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1 Structure-function dissection of the Pseudorabies virus glycoprotein B fusion
2 loops

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20

21 **ABSTRACT:**

22 Conserved across the *Herpesviridae* family, glycoprotein B (gB) is responsible for driving fusion
23 of the viral envelope with the host cell membrane for entry upon receptor binding and
24 activation by the viral gH/gL complex. Although crystal structures of the gB ectodomain of
25 several herpesviruses have been reported, the membrane fusion mechanism has remained
26 elusive. Here, we report the X-ray structure of the Pseudorabies virus (PrV) gB ectodomain,
27 revealing a typical class III post-fusion trimer that binds membranes via its fusion loops (FLs) in a
28 cholesterol-dependent manner. Mutagenesis of FL residues allowed us to dissect those
29 interacting with distinct sub-regions of the lipid bilayer and their role for membrane
30 interactions. We tested 15 gB variants for their ability to bind to liposomes, and further
31 investigated a subset of them in functional assays. We found that PrV gB FL residues *Trp187*,
32 *Tyr192*, *Phe275* and *Tyr276*, which were essential for liposome binding and for fusion in a
33 cellular and viral context, form a continuous hydrophobic patch at the gB trimer surface.
34 Together with reported results from other alpha-herpesvirus gBs, our data suggest a model in
35 which *Phe275* from the tip of FL2 protrudes deeper into the hydrocarbon core of the lipid
36 bilayer, while the side chains of *Trp187*, *Tyr192* and *Tyr276* form a rim that inserts into the
37 more superficial, interfacial region of the membrane to catalyze the fusion process.
38 Comparative analysis with gB from beta- and gamma-herpesviruses suggest that this
39 membrane-interaction mode is valid for gB from all herpesviruses.

40 **IMPORTANCE:**

41 Herpesviruses are common human and animal pathogens, which infect cells by entering via
42 fusion of viral and cellular membranes and which cause life-long and incurable infections.
43 Central to the membrane fusion event for entry is glycoprotein B (gB), which is the most
44 conserved envelope protein across the herpesvirus family. Like other viral fusion proteins, gB
45 anchors itself into the target membrane via two polypeptide segments called fusion loops (FL).
46 The molecular details of how gB FLs insert into the lipid bilayer have not been described. We
47 provide here structural and functional data regarding key FL residues of gB from *Pseudorabies*
48 *virus*, a porcine herpesvirus of veterinary concern, which allows us to propose, for the first time,
49 a molecular model to understand how the initial interactions by gB from all herpesviruses with
50 target membranes are established.

51 INTRODUCTION

52 The family *Herpesviridae* contains a large number of enveloped, double-stranded DNA
53 viruses, which are classified into alpha-, beta-, and gamma-herpesvirus subfamilies based on
54 their evolutionary relationship and biological properties (1). Pseudorabies virus (Suid
55 alphaherpesvirus 1), the etiological agent of Aujeszky's disease in swine (2), belongs to the
56 *Alphaherpesvirinae*, which also includes human pathogens such as herpes simplex virus 1 and 2
57 (HSV-1, HSV-2) and varicella zoster virus (VZV). PrV has been a useful model for studying the
58 biology of alphaherpesviruses in general (3).

59 Herpesviruses enter cells by fusion of their envelope with the host cell plasma
60 membrane or with the membrane of an endocytic vesicle, depending on the virus and the cell
61 type (4, 5). While many enveloped viruses require only one or two proteins to mediate receptor
62 binding and entry, herpesviruses rely on the concerted action of at least four glycoproteins.
63 Distinct viral proteins first engage specific cellular receptors in an interaction that provides a
64 trigger for membrane fusion (e.g. glycoprotein D (gD) in HSV-1/-2 and PrV, gO in the beta-
65 herpesvirus human cytomegalovirus (HCMV), gp42 in the gamma-herpesvirus Epstein-Barr virus
66 (EBV)) (6-9). The merger of the viral and cellular membranes is then catalyzed by a fusion
67 machinery, which is conserved across the herpesvirus family and consists of gB and a
68 heterodimeric complex of membrane-bound gH in association with the anchorless gL (gH/gL)
69 (reviewed in (10, 11)).

70 The molecular basis of the entry mechanism has been best described for
71 alphaherpesviruses. In HSV-1, the cascade begins with gD binding to cellular receptors such as
72 herpesvirus entry mediator (HVEM), nectin-1 or 3-O-sulfonated heparin sulfate (reviewed in

73 (12)). This interaction leads to a conformational change in gD (13, 14) that is believed to enable
74 its interaction with the gH/gL complex, which in turn triggers gB to carry out membrane fusion
75 (15, 16). It should be noted that unlike HSV-1, PrV does not require gD for direct viral cell-cell
76 spread and gB-induced cell:cell fusion (17-19). Moreover, PrV virions can acquire the ability to
77 infect cells in the absence of gD, gL or the N-terminal domain of gH, by compensatory
78 mutations in other envelope glycoproteins (20, 21).

79 gB was proposed to be the *bona fide* fusion protein of herpesviruses due to its structural
80 homology with the fusogenic G protein of the otherwise unrelated vesicular stomatitis virus
81 (VSV) (22). Together with VSV G and the baculovirus fusion protein gp64 (23), gB was classified
82 as a class III fusion protein. Viral fusion proteins are in general presented in a metastable pre-
83 fusion state on the viral membrane. Upon an activation signal they undergo conformational
84 rearrangements unmasking the initially buried hydrophobic regions (fusion peptide (FP) or
85 fusion loop(s) (FL)) for interactions with the target membrane, resulting in simultaneous
86 anchorage of the fusion protein in the viral and cellular membrane at opposite ends of the
87 protein. This extended intermediate is unstable and rapidly folds back into a “hairpin”, an
88 energetically more favorable post-fusion conformation (24, 25).

89 gB is the most conserved envelope glycoprotein of herpesviruses, although only ≈5% of
90 its residues are identical across the entire herpesvirus family. The level of conservation is higher
91 within each subfamily, and PrV and HSV-1 gB share 50% amino acid sequence identity. The X-
92 ray structures determined for the post-fusion gB ectodomains from HSV-1 (26), EBV (27) and
93 HCMV (28, 29) demonstrate conservation of the three-dimensional structural organization.
94 Although the crystal structure of gB in a pre-fusion state has not been determined yet, a low-

95 resolution structure of full-length HSV-1 gB in an intermediate state different from the post-
96 fusion conformation was obtained by cryo-electron tomography (30). VSV G protein has been
97 crystallized in both pre- and post-fusion conformations. The former revealed a trimeric
98 molecule with the FLs pointing towards the viral membrane, and with a different spatial
99 organization of domains compared to the post-fusion, low pH form (31). Hypothetical models of
100 the pre-fusion gB using the VSV G conformation as a template have been proposed (27, 32), but
101 supporting structural data are still lacking. The high structural conservation observed for gB also
102 applies to the gH/gL complex as illustrated by the X-ray structures of the gH/gL ectodomains
103 from HSV-2, EBV, VZV, and a core domain of PrV gH (33-36), which reveal a common fold
104 despite low sequence conservation (37). gH/gL has no structural resemblance to any known
105 fusion protein but was shown to play a role in regulation of the fusion activity of gB (37, 38).

106 Membrane-interacting regions of fusion proteins are typically well conserved within the
107 virus family, and are rich in hydrophobic and aromatic residues that insert into the outer leaflet
108 of a cell membrane (39). In class I fusion proteins, they are called FPs, which can be N-terminal
109 peptides or an internal loop near the N-terminus, as in the Ebola envelope glycoprotein GP2 for
110 example (40) (to avoid confusion, we reserve the term FL for the loops of class II and III fusion
111 proteins, and we refer to all class I membrane interacting regions as fusion peptides, even when
112 they correspond to an internal loop). FPs were shown to adopt a different structure upon
113 insertion into target membranes (reviewed in (41)). The FLs of class II and class III fusion
114 proteins, in contrast, are conformationally constrained by being a part of a larger structured β -
115 barrel domain, and appear to largely maintain their structure upon membrane insertion. In
116 class II fusion proteins, the membrane interacting surface can be composed of residues from

117 one (flaviviruses and alphaviruses (42, 43)), two (rubella virus, pheloboviruses (44, 45)) or three
118 FLs (hantaviruses (46)), while the known class III fusion proteins use two FLs to interact with
119 membranes. FLs of herpesviruses are unusual because their amino acid sequence is poorly
120 conserved even within subfamilies. Therefore, their identification was impossible until
121 structural data became available. Residues exposed at the tips of the recombinant crystallized
122 HSV-1 gB ectodomain (26) were proposed to form the FLs based on structural homology with
123 the well-defined FLs of the VSV G protein (47, 48). A similar organization of the FLs was
124 observed in the reported gB structures, with the difference that the aromatic and apolar
125 residues are predominantly presented at the sides of the HSV-1 FLs, while they protrude from
126 the tips of the EBV and HCMV FLs. These exposed hydrophobic residues in EBV and HCMV gB
127 caused the recombinant ectodomains to aggregate, forming rosette-like structures (49, 50).
128 Mutagenesis of the hydrophobic residues within the FL, while abolishing their fusion function,
129 was essential for solubility of these proteins for structure determination (50, 51). In contrast,
130 HSV-1 gB (52), and PrV gB (this study) exhibit less hydrophobic tips, form soluble trimers, and
131 can be studied and crystallized as functional, wild type proteins.

132 Extensive mutagenesis studies have been performed on HSV-1 gB, demonstrating that
133 the hydrophobic residues *Trp174*, *Phe175*, *Tyr179* in FL1, and *Ala261* in FL2 as well as polar and
134 charged residues, such as *His263* and *Arg264* presented at the sides of FL2, play an important
135 role in fusion (52-55). The hydrophobic residues were proposed to form a patch that inserts
136 into the membrane, while the *His* and *Arg* side chains were speculated to interact with
137 phospholipid head groups without penetrating deeper into the membrane. In support of this
138 model, the low-resolution structure of HSV-1 gB ectodomains bound to liposomes and obtained

139 by cryo-electron tomography, showed that the interactions of the HSV-1 gB ectodomains are
140 limited to the outer membrane leaflet. This model predicted that the FLs would insert into the
141 bilayer at an oblique angle indicating that the residues from β -strands leading to the FLs may be
142 involved in the interactions with lipids (56). HSV-1 gB FL residues *Trp174*, *Tyr179*, *His263* and
143 *Arg264* were furthermore shown to be the contact sites for interactions of the ectodomain with
144 liposomes (55).

145 Cholesterol (CH) is a lipid that is ubiquitously present in mammalian cell membranes,
146 where it plays an important role in entry of many viruses (57), including PrV and HSV-1
147 (reviewed in (58-60)). The HSV-1 gB ectodomain was shown to bind to liposomes only in the
148 presence of cholesterol (55), and full-length HSV-1 gB expressed in cells was found to associate
149 with lipid rafts (61).

150 While the current HSV-1 model has provided an important step forward in
151 understanding how gB binds to membranes, it has been unclear whether a similar model of
152 insertion would hold true for other gB proteins due to the poor conservation of the FLs,
153 highlighting the need to study each gB individually and to perform comparative analyses. With
154 this in mind, we sought to enlarge the structural and functional repertoire available for
155 alphaherpesviruses by carrying out studies on PrV gB. We report here the X-ray crystal
156 structure of the PrV gB ectodomain at 2.7Å resolution, and demonstrate that the recombinant
157 ectodomains bind to liposomes in a cholesterol-dependent fashion. The association with
158 liposomes occurs via the trimer tip that contains the FLs, resembling the shallow insertion
159 observed for HSV-1 gB. We designed the PrV gB mutagenesis to complement what had been
160 already reported for HSV-1 gB, and to target all aromatic and hydrophobic residues within the

161 FLs, some of which are unique to PrV and some that are conserved in HSV-1 gB as well. The
162 resulting pattern of active and inactive FL mutants matches in part what had been observed
163 previously for HSV-1 gB, but with some interesting differences that shine a new light on the way
164 the FLs insert into membranes. Based on our results, we propose a model in which the tip of
165 FL2 penetrates deeper into the lipid bilayer reaching into the hydrophobic core, while residues
166 on the sides of the FL1 and FL2 tips position their side chains within the same plane, inserting
167 into the amphipathic, interfacial region of the membrane. Comparative analysis with the FLs of
168 HCMV and EBV gB further suggests that in spite of considerable sequence divergence, this
169 model could be used to describe in general the way gB interacts with membranes for all herpes
170 viruses.

171

172 **RESULTS**

173 **PrV gB expression, crystallization and structure determination.**

174 The ectodomain expression construct of PrV gB (Suid alphaherpesvirus 1, Kaplan strain,
175 GenBank number AEM64049.1) encodes for residues 59-756 (Fig. 1A). The construct was
176 designed to exclude the gB signal sequence (residues 1-58), which was replaced by the
177 *Drosophila* Bip signal peptide, present in the expression vector and known to drive efficient
178 expression of heterologous proteins (62). The expression construct ends with the double strep
179 affinity tag added just before the hydrophobic membrane proximal region (MPR) (residues 757
180 – 800), which is followed by the transmembrane region (residues 801 - 821) and the
181 cytoplasmic domain (residues 822 - 916) in the intact protein.

182 The PrV gB ectodomain was expressed using stably transfected *Drosophila* Schneider 2
183 (S2) cell lines as described (63). After affinity and size exclusion chromatography (SEC), we
184 obtained 8-12 milligrams of pure protein from 1 liter of cell culture. The protein eluted as a
185 single peak from a SEC column, and exhibited no signs of aggregation. SDS-PAGE analysis of the
186 purified ectodomain showed a single band migrating just below the 100kDa marker under non-
187 reducing conditions (Fig. 1B, lane 1). PrV gB contains a furin cleavage site ⁵⁰¹RRARR⁵⁰⁵, and the
188 recombinant protein is cleaved by furin in S2 cells as demonstrated by the presence of two
189 protein bands of lower molecular masses under reducing conditions (~60kDa and ~40kDa
190 fragments labeled, respectively, as gB^b and gB^c) (Fig. 1B, lane 3).

191 Compared to its 100kDa apparent molecular mass, the polypeptide chain of the gB
192 ectodomain has a calculated mass of 82kDa, corresponding to the 49kDa N-terminal and 33kDa
193 C-terminal furin cleavage products, which indicates the presence of post-translational
194 modifications in the mature protein. There are six predicted N-glycosylation sites (four in the N-
195 terminal and two in the C-terminal fragment), and a shift to a lower molecular mass is indeed
196 observed for both fragments upon treatment with a deglycosidase (Fig. 1B, lanes 2 and 4).
197 *Drosophila* S2 cells add 1-2kDa simple mannose core structures to form N-linked sugars (64),
198 accounting for the presence of several N-linked sugars in the expressed protein.

199 The purified gB ectodomains crystallized easily under numerous conditions, but the
200 crystals were fragile and of poor diffraction quality. Upon enzymatic deglycosylation with Endo
201 D, we obtained diffraction-quality crystals in 0.1M Tris pH 8.5, 7% PEG 4,000, 0.6M LiCl.
202 Diffraction data were collected and processed as described in the Material and Methods
203 section. The crystals belong to the H3 space group (a=99.9Å, b=99.9Å, c=272.9Å; $\alpha=\beta=90^\circ$,

204 $\gamma=120^\circ$), with one gB protomer per asymmetric unit. Crystallographic statistics of data
205 processing and structure refinement are given in Table 1.

206

207 **PrV gB forms a typical class III post-fusion trimer.**

208 The PrV gB ectodomain folds into an ~16 nm x ~8 nm trimeric spike, resembling the
209 structures reported for the postulated post-fusion conformation of the HSV-1 (26), EBV (27) and
210 HCMV gB ectodomains (28, 29). Briefly, the N-terminus of the protein (amino acid 113) is
211 located at the top end of the trimer, with the polypeptide chain running down along the entire
212 length of the spike, folding into domain I, which carries the FLs exposed at the base of the
213 molecule (Fig. 1C). Domain II is positioned laterally, adopting a fold reminiscent of the pleckstrin
214 homology domains. The furin cleavage site is located in the flexible linker that connects
215 domains II and III, and could not be resolved in our structure. Domain III contains the
216 prominent, centrally located helix that extends to the top of the molecule, followed by domain
217 IV, also known as the ‘top’ or ‘crown’ domain. The residues 697-750 form a stretched chain that
218 packs tightly into the crevice formed by the other two protomers. This so-called ‘domain V’ has
219 the appearance of a zipper that seals the trimer (Fig. 1D), resulting in the C-terminus of the
220 ectodomain (amino acid 750) being brought into close vicinity of the FLs.

221 Although the expression construct encodes for gB residues 59-756, the first residue
222 resolved in the crystal structure was *Arg113*, consistent with the highly flexible N-terminal end
223 of the protein, which is rich in glycine and proline residues. The same applies to HSV-1, EBV and
224 HCMV gB, in which the N-terminal region of the crystallized construct was not resolved either.
225 The other PrV gB regions that were not built into the structure are the above mentioned

226 unstructured segment that contains the furin cleavage site and connects domains II and III
227 (residues 478-521), and the C-terminal six residues of the ectodomain (residues 750-756),
228 which are followed by the double strep tag in the expression construct. The PrV gB structure,
229 however, does reveal 3 residues at the very N-terminus (residues 113-116), which had not been
230 observed in the other gB structures. This segment forms a short β -strand (β 1) in domain IV that
231 packs against β 29 in an anti-parallel fashion (Fig. 1C, 1D).

232 Of six potential N-glycosylation sites, we observed density for the sugar moieties
233 attached to *Asn264* (domain I), *Asn444* (domain II) and *Asn636* (domain IV). The electron
234 density was of sufficient quality to allow placing one an N-acetyl glucosamine (NAG) residue
235 only to *Asn264* (Fig. 1C and D).

236 As anticipated, PrV gB shows higher structural conservation with the HSV-1 homolog
237 than with the more distant gB of EBV or HCMV. The root-mean-square deviation (rmsd) for the
238 superposition of the PrV and HSV-1 structures is 1.04 Å for $C\alpha$ atoms (Table 3). The main
239 difference between the PrV and HSV-1 ectodomains, regarding the gross organization of the
240 spike, resides in the position of domain IV, which appears slightly rotated when the structures
241 are superposed on domains I and II. Domain IV disposition is even more evident when
242 compared to HCMV and EBV gB (Fig. 2).

243

244 **PrV gB ectodomain requires cholesterol in the membrane for binding.**

245 The recombinant PrV gB ectodomain used for crystallization was tested for binding to
246 liposomes of different composition in co-floatation experiments in density gradients, as
247 described in detail in Materials and Methods. Briefly, the protein-liposome mixture, adjusted to

248 36% iodixanole (Optiprep), was loaded below the 20% iodixanole layer that was then overlaid
249 with buffer and subjected to ultracentrifugation. Liposomes float and are found at the top of
250 the gradient, while the protein alone sediments and remains at the bottom of the tube. Protein
251 bound to liposomes co-floats with liposomes, and in this case partitions to the top of the
252 gradient. All gradient fractions were analyzed for presence of the protein in the control
253 experiments, demonstrating that gB was found either at the top or bottom of the gradient,
254 which is why only these two fractions were analyzed further.

255 Liposomes containing 60% 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 40%
256 cholesterol (CH) were prepared. This is the same composition used for testing HSV-1 gB
257 ectodomain interactions with membranes (55, 65). The top and bottom fraction were analyzed
258 by Western blot (WB) with a reagent that specifically binds to the strep affinity tag. WT PrV gB
259 ectodomain was found in the top fraction only when liposomes were present, and sedimented
260 to the bottom of the tube in the absence of liposomes (Fig. 3A). Complexes made of WT PrV gB
261 ectodomain and liposomes containing DOPC and increasing molar amounts of CH (100% DOPC,
262 80% DOPC + 20% CH, or 60% DOPC + 40% CH) were prepared and tested next. gB ectodomains
263 bound only to the latter type of liposomes (Fig. 3B), suggesting that there is a threshold in CH
264 concentration required for the association. To determine if the lack of binding at lower CH
265 concentrations was due to the requirement for another lipid rather than insufficient CH
266 concentration, a series of liposomes made of mixtures of DOPC, 1,2-dioleoyl-*sn*-glycero-3-
267 phosphoethanolamine (DOPE), and sphingomyelin (SM) was prepared in the presence of 40%
268 CH, or without CH. DOPE alone or mixed with CH does not form liposomes, but assembles into
269 lipid nanotubes due to its inverted hexagonal shape (66), which is why these lipid mixtures

270 were omitted. The results of the floatation experiments showed that WT gB was present in the
271 top fraction i.e. bound to the liposomes only in the presence of CH (Fig. 3C), reinforcing the
272 conclusion that CH is essential for PrV gB ectodomain interactions with liposomes.

273 A sample of gB mixed with liposomes was visualized by negative stain electron
274 microscopy (EM) (Fig. 4A), revealing liposomes decorated by protein arrays, forming
275 honeycomb-like structures in which the individual trimers appear to be in contact with each
276 other. Cryo-EM images of the same sample (Fig. 4B) showed individual gB trimers packing
277 tightly and inserting into the liposomes via the spike end that contains the FLs (domain I), as
278 was observed for the HSV-1 protein (56).

279

280 **Selection of PrV gB FL residues and design of mutagenesis studies.**

281 The side chains of *Trp* and *Phe*, as well as smaller hydrophobic residues such as *Leu* or *Ile*
282 have favorable energies for penetration into the hydrophobic membrane core, made
283 exclusively of lipid tails (67), while amphipathic *Tyr* and *His* side chains preferentially partition in
284 the interfacial membrane region, localized between the hydrocarbon core and the aqueous
285 phase (67, 68). *Trp* is also often found in this region (69). The interfacial membrane region is
286 amphipathic itself due to the presence of carbons from the beginnings of the apolar lipid tails
287 and of polar phosphate and glycerol groups (70). A *Phe* side chain can penetrate into the
288 membrane core, and could thus be emulated by *Trp* but not by *Tyr* due to the presence of a
289 polar hydroxyl group at the distal tip of the side chain. The interfacial region on the other hand
290 would be expected to accommodate aromatic residues as *Tyr*, *Trp*, *Phe* or *His* (the latter in its
291 unprotonated form).

292 To shed light on how the side chains of individual FL residues interact with membranes,
293 several changes were introduced: each of the aromatic or hydrophobic residues found in PrV gB
294 FLs (*Trp187, Tyr192, Tyr267, Ile270, Phe275, Tyr276, and His277*) (Fig. 5) was individually
295 mutated to *Ala*, a change that resulted in the replacement of the bulky hydrophobic side chain
296 for a methyl group, or to a different aromatic residue with similar chemical structure: *Trp* was
297 changed to *Phe* or *His*; *Phe* to *Trp* or *Tyr*; *His* to *Trp*, and *Tyr* to *Phe* (Table 2). This was done to
298 probe if the residue was more likely to insert deeper into the hydrocarbon core or remain in the
299 polar region of the membrane. Three of the mutagenized residues are unique to PrV gB (*Ile270,*
300 *Tyr276, and His277*), *Tyr267* is conserved but has not been mutated, and the changes
301 introduced at the conserved positions have not been analyzed earlier.

302

303 **Side chains of PrV gB FL residues *Trp187, Tyr192, Phe275* and *Tyr276* mediate binding to**
304 **liposomes and form a hydrophobic patch.**

305 To facilitate generation of mutant proteins, recombinant PrV gB ectodomains were
306 expressed after transient transfection of Expi293F mammalian cells, and the same type of
307 liposomes (60% DOPC and 40% CH) was used in the liposome floatation assay as reported for
308 the HSV-1 gB ectodomain variants (55). The variants did not differ from the WT protein in terms
309 of expression yields, behavior during affinity and size exclusion chromatography, and cleavage
310 by cellular furin (Fig. 6).

311 Substitution of the selected residues to alanine had three consequences (Table 2, Fig.
312 7A). W187A, Y192A, F275A and Y276A variants completely lost the ability to bind to liposomes
313 and were present exclusively in the bottom fraction. I270A and H277A floated as the WT

314 protein, indicating that the mutation exerted no effect on liposome binding. Y267A showed an
315 intermediate behavior, with very low binding to liposomes, at the limit of the WB detection.

316 Mutation to other aromatic residues also had different outcomes. *Trp187* was essential
317 for association of the ectodomain with liposomes, and changes to *Phe* or *His* yielded a protein
318 that did not co-float with liposomes. Y192F was only weakly detected in the liposome fraction,
319 similar to what was observed for Y267A, while Y267F and F275W bound to liposomes
320 comparable to WT gB. Interestingly, in contrast to F275W, F275Y did not associate with
321 liposomes, indicating that the presence of the hydroxyl group in F275Y may impair the ability of
322 the protein to interact with lipids. Y276F showed very weak binding, resembling Y192F and
323 Y267A. The amino acid at position 277 does not seem to be important for liposome binding,
324 since the substitutions H277A and H277W had no effect (Fig. 7A).

325 The four residues *Trp187*, *Tyr192*, *Phe275* and *Tyr276* that did not tolerate substitutions
326 to alanine, and must have a bulky aromatic side chain in order to bind to liposomes, form a
327 continuous hydrophobic and electrostatically neutral patch at the surface of the trimeric post-
328 fusion spike as shown in Fig. 7B. The phenyl group of *Phe275* appears the most protruding,
329 while *Trp187*, *Tyr192* and *Tyr276* form a rim above (Fig. 5B).

330

331 **PrV gB FL variant binding to liposomes correlates with fusion activity.**

332 The fusogenic potential of a subset of PrV gB FL mutants, including all the *Ala* variants,
333 was assessed in eukaryotic cells using the corresponding full-length gB constructs, generated by
334 site directed mutagenesis of pcDNA-gB Ka. Correct mutagenesis was verified by sequencing,
335 and protein expression and processing were analyzed, respectively, by indirect

336 immunofluorescence of permeabilized gB expressing cells (Fig. 8A) and WB of whole cell lysates
337 (Fig. 8B). The PrV gB FL variants revealed WT-like behavior both in subcellular localization and
338 processing by furin, with the exception of W187A and Y192A gB. These two variants
339 accumulated in larger structures in the cytoplasm and showed impaired furin-cleavage as
340 indicated by lower levels of the furin-cleaved subunit (gB^b) and more abundant uncleaved gB
341 (gB^a) (Fig. 8B).

342 To test the fusogenic potential of the mutated gB proteins, RK13 cells were co-
343 transfected with plasmids encoding WT gB (Ka) or the mutated gB, and gH/gL as described (71,
344 72). In addition, an enhanced green fluorescent protein (EGFP) expression plasmid was co-
345 transfected to facilitate evaluation of the assays by fluorescence microscopy (73). Transfection
346 with plasmids encoding the WT proteins served as positive control and the results were set as
347 100%, while the empty expression vector pcDNA3 was used as negative control.

348 Fusion assays revealed a good correlation between the ability of the protein to bind to
349 liposomes *in vitro*, and its activity in fusion (Table 2, Fig. 9A). Most of the variants that did not
350 show association with liposomes were unable to induce cell-cell fusion, and all variants with
351 liposome-binding properties supported efficient cell-cell fusion. Surprisingly, the three variants
352 that showed only weak binding to liposomes, Y192F, Y267A and Y276F, mediated fusion to a
353 similar extent as the WT gB.

354 To test whether this discrepancy was due to the liposome composition used in the *in*
355 *vitro* experiments (60% DOPC, 40% CH, referred to as '2L' for two lipids present), , the co-
356 floatation experiments were repeated with liposomes whose composition resembles better the
357 composition of the plasma membrane (20% DOPC, 20% DOPE, 20% SM and 40% cholesterol,

358 referred to as '4L' for four lipids present) (74-76). The three variants, Y192F, Y267A and Y276F,
359 indeed floated with '4L' liposomes like the WT protein (Fig. 9B), demonstrating that the
360 previously observed weak association was due to the inadequate liposome composition, and
361 not the mutated protein. The association of the other FL variants did not change as a function
362 of liposome composition (Fig. 9C). This data for the first time demonstrates significant
363 differences in capabilities of gB mutants to bind to liposomes as a function of lipid composition,
364 underlining the importance of testing different liposome compositions and cross-checking
365 biochemical with functional data.

366 Since W187A and Y192A variants were inactive, and showed impaired furin cleavage, we
367 wanted to establish whether furin-processing was important for the function of PrV gB in the
368 particular functional assays used in this study. A gB variant with a 5-residue deletion (RRARR) in
369 the furin site (gB- Δ furin) was created. WB analysis of gB- Δ furin expressed in RK13 cells
370 confirmed that the protein was not cleaved as indicated by a prominent band for uncleaved gB
371 (gB^a), and absence of bands corresponding to the furin-cleaved subunits (Fig. 10). gB- Δ furin was
372 functional in cell-cell fusion assays and able to complement gB-negative PrV to levels
373 comparable to WT-gB (Fig. 10C). These data demonstrate that furin-cleavage of PrV gB is not
374 necessary for its function in cell-cell fusion and complementation. The observed fusion
375 deficiency of W187A and Y192A variants thus cannot be attributed to the reduced furin-
376 cleavage alone, but could still be caused by impaired trafficking and low surface expression.
377 Unlike HSV-1 gB, which is highly abundant at the cell surface, WT PrV gB exhibits rather low
378 surface expression. Only around 4% of WT PrV gB is targeted to the plasma membrane as
379 determined by FACS analysis (data not shown), making robust quantification complicated.

380 Next, the function of the mutated proteins during virus entry was tested in trans-
381 complementation assay. RK13 cells were transfected with the different gB expression plasmids
382 and infected one day later with a PrV mutant lacking the gB gene (PrV-ΔgB) (77). Cells and
383 supernatant were harvested 24h p.i. and progeny viral titers were determined on RK13-gB cells
384 (Fig. 9A). Cells transfected with expression plasmids for gBs which efficiently mediated cell-cell
385 fusion also complemented the defect of PrV-ΔgB during entry comparable to WT gB. In
386 contrast, no infectious progeny was derived from cells transfected with the empty vector
387 (“mock”). Infectious progeny was also not produced after infection of cells transfected with the
388 expression plasmid for W187A indicating that this gB mutant is not functional. Surprisingly, gB
389 mutants Y192A, F275A, and F275Y which were unable to mediate cell-cell fusion supported
390 production of a low titer of infectious virions (10^2 PFU/ml), while expression of Y276A resulted
391 in titers of 10^4 PFU/ml, indicating that presence of other viral proteins might have partially
392 compensated for the impairment in membrane binding and fusion.

393

394 DISCUSSION

395 **Comparison with the available gB structures from other herpes viruses.** The structural
396 alignment of PrV gB with the homologous HSV-1, EBV and HCMV proteins demonstrates that
397 the post-fusion conformation is well preserved, with the main difference residing in the
398 positioning of domain IV relative to the rest of the protein (Fig. 2). The low-resolution structure
399 obtained for the full-length HSV-1 gB in a conformation distinct from the post-fusion
400 conformation localizes domain IV to the interior of the spike (30), implying that a large
401 movement would occur during the conformational change. Insertion of a foot-and-mouth

402 disease virus (FMDV) epitope in PrV gB domain IV, right after the residue *Arg685*, resulted in
403 high titers of neutralizing antibodies against FMDV (78), suggesting that at least some of the
404 antigenic determinants on PrV gB domain IV are presented at the surface when the protein is
405 expressed in its pre-fusion form. It is also possible, however, that the epitope is available
406 because a fraction of gB at the virion surface may have adopted the post-fusion conformation.

407

408 **Cholesterol dependence.** HSV-1 gB binding to membranes was previously concluded to be
409 cholesterol-dependent based on experiments using binary liposomes made of DOPC and CH
410 (55). As we show in this study, the ability of PrV gB to bind to liposomes is not only influenced
411 by its FL residues, but also by the lipid membrane composition. WT PrV gB ectodomains, as well
412 as Y192F, Y267A and Y276F variants, showed increased binding to '4L' compared to '2L'
413 liposomes, whose composition does not emulate well a real biological membrane (Fig. 9B). We
414 show here that liposomes, either 2L or 4L, need to contain at least 40% CH for gB to bind (Fig.
415 3C), which is in agreement with 30-40% CH being present in plasma membrane and secretory
416 vesicles (76), and with PrV entering cells via fusion with the plasma membrane. In case of
417 herpesviruses that enter cells by endocytosis, the requirement for high CH concentration might
418 also indicate that the fusion would occur within the internal compartments enriched in CH. A
419 possible role for CH could be to fill the voids in the leaflet introduced by displacement of the
420 bulkier head-groups of the other lipids upon insertion of the FLs.

421 Cholesterol has been shown previously to induce lipid curvature and promote formation
422 of lipid stalks in fusion intermediates (25). While CH depletion was reported not to affect PrV
423 attachment to cells, the virus was observed as stalled at the plasma membrane and entry was

424 significantly reduced (59). It was speculated that the particles might have been blocked due to
425 the inability to resolve the hemifusion intermediate in the absence of cholesterol, which is
426 contrary to our observation that CH is required for the initial insertion of FLs into the
427 membrane i.e. for an early event that must take place prior to reaching the hemifusion state.

428 HSV-1 gB full-length protein expressed on cells was shown to associate specifically with
429 CH- and SM-enriched lipid rafts (61), in which the two lipids are organized in the so-called
430 ordered lipid domains (Lo) (79). The borders between these Lo and the more fluid, disordered
431 membrane domains (Ld) were identified as the preferred sites for insertion of the HIV Env class
432 I fusion protein FP (80), presumably because they impose the least energy penalty for insertion
433 due to the line tension caused by the discontinuity between the domains. Desplanques *et al.*
434 had previously observed a significant fraction of PrV virions juxtaposed to the lipid raft marker
435 GM1 (59), which could, in the light of the Env insertion mode, indicate that PrV may use Lo-Ld
436 boundaries as sites of attachment as well. Although the FP of HIV Env is a short hydrophobic
437 sequence that has a free N-terminus, in contrast to the bipartite internal FLs of gB, it is
438 tempting to speculate that despite the different nature of the inserted segments, similar
439 energetic constraints might apply to membrane insertion of these divergent proteins. Further
440 experimental studies on herpesvirus gB are needed to test these hypotheses, and to establish if
441 CH is required for binding of herpesviruses to membranes, for membrane fusion or for both. It
442 is worth noting out that our EM data show an even distribution of the PrV gB ectodomains
443 bound to liposomes, opposing the idea of gB having preferred membrane insertion sites. This
444 could be due to the properties of the liposomes used for EM, which were made of 60% DOPC
445 and 40% CH, a binary mixture that would be expected to form the Lo phase only (81).

446

447 **Furin processing.** Proteolytic cleavage by cellular furin had been previously reported to be
448 dispensable for gB function in herpesviruses encoding cleavable gBs, as its absence did not have
449 an effect on viral replication or penetration kinetics, although smaller syncytia were detected
450 (82-86). A similar observation was made when gB was expressed in LoVo cells, which are
451 naturally deficient in furin, suggesting that PrV gB cleavage may play a role in cell-cell fusion
452 (84). Our data demonstrate that furin-cleavage of PrV gB was not required for its activity in cell-
453 cell fusion and virus trans-complementation assays used in this study (Fig. 10). The discrepancy
454 between our results regarding the gB- Δ furin variant function in cell-cell fusion and those
455 reported by Okazaki (84) could be due to the different cells types used for fusion.

456

457 **PrV gB FL variants in liposome binding and functional assays.** The liposome binding
458 experiments performed with the 15 recombinant gB ectodomain variants studied here allowed
459 the selection of a subset of variants to be followed up by studies in the context of the full-
460 length protein, both in cell-cell fusion and after incorporation into virus particles. The gB
461 ectodomains in interaction with liposomes provided snapshots of the protein already in the
462 post-fusion form, (Figs. 6A, 8B, 8C). The functional assays, on the other hand, illuminated the
463 function of full-length protein mutants expressed at the cell surface or incorporated into virus
464 particles in their pre-fusion state (Fig. 9A). This may explain certain discrepancies for instance
465 with variants that expressed well as recombinant ectodomains but that failed to be folded and
466 transported to the cell surface in cells. The selected variants of full-length gB were assessed for

467 their ability to mediate fusion in two functional assays: virus-free cell-cell fusion assay and
468 trans-complementation of gB-negative PrV (18, 19, 21, 72).

469 To simplify comparisons with gB from other herpesviruses, we have numbered the FL
470 residues from 1 to 14 (Fig. 5A). Our mutagenesis data are consistent with a model in which
471 residues *Trp187* (FL1-3), *Tyr192* (FL1-8) and *Tyr276* (FL2-10) form an aromatic surface
472 compatible with insertion into the polar region of the membrane, establishing an 'interfacial
473 rim' structure that would provide multiple interactions with the lipid head groups, while
474 *Phe275* (FL2-9) would reach deeper into the hydrocarbon core (Fig. 11A). gB sequence
475 conservation in alpha herpesviruses shows that residues with similar membrane-partitioning
476 preferences are found at these four positions (supplemental material Fig. S1). Hydrophobic
477 residues compatible with the insertion into the membrane core are present at FL2-9 (*Phe, Val,*
478 *Leu, Trp*), while amphipathic side chains that would favor the interfacial region (*Tyr, His, Trp*)
479 are found at positions FL1-3, FL1-8 and F2-10. This suggests that a common mode of gB
480 insertion into membranes may have evolved within the alpha herpesvirus subfamily, although
481 the residues interacting with lipids may not be identical. Charged residues such as *Arg* and *Glu*
482 are present in the FLs of HSV-1 but not of PrV gB (Fig. 5A), highlighting intrinsic differences that
483 exist despite potentially shared principles for insertion of aromatic and hydrophobic residues.

484 The FL2-9 position is always occupied with a hydrophobic side chain, such as *Phe275* in
485 PrV gB. Mutation of the corresponding residue in HSV-1 gB (*Phe262*) to *Asp* or *Leu*, respectively,
486 resulted in a poorly expressed protein and a protein that could mediate fusion at 70% of the
487 level measured for the WT protein (52, 55). The tolerance for *Leu* (but not for *Asp*) in HSV-1 gB

488 and for *Trp* (but not for *Tyr*) in PrV gB indicates that FL2-9 has the potential to insert into the
489 membrane core, i.e. deeper than the interfacial region.

490 Positions FL1-3 (*Trp187*), FL1-5 (*Gly189*) and FL1-8 (*Tyr192*) are occupied by the best
491 conserved residues in FL1 of alphaherpesviruses (supplemental material Fig. S1, Fig. 12). FL1-5
492 *Gly* has already been shown to be important for function of HSV-1 gB (55), and was not tested
493 here. Substitution of FL1-3 (*Trp*) of HSV-1 for a *Tyr* resulted in a protein that was 50% active in
494 fusion (52). When the same residue was changed to *Phe* in PrV gB, the resulting ectodomain did
495 not co-float with liposomes. It is possible that the FL1-3 position requires *Trp* because its indole
496 ring may be involved in interactions with a specific lipid, and/or because its central location in
497 between FL1-8 and FL2-10 might be important for the structural integrity and docking of the
498 trio of rim residues into the membrane interface. HSV-1 gB variants containing a *Ser* and *Arg* at
499 positions FL1-3 and FL1-8, respectively, were shown to be defective in fusion and in liposome-
500 binding (55). These variants were subsequently crystallized, demonstrating that the mutations
501 did not introduce any structural changes in FL1, and that the loss of binding to liposomes was
502 solely due to the elimination of the hydrophobic side chains (87). While we anticipate that the
503 same is true for the PrV gB ectodomains with mutations in FL1-3 and FL1-8, it is important to
504 note that when expressed in cells as full-length proteins, W187A and Y192A accumulated in
505 intracellular compartments and showed impaired processing (Fig. 8). We speculate that furin
506 site in the misfolded gB variants may not be accessible for cleavage, giving rise to the observed
507 decrease in the amount of cleaved protein. The possibility therefore remains that these two FL1
508 variants were not active in functional assays due to the altered cellular localization, even
509 though the production of the ectodomains with the same mutations in insect cells led to

510 efficient protein secretion into the medium, suggesting that these residues are at least not
511 important for correct folding of the post-fusion ectodomain.

512 The FL2-10 residue is predicted to insert into the interfacial region, and while PrV gB
513 Y276F was functional, Y276A did not associate with liposomes and failed to mediate cell-cell
514 fusion, although it unexpectedly partially rescued gB-null virus in the complementation assay.
515 This result emphasizes distinct functional requirements for cell-cell fusion and viral entry
516 processes, and that the two do not follow identical mechanisms. This is highlighted by the
517 requirement for gD in PrV entry, but not for cell-cell spread (18, 88). The presence of other viral
518 proteins during infection may compensate for the reduced membrane binding activity, and/or
519 the protein may be present in higher density in the virion envelope than at the cell surface,
520 favoring the fusion function.

521 FL2-1 is the most conserved FL2 residue in alphaherpesviruses, being *Tyr* in 75% of the
522 sequences (supplemental material Fig. S1, Fig. 12). This residue was not mutagenized in HSV-1
523 gB, but was analyzed here for PrV gB, revealing to our surprise that changes to *Ala* or *Phe* had
524 no effect. This suggests that *Tyr267* is not directly involved in interactions with liposomes, and
525 is not essential for the protein function in fusion. Interestingly, Y267F bound indiscriminately to
526 '2L' and '4L' liposomes, while Y267A was sensitive to the lipid composition and associated
527 better with the '4L' liposomes. Membrane composition has been shown to influence binding of
528 other viral fusion proteins of flaviviruses (89, 90), bunyaviruses (91), influenza virus (92) and HIV
529 (93). In the latter case, the FP of gp41 was shown to adopt an α -helical or β -strand structure,
530 depending on CH concentration, indicating alternative modes of insertion into membranes of
531 the same protein.

532 Position FL2-11 is occupied by *Arg* in most alphaherpesviruses, and with *His* in PrV gB.
533 The substitution of this residue for *Ala* or larger *Trp* in PrV gB resulted in a functional protein
534 that associated with liposomes, indicating that this aromatic side chain is not important for
535 interactions with lipids. In contrast, *Arg* present at the same position in HSV-1 gB and in 26
536 other analyzed sequences (supplemental material Fig. S1), was implicated in binding to
537 liposomes, possibly by interacting with the negatively charged phosphate head-groups of
538 membrane lipids (55). The *His* and *Arg* side chains present at position FL2-11 obviously play
539 different roles in binding to lipids.

540

541 **Model of PrV gB interactions with lipids.** We show here that PrV gB residues *Trp187*, *Tyr192*,
542 *Gly274*, *Phe275* and *Tyr276* form a continuous, electrostatically neutral hydrophobic surface
543 (Fig. 7B) reminiscent of the hydrophobic patch reported for HSV-1 gB (55). EBV and HCMV gB
544 ectodomains used for crystallization had polar and charged residues inserted in place of the
545 hydrophobic WT FL residues, but similar hydrophobic patches at the base of the trimers are
546 observed when the WT residues are modeled back on the structure (data not shown). This
547 strongly suggests functional conservation of the areas involved in membrane interactions.
548 Closer inspection of the HCMV and EBV gB FL residues found at the positions identified as the
549 membrane contact sites in PrV gB reveals that two of the three rim positions, FL1-3 and FL2-10,
550 are occupied by *Tyr*, similar to gB of alphaherpesviruses, while *Thr* and *Ala* are found at FL1-8 in
551 HCMV and EBV gB, respectively (Fig. 11B, C). The *Tyr* residues at FL1-3 and FL2-10 are highly
552 conserved in beta- and gamma herpesviruses (data now shown), while FL1-8 is not. In addition,
553 the side chain of *Tyr*, found at neighboring FL1-10 in HCMV and at FL1-7 in HCMV and EBV gB,

554 could reach the plane in which FL1-3 and FL2-10 side chains insert, contributing to formation of
555 the interfacial rim. FL2-9, which according to our model inserts deeper, is *Leu* in HCMV and *Thr*
556 in EBV gB, while *Trp* is found at FL2-8 in both proteins. This *Trp* is strictly conserved within beta-
557 and partially within gamma-herpesviruses, while the latter have in addition a well-conserved
558 *Trp* inserted between FL2-5 and FL2-6 (designated as FL2-5*6 on Fig. 11B). There are also other
559 hydrophobic residues in the FLs on HCMV and EBV gB, whose side chains could insert into the
560 lipid bilayer (FL1-4, FL1-5, and FL1-6 in HCMV, and FL1-6, FL2-6 and FL2-7 in EBV gB; not shown
561 on Fig. 11B for clarity reasons). Thus, although they have more hydrophobic residues at the tips
562 of the FLs, our model suggests that beta- and gamma-herpesviruses gB interacts with the
563 membranes in a similar way as alpha-herpesvirus gB, with exposed hydrophobic residues in FL2
564 penetrating into the hydrocarbon core (FL2-9 and/or FL2-8 and/or FL2-5*6), while the rim
565 formed by residues from both FLs (FL1-3, FL1-7, FL2-10) would secure protein insertion into the
566 interface. The exact molecular mechanism through which this is achieved may vary, pointing to
567 differences between the herpesvirus subfamilies that might be related to the different host
568 cells and the specific composition of the target membranes.

569 **Materials and Methods.**

570 **Expression and purification of PrV gB ectodomain in insect cells for crystallization studies**

571 The synthetic gene encoding gB of Suid herpesvirus 1, Kaplan strain (GenBank number
572 AEM64049.1) was codon optimized for protein expression in *Drosophila* Schneider 2 (S2) cells
573 and purchased from Genscript. The gene segment coding the ectodomain (PrV gB residues 59-
574 756) was cloned into the expression vector pT350 (62), so that the ectodomain was flanked by
575 the *Drosophila* Bip secretion signal, driving efficient protein secretion (94) at the N-terminus,
576 and the enterokinase-cleavable double strep tag (sequence SRFESDDDDKAG WSHPQFEK
577 GGGSGGGSGGGG WSHPQFEK) at the C-terminus. Protein expression was induced by addition of
578 0.5mM CuSO₄, and the protein was harvested from the culture supernatant 7 days post-
579 induction (detailed protocol available in (63)). Standard protocols were applied to purify the
580 protein by affinity (Streptactin resin, IBA Technologies) and then by size exclusion
581 chromatography using 10mM Tris, 50mM NaCl pH 8 buffer and Superdex S200 column.
582 Extinction coefficient of 1.2 ml/mg for protein absorbance at 280nm was used for calculation of
583 protein concentration.

584

585 **Crystallization and structure determination of PrV gB ectodomain**

586 Purified PrV gB ectodomains were deglycosylated with endo- β -N-acetylglucosaminidase (Endo
587 D) from *Streptococcus pneumoniae* (95), by an overnight incubation of gB and Endo D in 10:1
588 ratio (weight: weight) in 50mM sodium-phosphate buffer pH 7.5 at 25°C. Reaction mixture was
589 then loaded onto Superdex S200 column to separate deglycosylated gB from Endo D, using

590 10mM Tris, 50mM NaCl pH 8 buffer. Fractions corresponding to gB were pooled, and the
591 protein was concentrated to 9.2 mg/ml in 50kDa cut-off Vivaspin concentrator.

592 Crystals were grown by vapor diffusion in hanging drops in 0.1M Tris pH 8.5, 7% PEG
593 4,000, 0.6M LiCl, and then flash frozen in liquid nitrogen using 20% glycerol as the cryo-
594 protectant. Data was collected at the ESRF synchrotron source ID29 beamline, and processed
595 using XDS (96). Molecular replacement was done with Phaser (97) using HSV-1 gB structure
596 (PDB 2GUM) as a search model. Structure was refined using BUSTER (98).

597

598 **Electron microscopy**

599 Samples for negative stain EM were prepared by mixing 0.5 μ M protein and 1mM liposomes as
600 described in more detail in the 'liposome floatation assay' below. Liposomes were made of 60%
601 DOPC and 40% CH. Staining was done with 2% uranyl-acetate, and images were collected on
602 Tecnai G2 Spirit Biotwin microscope 5 (FEI, USA) operating at an accelerating voltage of 120 kV.
603 Samples for cryo-EM analysis were made by incubating 4 μ M protein and 2mM liposomes. Cryo-
604 fixation was done using Lacey grids using in EMGP (Leica, Austria). Data were recorded on
605 Tecnai F20 operating at 200kv, equipped with a Falcon II direct detector (FEI, USA) and under
606 low dose conditions.

607

608 **Generation of expression constructs and production of PrV gB recombinant ectodomains in** 609 **mammalian cells**

610 The gene encoding PrV gB ectodomain residues 59-756, followed by a double strep tag, was
611 cloned into pcDNA4 (Invitrogen™) expression vector using Gibson Assembly Master Mix® (New

612 England Biolabs). Quikchange mutagenesis was applied to introduce single point mutations in
613 the FLs, following the standard protocols (Agilent Technologies, QuikChange II site-directed
614 mutagenesis kit™). The presence of desired mutations was confirmed by DNA sequencing. The
615 expression constructs were transfected into Expi293F™ mammalian cells (Gibco) using
616 Expifectamine transfection agent, and as described in the manufacturer's manual. Cell
617 supernatants were collected 5 days post-transfection, and the protein was purified by affinity
618 chromatography on Streptactin resin (IBA Technologies) followed by SEC.

619

620 **Liposome floatation assay**

621 Lipids, DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), DOPE (1,2-dioleoyl-sn-glycero-3-
622 phosphoethanolamine), SM (from bovine brain) and CH (from ovine wool), were purchased
623 from Avanti Polar Lipids. Liposomes were prepared by the freeze-thaw and extrusion method
624 (99), using 200nm membranes for extrusion. '2L' liposomes were made of 60% DOPC, 40% CH
625 (mol%), and '4L' liposomes of 20% DOPC, 20% DOPE, 20% SM and 40% CH (mol%).
626 Recombinant 0.5µM gB ectodomains were mixed with 1mM liposomes in PBS buffer in 100µl
627 volume, incubated overnight (12-15h) at 4°C and then at 37°C for 15 minutes (the overnight
628 incubation can be omitted, and was done for convenience reasons). OptiPrep medium (Axis-
629 Shield PoC AS, Oslo Norway) was used for density gradient preparation. Protein-liposome
630 samples were adjusted to the volume of 300µl by addition of 30µl of PBS and 170µl of 60%
631 OptiPrep (final Optiprep concentration 36%), and deposited with a syringe on the bottom of the
632 ultracentrifuge tube already containing 4.5ml of 20% Optiprep solution in PBS. 200µl of PBS was
633 added to the top, and the samples were centrifuged for 1h at 40,000 x g and 4°C, in SWTi 55

634 swinging bucket rotor in Beckman Coulter ultracentrifuge. The 5ml gradients were fractionated
635 into 2 x 400 μ l fractions (the first one representing the 'top' fraction), 3 x 1.2ml and 1 x 600 μ l
636 ('bottom') fraction. 30 μ l aliquots of the top and bottom fractions were analyzed on SDS-PAGE
637 4-20% gradient gels (GenScript), and Western blotting to detected the double strep tag on gB
638 was done with the Streptactin-HRP conjugate (IBA Technologies) following the manufacturer's
639 instructions.

640

641 **Viruses and cells.** Rabbit kidney (RK13) and RK13-gB cells were grown in Dulbecco's modified
642 Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum at 37°C and
643 5 % CO₂. PrV mutant lacking gB (PrV- Δ gB) (77), which was derived from PrV strain Kaplan (PrV-
644 Ka), was propagated in RK13-gB cells.

645

646 **Expression plasmids for cell-cell fusion assay.** Generation of expression plasmids for PrV-
647 Kaplan (PrV-Ka) gB, gH, and gL has been described previously (71). Expression plasmid pcDNA-
648 gB^{Ka} containing the gB open reading frame (ORF UL27) was used for site-directed mutagenesis
649 (QuikChange II XL kit, Agilent) with the complementary pair of oligonucleotide primers. Correct
650 mutagenesis was verified by sequencing.

651

652 ***In vitro* cell-cell fusion assays.** Fusion activity of the different gB-mutants was analyzed after
653 transient transfection of RK13 cells as described recently (73). Briefly, approximately 1.8×10^5
654 RK13 cells per well were seeded onto 24-well cell culture plates. On the following day, cells
655 were transfected with 200 ng each of expression plasmids for EGFP (pEGFP-N1; Clontech), and

656 for PrV glycoproteins gB^{Ka} or mutant gB, gL^{Ka} and gH^{Ka} (21, 71, 100) in 100 μ l Opti-MEM using 1
657 μ l Lipofectamine 2000 (Thermo Fisher Scientific). Empty vector (pcDNA3) served as negative
658 control. The mixture was incubated for 20 min at room temperature and added to the cells.
659 After 3 hours, cells were washed with PBS and incubated in MEM supplemented with 2% FCS
660 for another 24 h at 37°C. Thereafter, cells were fixed with 3% paraformaldehyde (PFA). Syncytia
661 formation was analyzed using an Eclipse Ti-S fluorescence microscope and the NIS-Elements
662 Imaging Software (Nikon). Total fusion activity was determined by multiplication of the area of
663 cells with three or more nuclei with the number of syncytia within 10 fields of view (5.5 mm²
664 each). The experiment was repeated three times and average percent values of positive control
665 transfections as well as standard deviations were calculated.

666

667 **Trans-complementation assay.** Function of the different gB mutants in virus entry was
668 determined by trans-complementation of PrV- Δ gB (77). Approximately 1.8×10^5 RK13 cells per
669 well were seeded onto 24-well cell culture plates. On the following day, cells were transfected
670 with 200 ng of the corresponding gB-expression plasmid as described above. One day post
671 transfection cells were infected with phenotypically gB-complemented PrV- Δ gB at an MOI of 3,
672 and consecutively incubated on ice for 1 h and at 37°C for 1 h. Subsequently, the inoculum was
673 removed, non-penetrated virus was inactivated by low-pH treatment (101), and 1 ml fresh
674 medium was added. After 24h at 37°C, the cells were harvested together with the supernatants
675 and lysed by freeze-thawing (-80°C and 37°C). Progeny virus titers were determined on PrV gB-
676 expressing cells (RK13-gB) (77). Shown are the mean values of three independent experiments
677 with the corresponding standard deviation.

678

679 **Indirect immunofluorescence tests.** To test for subcellular localization of the mutated proteins,
680 RK13 cells were transfected with the gB expression plasmids as described above. After 24 h,
681 cells were fixed with 3% paraformaldehyde (PFA) for 20 min and permeabilized in PBS
682 containing 0,1 % Triton X-100 for 10 min at room temperature. Subsequently, cells were
683 incubated with a rabbit antiserum specific for PrV gB (102), which was diluted 1:1000 in PBS.
684 After 1 hour at room temperature, bound antibody was detected with Alexa 488-conjugated
685 goat anti-rabbit antibodies (1:1000 in PBS) (Invitrogen). After each step, cells were washed
686 repeatedly with PBS. Green fluorescence was excited at 488 nm, and recorded with a laser
687 scanning confocal microscope (SP5; Leica, Mannheim, Germany).

688

689 **Western blot analyses.** RK13 cells were harvested 24 h after transfection with the different gB
690 expression plasmids, as described above. Cells were lysed and protein samples were separated
691 by sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred
692 to a nitrocellulose membrane. The membrane was incubated with a monoclonal gB-antibody
693 (C15-b1 at 1:500 dilution) (102, 103). Binding of peroxidase-conjugated secondary antibody
694 (Jackson ImmunoResearch) was detected with Clarity™ Western ECL substrate (Bio-Rad) and
695 recorded by VersaDoc 4000 MP imager (Bio-Rad).

696

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991 **FIGURE LEGENDS AND TABLES.**

992 **Figure 1. PrV gB ectodomain structure. A) Schematic representation of the PrV gB expression**
993 **construct.** The PrV gB signal peptide (residues 1-58) was replaced by the *Drosophila* BiP
994 secretion signal, which was cleaved off and not part of the secreted protein. The secreted PrV
995 gB ectodomain used for crystallization contains residues 59-756, followed by a double strep tag
996 (DST). Regions forming the five gB domains are labeled with Roman numbers I to V below the
997 bar, and are colored as follows: domain I (blue), domain II (green), domain III (yellow), domain
998 IV (orange) and domain V (red). Two linker regions (residues 147-154 and 493-528) are shown
999 in grey. Dashed lines mark the regions that were unresolved in the gB structure. Location of the
1000 furin cleavage site ('RRARR' residues 501-505) is indicated. **B) SDS-PAGE analysis of the**
1001 **recombinant PrV gB.** Coomassie-blue stained 4-20% SDS-PAGE gel showing purified PrV gB
1002 ectodomain under non-reducing (lanes 1 and 2), and reducing (lanes 3 and 4) conditions.
1003 Samples in lanes 2 and 4 were treated with Endo D. The N-terminal and C-terminal fragments,
1004 generated by furin cleavage, are labelled as gB^b and gB^c, and the small fraction of uncleaved gB
1005 is marked as gB^a. **C) Structure of the PrV gB monomer.** The molecule is colored starting from
1006 blue (N-terminus) to red (C-terminus). The location of the N- and C-termini is indicated with the
1007 letters 'N' and 'C', and domains are labeled with Roman numbers I to V. The C-terminus is
1008 followed by the 50-residue long membrane-proximal region (MPR), not present in the
1009 expression construct, leading to the transmembrane anchor; the anticipated location of the
1010 membrane is indicated with the arrow. Fusion loops presented by domain I are marked with the
1011 star symbol (*) and 'FL'. The extra N-terminal residues that were resolved for the first time in
1012 this structure form a strand labeled as β 1. The linker connecting domains II and III, which is not

1013 visible in our structure, is plotted as a yellow dotted line to indicate the putative location of the
1014 furin cleavage site (orange star). The location of the glycosylation site *Asn264*, to which a single
1015 NAG residue is attached, is indicated with the yellow star. **D) Structure of the PrV gB trimer.**
1016 The same coloring for the protomers as in (C) applies. Ribbon and molecular surface
1017 representations are shown. The N- and C-termini of the same protomer represented in (B) are
1018 labeled with 'N' and 'C', respectively. Strand β 29 that runs in anti-parallel direction to strand β 1
1019 is indicated (domain IV). PyMOL was used to create structures shown in panels (C) and (D)
1020 (104).

1021

1022 **Figure 2. Structural comparison of gB ectodomains.** PrV gB ectodomain structure, shown in
1023 grey cartoon representation, was superposed with those of HSV-1 (PDB 2GUM; light blue), CMV
1024 (PDB 5C6T; green) and EBV gB (PDB 3FVC; purple), using the residue range that corresponds to
1025 domains I and II (residues 167-458, 154-473, 89-388 and 133-415) of PrV, HSV-1, CMV, and EBV
1026 gB, respectively). The alignment was done by superposing domains I and II to highlight the
1027 disposition of the top, domain IV, which is not as obvious when the superposition is applied to
1028 the entire molecule. The alignments were done in PyMol (104). Domains are labeled with
1029 Roman numbers. Each superposition is shown in two orientations, looking at the monomer
1030 from the front (upper panel) and at domain IV from the top of the spike (lower panel). Red
1031 pentagon symbol is placed in the same loop of PrV gB to serve as a reference point for the
1032 movement of the superposed domain IV, the direction of which is indicated with the black
1033 arrow.

1034

1035 **Figure 3. WT PrV gB ectodomains interactions with liposomes. A) WT gB ectodomains bind to**
1036 **the DOPC-CH liposomes.** Presence of liposomes ('L') and protein is indicated with + and – signs
1037 on the top. Aliquots of the top ('t') i.e. liposome, and bottom ('b') i.e. unbound protein,
1038 fractions were analyzed by SDS-PAGE and the Strep tag present on gB was detected by WB
1039 using Streptactin-HRP conjugate. The WT protein is found in the top fraction only in the
1040 presence of liposomes with 60% DOPC and 40% CH. **B) Co-floatation of WT gB and DOPC**
1041 **liposomes with increasing amount of cholesterol.** Liposome composition is indicated on the
1042 top. gB is detected in the top fraction only when 40% CH is present. **C) Co-floatation of WT gB**
1043 **and liposomes made of combinations of lipids found in plasma membrane.** The CH
1044 concentration was fixed to 40% or 0% (upper and lower panels, respectively), and one or two
1045 more lipids were added, resulting in total of 5 lipid compositions. The protein was detected
1046 bound to all of the CH-containing liposomes, while it was found in the unbound fraction in the
1047 absence of CH.

1048

1049 **Figure 4. A) Negative stain EM image of liposomes incubated with PrV gB ectodomains.**
1050 Characteristic hexagonal structures made of gB covering the liposomes (60% DOPC, 40% CH)
1051 are observed, representing the top view on the gB spikes. Individual trimers are establishing an
1052 extensive network of lateral interactions giving rise to the array appearance of the protein coat.
1053 Liposomes showing side views of the gB trimers projecting away from the liposome surface are
1054 marked with arrows. A blow-up of the area within the black rectangle is shown in the upper
1055 right corner. **B) Cryo-EM of PrV gB ectodomains bound to liposomes.** Arrows indicate
1056 individual gB trimers in which the 'top' end of the spike is better resolved, indicating that the

1057 molecule binds to the liposomes via the other end i.e. the one carrying the FLs. Insert in the
1058 upper right corner depicts the shape of a gB trimer with the 'top' representing domain IV end of
1059 the spike, and 'FL' marking the base of domain I and location of FLs.

1060

1061 **Figure 5. A) Sequence alignment of PrV and HSV-1 gB fusion loops regions.** Identical residues
1062 are shown as white letters on red background, and residues with similar physico-chemical
1063 properties are colored in red on white background. Residues mutated in this study (*Trp187*,
1064 *Tyr192*, *Tyr267*, *Ile270*, *Phe275*, *Tyr276* and *His277*) are boxed in green. Numbers 1-14 above
1065 the alignment designate the position of each residue in FL1 or FL2. The residues found to be
1066 important for binding to liposomes are indicated with vertical arrows and their corresponding
1067 numbers (3, 8 in FL1 and 9, 10 in FL2) are colored according to the scheme used in panel B. **B)**

1068 **Structural representation of the mutated PrV gB FL residues.** The aromatic residues in FL1 and
1069 FL2 are indicated with their side chains in stick representation. These residues were individually
1070 mutated as shown in Table 2. For clarity reasons the two views of the FLs are presented, and
1071 the remaining of the protein was omitted. The residues shown to be required for binding to
1072 liposomes are also indicated with their corresponding positions within FL1 and FL2.

1073

1074 **Figure 6. SDS-PAGE analysis of purified PrV gB ectodomain variants.** SDS-PAGE analysis of the
1075 PrV gB recombinant ectodomains under non-reducing (upper panel) and reducing (lower panel)
1076 conditions. 2µg of each purified protein were loaded on a 4-20% gradient gel. Proteins were
1077 stained with Coomassie blue stain. The three bands resolved under reducing conditions
1078 correspond to the uncleaved protein, the N-terminal and C-terminal fragments produced by

1079 furin cleavage. There are no observable differences in purity and cleavage pattern between the
1080 FL variants and the WT PrV gB.

1081

1082 **Figure 7. A) Liposome binding of PrV gB FL variants.** WB analysis of gradient fractions, 't' - top
1083 fraction (liposomes and bound gB) and 'b'-bottom fraction (unbound protein). Liposomes were
1084 made of 60% DOPC and 40% CH. Arrows indicate presence of very weak signals in the top
1085 fractions observed for Y192F, Y267A and Y276F. Streptactin-HRP conjugate was used for gB
1086 detection. **B) Solvent-accessible surface representation of the bottom tip of the PrV gB spike**
1087 **carrying fusion loops. Left panel:** The FL residues mutated here are shown in the same colors
1088 as in Figure 4B. Star signs (*) indicate position of the conserved *Gly274*. The black contour
1089 designates the edges of the continuous, hydrophobic surface formed by the side chains of
1090 *Trp187, Tyr192, Gly274, Phe275, and Tyr276*. **Middle panel: Solvent-accessible surface colored**
1091 **according to hydrophobicity.** The same PrV gB surface as in the left panel is shown, with the
1092 colors corresponding to the hydrophobicity of the individual side chains and calculated using
1093 Eisenberg hydrophobicity scale (105), with white corresponding to the most hydrophobic, and
1094 dark yellow to the most hydrophilic/charged side chains. **Right panel: Electrostatic solvent-**
1095 **accessible surface.** The same surface as in the left panel is colored according to the
1096 electrostatic potential calculated by ABPS tool (106), with the blue and red corresponding to
1097 negative and positive potentials, respectively. All the images were generated in PyMol (104).

1098

1099 **Figure 8. Expression of full-length PrV gB variants in RK13 cells (A) Subcellular localization.**
1100 RK13 cells were transfected with expression plasmids for wild type gB (gB Ka) or the PrV gB FL

1101 mutants and analyzed by indirect immunofluorescence. One day after transfection, cells were
1102 fixed with 3% paraformaldehyde and permeabilized with 0.1 % Triton X-100. gB was detected
1103 using a gB specific rabbit antiserum and Alexa Fluor 488-conjugated secondary antibodies.
1104 Green fluorescence was excited at 488 nm, and recorded with a laser scanning confocal
1105 microscope (SP5; Leica, Mannheim, Germany). **(B) Western blot analyses.** Lysates of RK13 cells
1106 transfected with expression plasmids for wild type gB (gB Ka) or gB variants containing FL single
1107 point mutations were separated by SDS-PAGE under reducing conditions. Cells transfected with
1108 the empty vector pcDNA3 served as negative control (mock). The blots were incubated with the
1109 gB specific monoclonal antibody C15-b1. Signals of uncleaved gB (gB^a) or furin-cleaved gB
1110 subunit (gB^b) are labeled by arrows, and molecular masses of marker proteins are indicated. As
1111 loading control, the blot was incubated with an anti-alpha-tubulin monoclonal antibody.

1112

1113 **Figure 9. A) Functional data obtained for the PrV gB FL variants.** The results of the cell-cell
1114 fusion assay are plotted as black bars, and expressed as percent of the activity of the WT gB in
1115 combination with gH/gL which was set to 100% (left y-axis). Viral titers obtained in the trans-
1116 complementation assay are shown as grey bars, and correspond to the number of plaque-
1117 forming units (PFU) per ml (right y-axis). 'Ka' stands for PrV strain Kaplan and designates the WT
1118 protein, used as a positive control. The inverted triangles (▼) mark the three variants that
1119 showed poor binding to liposomes, while being functional in fusion and complementation
1120 assay. The ▲ triangle labels Y276A that had fusion-null phenotype in cell-cell assay, but was
1121 partially functional in the virus complementation assay. **B) Effect of liposome composition on**
1122 **binding of WT, Y192F, Y267A and Y276F PrV gB variants.** Two types of liposomes were used:

1123 '2L': 60% DOPC, 40% CH (left), and '4L': 20% DOPC, 20% DOPE, 20% SM and 40% CH (right), and
1124 the top ('t') and bottom ('b') fractions were analyzed by WB, showing higher amount of all the
1125 variants bound to the more complex '4L' liposomes. C) Floatation of PrV gB variants whose
1126 binding to liposomes is not affected by lipid composition. These variants showed the same
1127 binding pattern to '4L' liposomes, as for '2L' liposomes (Fig. 7A). Aliquots from the 'top' (t) and
1128 'bottom' fractions were analyzed by WB.

1129

1130 **Figure 10. Functional characterization of gB Δ furin. A) Western blot analysis.** Lysates of RK13
1131 cells transfected with expression plasmids for wild type gB (gB Ka) or the gB furin deletion
1132 mutant (gB- Δ furin) were separated by SDS-PAGE under reducing conditions. Cells transfected
1133 with the empty vector pcDNA3 served as negative control (mock). Blots were incubated with gB
1134 specific monoclonal antibody C15-b1. Signals of uncleaved gB (gB^a) or furin-cleaved gB subunit
1135 (gB^b) are labeled by arrows, and molecular masses of marker proteins are indicated. Signals of
1136 α -tubulin served as loading control. **B) Subcellular localization.** RK13 cells were transfected
1137 with expression plasmids for wild type gB (gB Ka) or gB Δ furin mutants and analyzed by indirect
1138 immunofluorescence. gB was detected using a gB specific rabbit antiserum and Alexa Fluor 488-
1139 conjugated secondary antibodies. Green fluorescence was excited at 488 nm, and recorded
1140 with a laser scanning confocal microscope (SP5; Leica, Mannheim, Germany). **C) Functional**
1141 **data.** Results from cell-cell fusion assay are plotted as black bars, and expressed as the percent
1142 of the signal measured for the WT protein set to 100% (left y-axis). The viral titers obtained in
1143 the trans-complementation assay are shown as grey bars, and correspond to the number of
1144 plaque-forming units (PFU) per ml (right y-axis). 'Ka' stands for PrV strain Kaplan and designates

1145 the WT protein, used as a control. pcDNA-3 transfected cells were used as negative control
1146 (mock).

1147

1148 **Figure 11. A) Hypothetical model of PrV gB ectodomain interactions with membranes.** The
1149 model displays the base of the PrV gB trimer, illustrating putative location of the FL residues
1150 investigated in this study, shown with their side chains as sticks, and colored as in Fig. 5B. The
1151 arrows and numbers are used to indicate the deeper insertion of *Phe275* (FL2-9) into the apolar
1152 region containing the lipid tails ('1'), and the 'interfacial rim' made of *Tyr192*, *Trp187*, *Tyr276*
1153 ('2') (FL1-8, FL1-3, FL2-10) that would be located within the more polar part of the membrane.

1154 A single molecule of DOPC and its putative localization in the membrane is shown in stick
1155 representation on the right. Carbon, oxygen, phosphorous and nitrogen atoms are colored as
1156 white, red, orange and blue. **B) Hypothetical model of HCMV and EBV gB interactions with
1157 membranes.** HSV-1 (PDB 2GUM), HCMV (PDB 5CXF) and EBV gB (PDB 3FVC) structures were
1158 superposed onto the PrV structure using Dali pairwise alignment algorithm (107). The latter two
1159 structures contain WT residues (YIY¹⁵⁵⁻¹⁵⁷ and WLY²⁴⁰⁻²⁴² in HCMV, WY¹¹²⁻¹¹³ and WLIW¹⁹³⁻¹⁹⁶ in
1160 EBV gB) that were modeled back onto the FL residues that had been mutated in the crystallized
1161 constructs (GHR¹⁵⁵⁻¹⁵⁷ and ATH²⁴⁰⁻²⁴² in HCMV, and HR¹¹²⁻¹¹³ and RVEA¹⁹³⁻¹⁹⁶ in EBV). The
1162 residues at positions FL1-8, FL1-3, FL2-10 and FL2-9 are indicated and colored as in Fig. 5B, with
1163 their side chains shown as sticks. *Trp* found at FL2-8 in HCMV and EBV gB are colored in salmon,
1164 and *Tyr* side chains at FL1-10 and FL1-7 positions in HCMV and EBV gB are colored in light blue.
1165 Grey transparent boxes are highlighting the putative interfacial rim in each structure. The side
1166 chain of *Trp*¹⁹³ inserted between FL2-5 and FL2-6 residues in EBV gB is labeled as 'FL2-5*6' and

1167 colored in magenta. Other hydrophobic residues are not shown as sticks for clarity reasons. **C)**
1168 **Sequence alignment of the FL regions.** Residues with similar physico-chemical properties that
1169 are 75% or more conserved are colored in red. Secondary structure elements corresponding to
1170 the PrV gB ectodomain structure are displayed on the top, and residues identified as membrane
1171 contact sites in PrV gB (FL1-3, FL1-8, FL2-9 and FL2-10) are indicated with black arrows. The
1172 numbering on the top corresponds to that of the PrV gB protein. The alignment was generated
1173 in Clustal Omega (108) and displayed using ESPrpt (109).

1174

1175 **Figure 12. Conservation of gB in alphaherpesviruses plotted on the PrV gB ectodomain**
1176 **structure. A) Solvent-accessible surface of PrV gB in multiple orientations is shown.** Residues
1177 showing identity lower than 75% are colored in white, while the residues that are strictly
1178 conserved in more than 75% of the analyzed sequences are colored in increasing purple
1179 gradient. The alignment shown in supplemental material Fig. S1 was used. **B) PrV gB FLs.**
1180 Fusion loop residues investigated in this study are shown with the side chains in stick
1181 representation. Two orientations are presented for clarity reasons.

1183

1184

1185

Table 1. X-ray diffraction data collection and refinement for PrV gB

X-ray data processing			
Beamline	ESRF ID23		
Space group	H3		
Cell constants	99.89 Å	99.89 Å	272.89 Å
a, b, c, α , β , γ	90.00°	90.00°	120.00°
Resolution (Å)	46.15-2.69 (2.85-2.69) ^a		
No. of reflections	147396 (23196)		
Multiplicity	5.3		
$\langle I/\sigma(I) \rangle$	14.99 (2.33)		
Completeness (%)	99.6% (98.3%)		
R_{merge}	7.4% (52.3%)		
R_{pim}	5.5% (38.6%)		
CC _{1/2}	0.98 (0.82)		
Wilson B factor (Å ²)	55.7		
Refinement			
Program	BUSTER 2.10.2		
Resolution (Å)	46.15-2.70 (2.80-2.70)		
No. of reflections	27695 (2738)		
R_{work}/R_{free} ^b	0.193 / 0.236		
No. of atoms ^c	4759 / 92		
B-atomic factors (Å ²) ^d	73 / 73.8 / 59.6		
Geometry			
<i>rmsd</i> bond lengths (Å)	0.010		
<i>rmsd</i> bond angles (°)	1.16		

51

	Ramachandran plot ^e (%)	95.25 / 4.24 / 0.51
1186		
1187	^a – Outer-shell values are given in parentheses	
1188	^b – R_{free} test set was composed of 5% randomly chosen reflections	
1189	^c – Refers to number of protein / water atoms	
1190	^d – Refers to overall / protein / water B-factors	
1191	^e – Preferred / allowed / outliers as calculated by Coot	
1192		

1193 **Table 2. PrV gB mutants with single point mutations in fusion loops in liposome binding and**
 1194 **functional assays.**

	PrV gB variant	Binding to 2L ^a liposomes	Cell-cell fusion	Trans-complement.	Binding to 4L ^b liposomes	Ectodomain yield /protein purity ^c
FL1	W187A	-	-	-	-	n.c.
	W187H	-	n.t.	n.t.	-	n.c.
	W187F	-	n.t.	n.t.	-	n.c.
	Y192A	-	-	-/+	-	n.c.
	Y192F	-/+	+	+	+	n.c.
FL2	Y267A	-/+	+	+	+	n.c.
	Y267F	+	n.t.	n.t.	+	n.c.
	I270A	+	+	+	+	n.c.
	F275A	-	-	-/+	-	n.c.
	F275W	+	+	+	+	n.c.
	F275Y	-	-	-/+	-	n.c.
	Y276A	-	-	-/+	-	n.c.
	Y276F	-/+	+	+	+	n.c.
	H277A	+	+	+	+	n.c.
	H277W	+	n.t.	n.t.	+	n.c.

1195

1196 Single point mutations introduced in the FL1 and FL2 of PrV gB are indicated. Sign '-/+' in the
 1197 second column designates presence of a faint band in the liposome fraction (Fig. 7A), indicating
 1198 weak binding of the protein to liposomes, or in the fourth column, marginal complementation
 1199 of PrV-ΔgB virus (Fig. 8A). Highlighted in grey are the variants containing mutations to alanine.
 1200 'n.t.' stands for not tested.

1201 ^a – 2L indicates that liposomes were made of 2 lipids: 60% DOPC, 40% CH

1202 ^b - 4L indicates that liposomes were made of 4 lipids: 20% DOPC, 20% DOPE, 20% SM, 40% CH

1203 ^c – expression yield and purity of the recombinant ectodomains is indicated relative to the WT
 1204 protein; 'n.c.' stands for 'no change'.

1205

1206 **Table 3. Sequence and structural comparison of PrV gB and HSV-1 / CMV / EBV ectodomains**

1207

1208

1209

1210

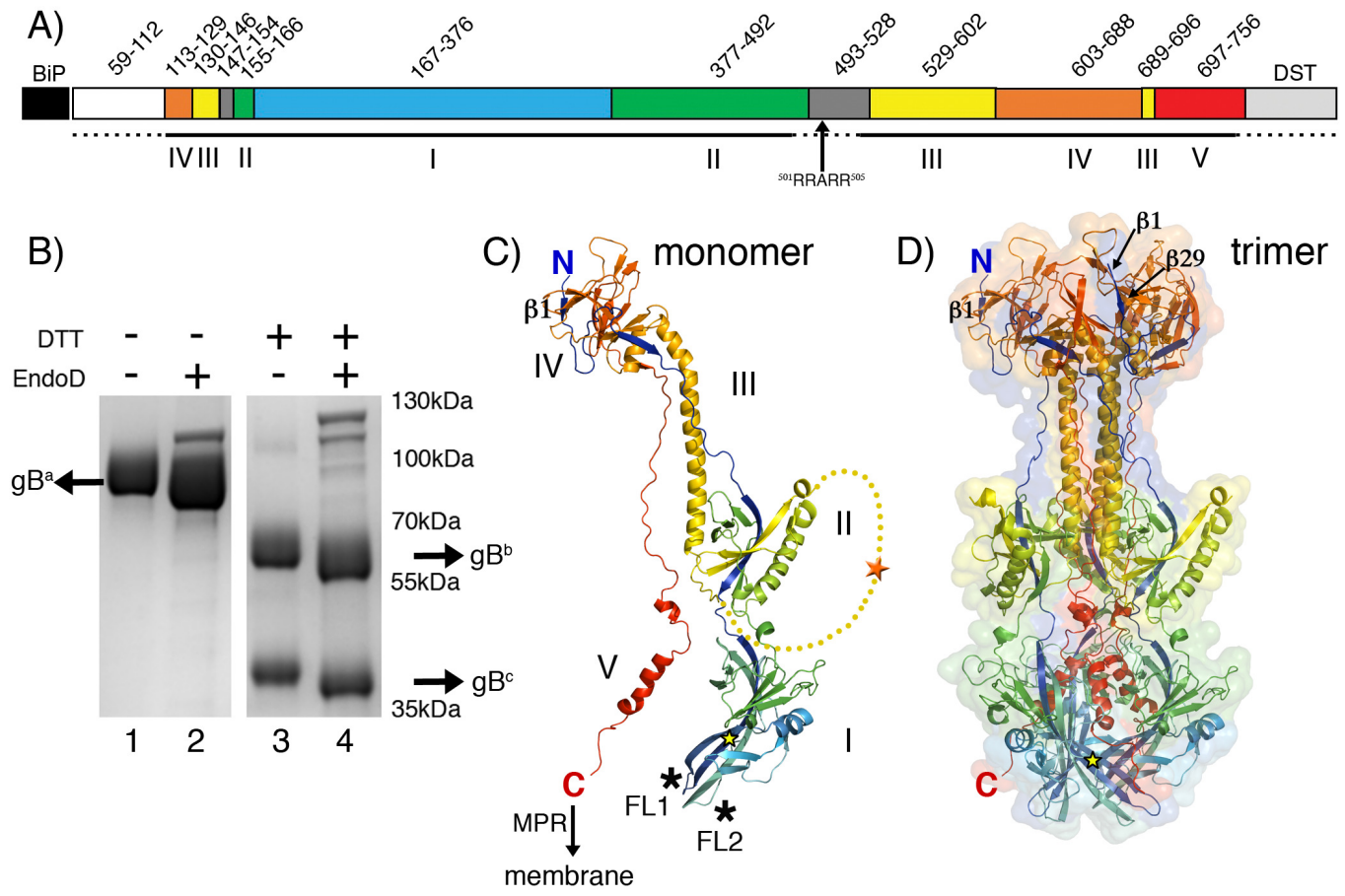
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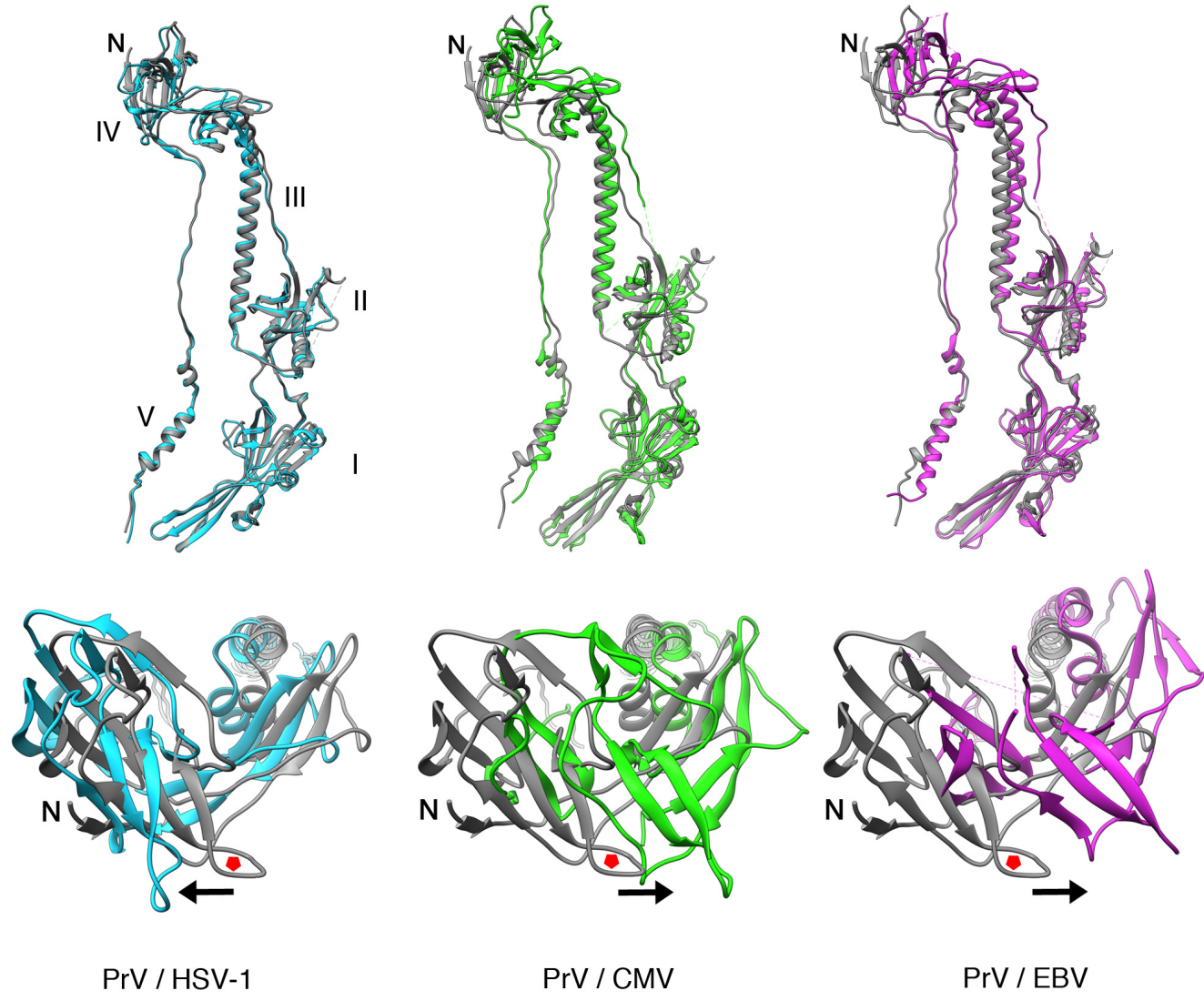
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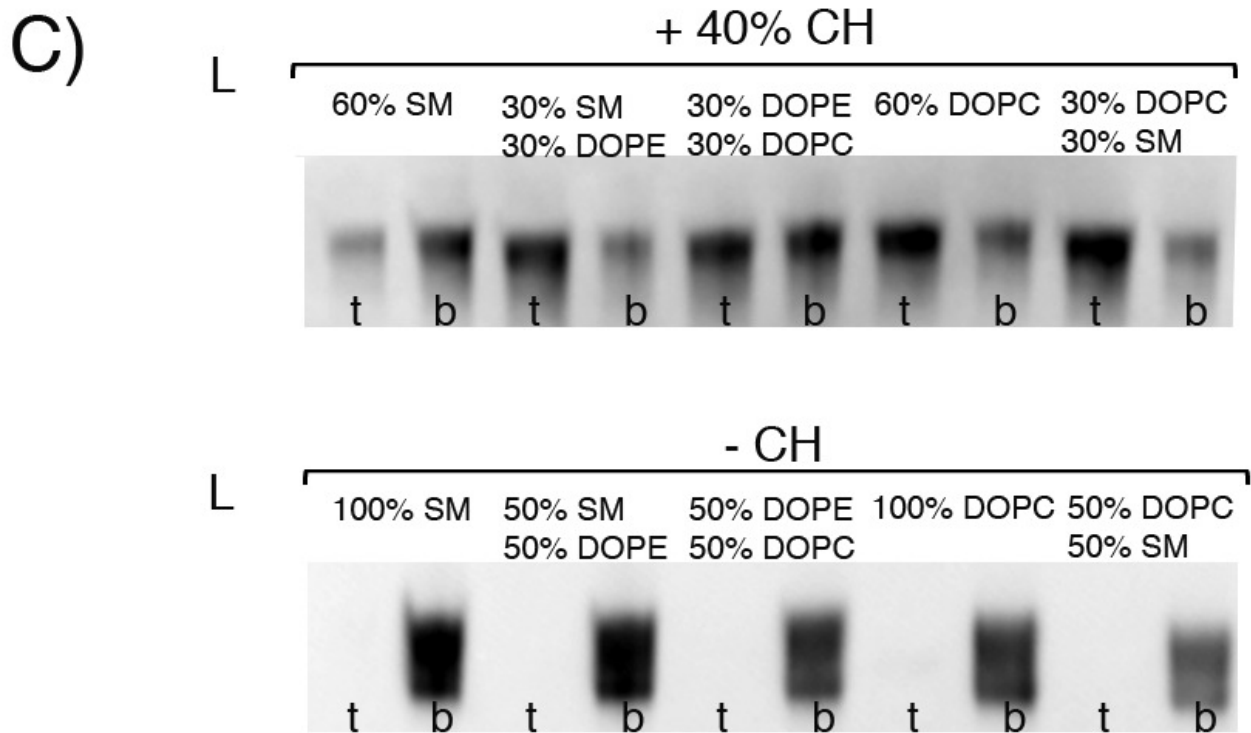
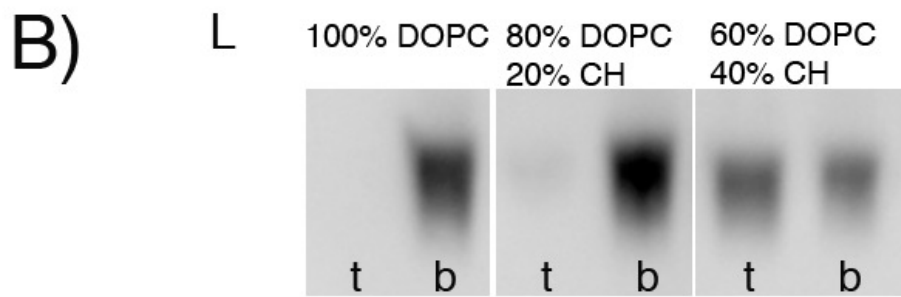
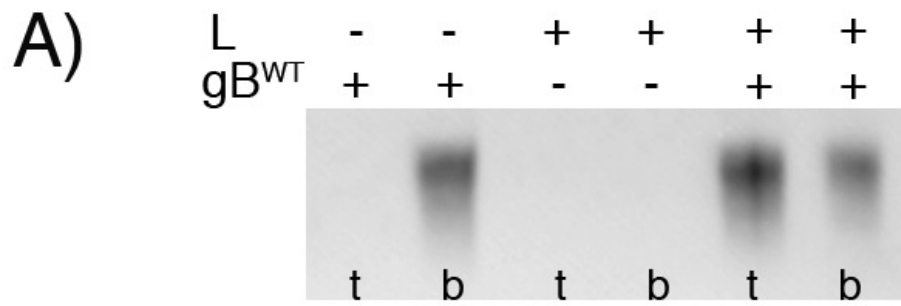
	Sequence identity (%) ^a	n _A /n _T ^b	rmsd (Å) ^c
PrV vs HSV-1	50.1	524/568	1.04
PrV vs CMV	25.6	508/535	3.39
PrV vs EBV	25.3	496/530	3.72

1213 ^a – percent identity has been calculated using the shorter sequence as denominator1214 ^b – n_A and n_T indicate, respectively, the number of aligned and total atoms1215 ^c – the root-mean-square deviations (rmsd) were calculated for C α atoms in PyMOL (104)

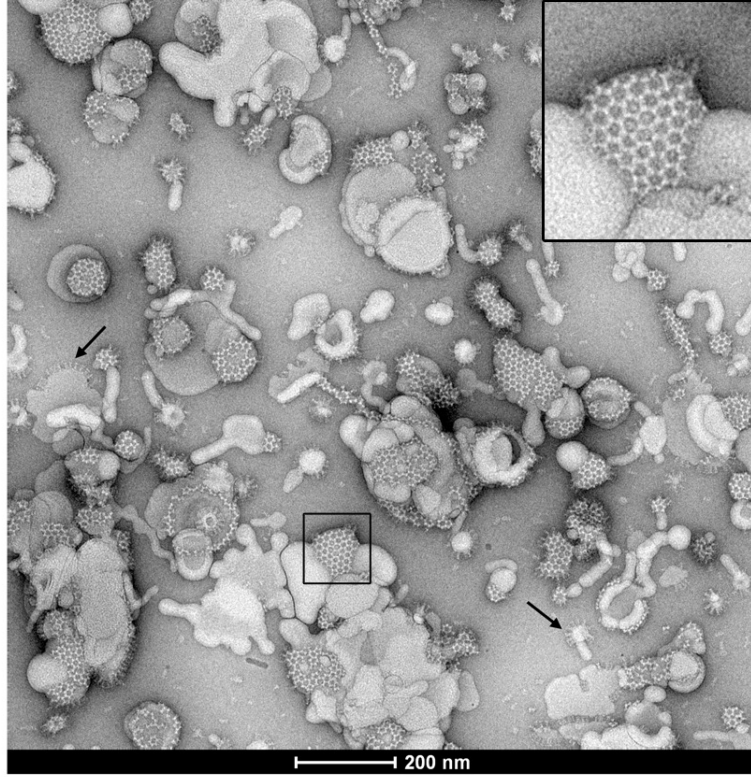
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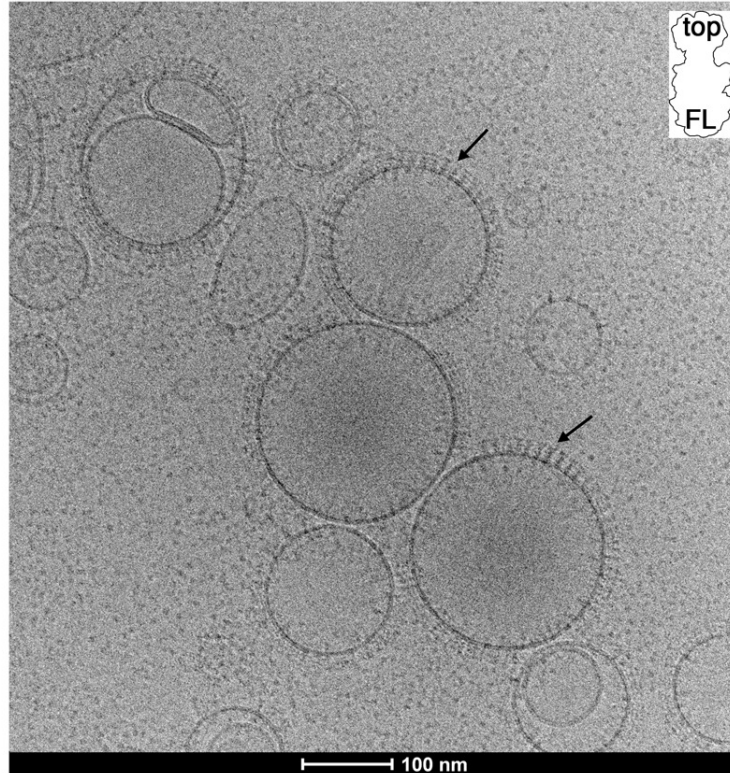




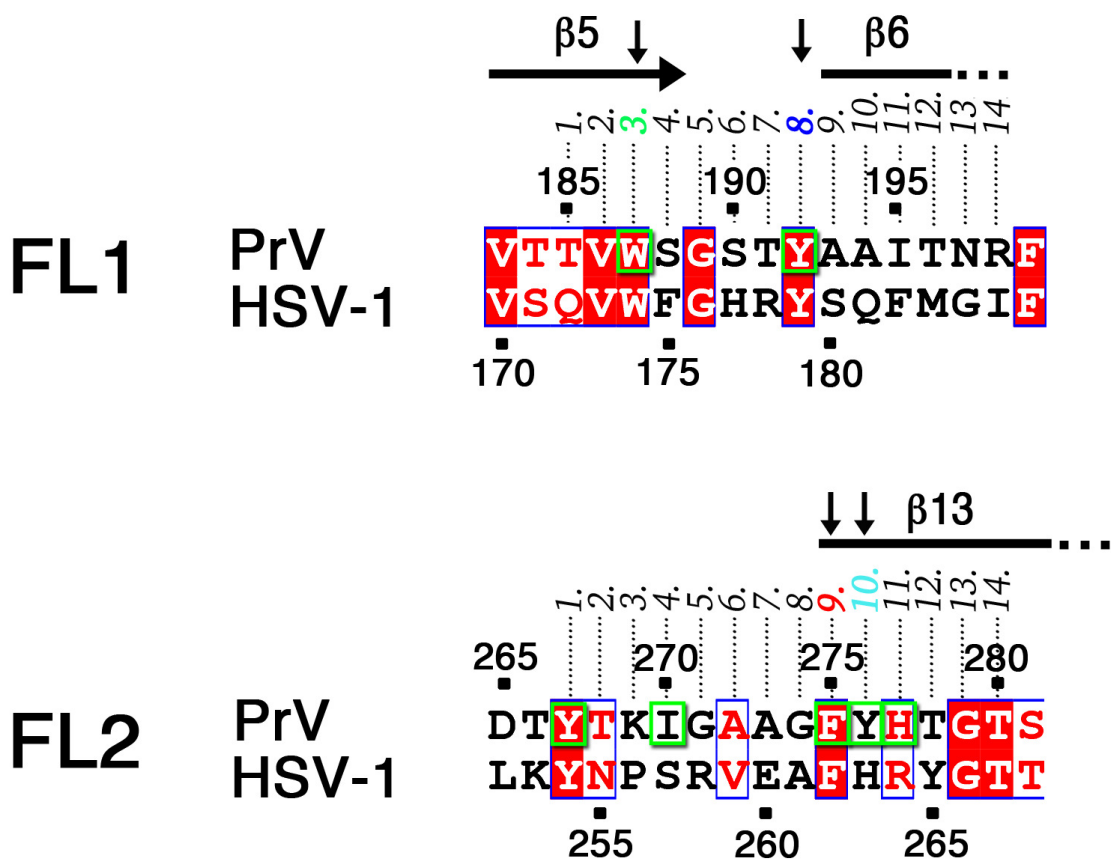
A) Negative stain



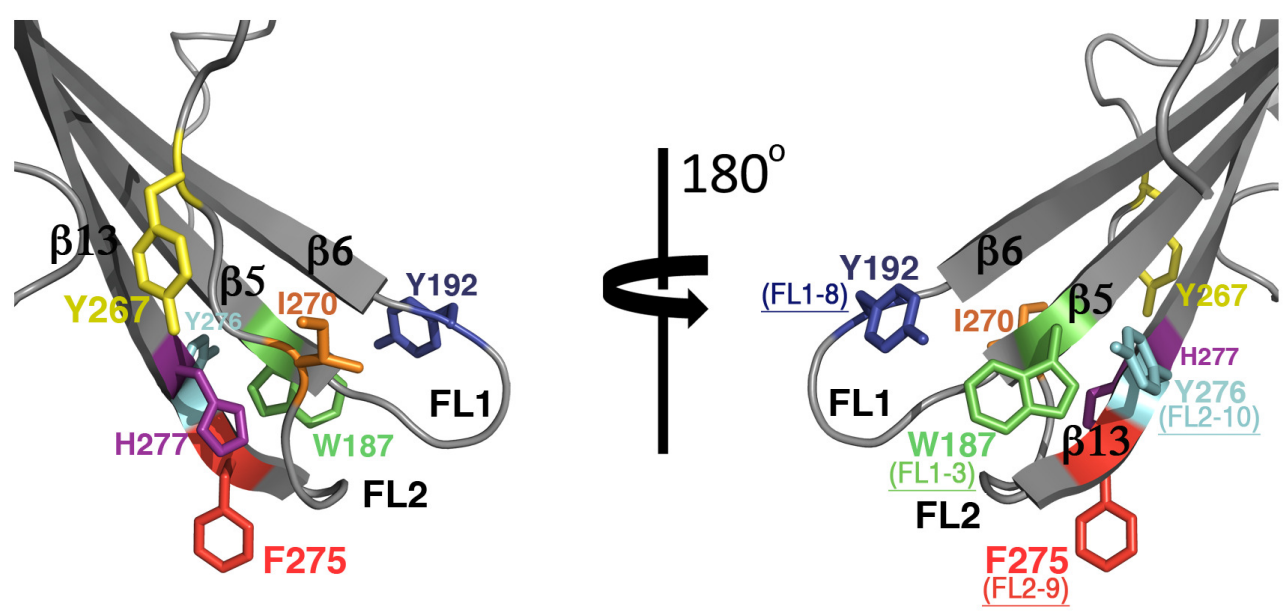
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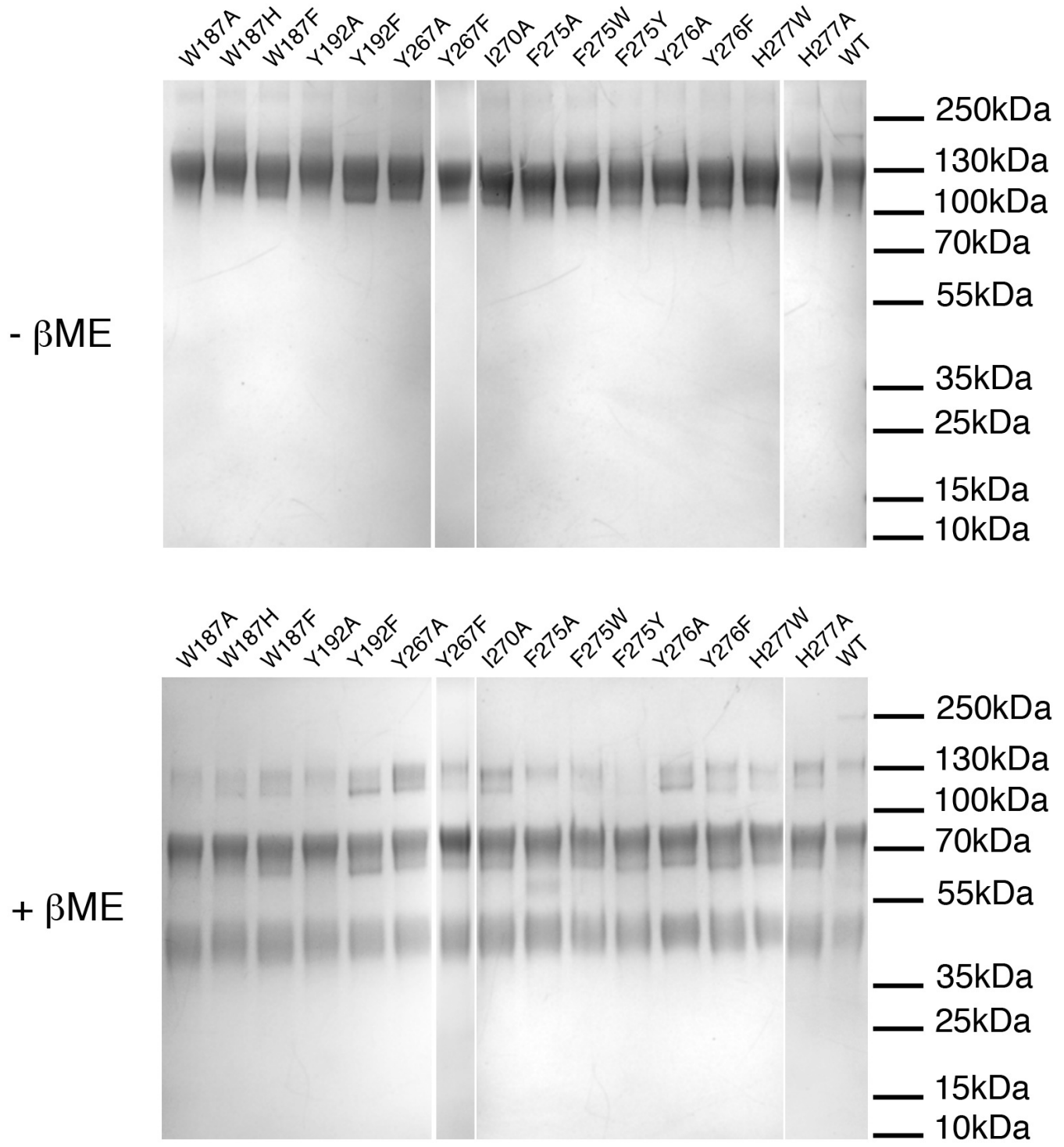


A)

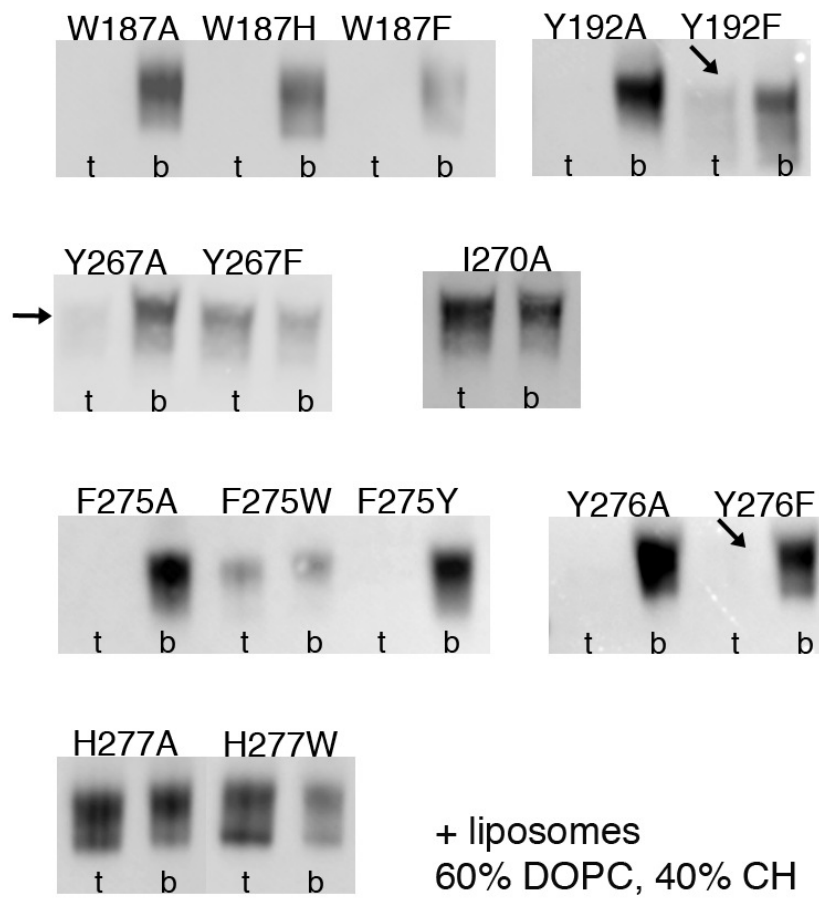


B)





A)



B)

