot probing for MEGO, actin, major capsid protein (MCP), or gL as indicated to the right.

## Zhang et al., Figure 9



**Figure 9. Specific infectivity of ME virions produced under conditions of gO overexpression.** nHDF or HFFF-tet cells were infected with ME for 2 days, then superinfected with Ad vectors expressing either GFP or MEgO for an additional 4 days. Extracellular virions from nHDF (ME) or HFFFtet (ME-T) were analyzed by quantitative PCR for viral genomes, and PFUs determined by plaque assay on nHDF. Shown are average particle-PFU ratios of virions produced in 2 independent experiments, each analyzed in triplicate. Error bars represent the standard deviation. Asterisks (\*) above fold differences indicate  $P < 0.03$  (Student's unpaired T-test (2-tailed)).

**Table 1. Quantitative comparison of glycoprotein expression in TR and ME infected cells.**

IP Antibody <sup>a</sup>	Extract <sup>b</sup> (mL)	Strain		Fold <sup>f</sup>		Mean Fold <sup>g</sup>
		TR		ME		
		Density <sup>c</sup>	Adjusted Density <sup>e</sup>	Density	Adjusted Density	
anti-gH	0.04	n.d. <sup>d</sup>	n.d.	n.d.	n.d.	1.4(+/-0.1)
	0.13	136.7	4.6	106.6	3.4	
	0.40	476.9	15.9	337.8	10.9	
	1.20	1200.7	40.0	872.9	28.2	
anti-gL	0.04	143.8	11.1	n.d.	n.d.	4.0 (+/-1.2)
	0.13	679.1	52.2	127.2	9.8	
	0.40	1627.3	125.2	509.3	39.2	
	1.20	6071.3	467.0	1809.9	139.2	
anti-gO	0.04	267.0	12.1	n.d.	n.d.	27.2 (+/-9.4)
	0.13	1008.6	45.8	n.d.	n.d.	
	0.40	3805.4	173.0	120.8	5.0	
	1.20	9251.8	420.5	478.9	20.0	

a. 7uL of rabbit anti-peptide serum per immunoprecipitation reaction

b. Preparation of radiolabeled cell extracts described in legend to Figure 6, and in materials and methods.

c. Densities of bands shown in Figure 6A as determined using Image J v. 1.48

d. Band density not detected.

e. Density divided by the predicted number of methionine and cysteine residues: TRgH(30), MEgH(31), TRgL(13), MEgL(13), TRgO(22), MEgO(24)

f. Adjusted density TR divided by adjusted density ME.

g. Average fold difference between TR and ME +/- standard deviation.

**Table 2. Quantitative comparison of glycoprotein expression in TR\_MEgO and ME\_TRgO infected cells.**

IP Antibody <sup>a</sup>	Extract <sup>b</sup> (mL)	Strain				Fold <sup>f</sup>	Mean Fold <sup>g</sup>
		TR_MEgO		ME_TRgO			
		Density <sup>c</sup>	Adjusted Density <sup>e</sup>	Density	Adjusted Density		
anti-gH	0.04	68.4	2.3	29.5	1.0	2.4	1.6(+/-0.5)
	0.13	181.6	6.1	163.1	5.3	1.1	
	0.40	539.5	18.0	410.6	13.2	1.4	
	1.20	1697.7	56.6	1064.0	34.3	1.6	
anti-gL	0.04	n.d. <sup>d</sup>	n.d.	n.d.	n.d.	n.d.	1.5 (+/-0.4)
	0.13	196.6	15.1	153.8	11.8	1.3	
	0.40	645.0	49.6	508.3	39.1	1.3	
	1.20	2547.5	196.0	1269.6	97.7	2.0	
anti-gO	0.04	187.8	7.8	n.d.	n.d.	n.d.	19.7 (+/-1.7)
	0.13	945.8	39.4	n.d.	n.d.	n.d.	
	0.40	2580.3	107.5	127.8	5.8	18.5	
	1.20	10502.7	437.6	460.1	20.9	20.9	

a. 7uL of rabbit anti-peptide serum per immunoprecipitation reaction

b. Preparation of radiolabeled cell extracts described in legend to Figure 6, and in materials and methods.

c. Densities of bands shown in Figure 6B as determined using Image J v. 1.48

d. Band density not detected.

e. Density divided by the predicted number of methionine and cysteine residues: TRgH(30), MEgH(31), TRgL(13), MEgL(13), TRgO(22), MEgO(24)

f. Adjusted density TR\_MEgO divided by adjusted density ME\_TRgO.

g. Average fold difference between TR\_MEgO and ME\_TRgO +/- standard deviation.