Subcellular trafficking and functional importance of herpes simplex virus type 1 glycoprotein M domains

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Herpes simplex virus type 1 (HSV-1) glycoprotein M (gM/UL10) is a 473 aa type III transmembrane protein that resides in various membrane compartments. HSV-1 gM contains several putative trafficking motifs, but their functional relevance remains to be elucidated. We show here that transiently expressed gM 19–343 was sufficient for transport to the trans-Golgi network (TGN), whilst gM 133–473, where the first two transmembrane domains were deleted, and gM 1–342, which lacked the final residue of the last transmembrane domain, were retained in the endoplasmic reticulum (ER), indicating that all transmembrane domains are required for proper folding and ER exit. A series of bacterial artificial chromosome mutants revealed that in addition to the authentic start codon, translation of gM can be initiated at methionine 19 and 133/135. Whilst a protein lacking the first 18 residues supported WT-like growth, gM 133/135–473 resulted in reduced plaque diameters resembling a UL10 deletion mutant. An HSV-1 mutant encoding gM 1–342 showed similar growth characteristics and accumulated non-enveloped cytoplasmic particles, whilst gM 1–343 resulted in a gain of function, indicating that all transmembrane domains of the protein are important for viral growth. A C-terminal extension further supported viral propagation; however, the C-terminal trafficking motifs (residues 423–473) were completely dispensable. We propose a functional core within gM 19–343 comprised of all transmembrane domains that is sufficient to target the protein to the TGN, a favoured site for envelopment, and to support viral functions.

INTRODUCTION

Herpesviruses have evolved a life cycle that strongly depends on membrane-associated processes (Johnson & Baines, 2011; Mettenleiter et al., 2009) involving a number of virally encoded transmembrane proteins. A small set consisting of glycoproteins (g) B, gH, gL, gM and gN is evolutionarily conserved, indicating important roles in viral entry and maturation. Whilst gB, gH and gL are essential in all herpesviruses, gM of most alpha-herpesviruses is not essential for viral growth in cultured cells (Baines & Roizman, 1991; Browne et al., 2004; Dijkstra et al., 1996; Fuchs & Mettenleiter, 1999; Lehee et al., 2009; Osterrieder et al., 1996; Ren et al., 2012; Sadaoka et al., 2010).
Herpes simplex virus type 1 (HSV-1) gM encoded by the UL10 ORF is a type III transmembrane protein comprised of 473 aa. Whilst detailed topological data are lacking, a model predicts that it folds into eight membrane-spanning domains with the N- and C-terminal ends oriented towards the cytosol (Crump et al., 2004), and this has been supported by recent evidence (Raschbichler et al., 2012). HSV-1 gM is expressed as a 47 kDa precursor that matures into a family of 53–63 kDa glycosylated proteins (Baines & Roizman, 1993). An additional layer of complexity is generated by alternative start codons at positions 19 and 133/135 that are used in addition to the authentic start codon, leading to gM proteins varying in length (Chouljenko et al., 2012; El Kasmi & Lippe´, 2014). Although the function of gM remains unclear, its presence in extracellular virions (Baines & Roizman, 1991; Loret et al., 2008) requires that gM participates in secondary envelopment (Crump et al., 2004; Klupp et al., 2000; Maringer et al., 2012; Ren et al., 2012; Sadaoka et al., 2010). Its ability to modulate membrane fusion induced by the viral proteins gB, gH/gL and gD hints at a role of gM in entry (Crump et al., 2004; Kim et al., 2013; Klupp et al., 2000; Koyano et al., 2003; Lau & Crump, 2015). In transfected cells, gM drives the internalization of several proteins located to the plasma membrane, including gH/gL and gD (Crump et al., 2004), a function that is redundant in the viral context (Lau & Crump, 2015; Ren et al., 2012).

A striking feature of HSV-1 gM is its targeting to various membrane compartments (Baines et al., 2007; Crump et al., 2004; El Kasmi & Lippe´, 2014; Lau & Crump, 2015; Stylianou et al., 2009; Wills et al., 2009; Zhang et al., 2009). Generally, proteins are selectively transported to their destination based on signal sequences that are recognized by transport machineries. The cytoplasmic tail of gM harbours several potential trafficking sequences, including four Y-motifs matching the consensus YXXφ (Y, tyrosine; X, any amino acid; φ, bulky hydrophobic amino acid) as well as one acidic cluster (DExxDDxxD) (Figs 1a and 2a) (Crump et al., 2004) that were reported to serve a function in endoplasmic reticulum (ER) exit and endocytic retrieval of transmembrane proteins (Favoreel, 2006). However, the role of these motifs for HSV-1 gM has yet to be clarified.

We aimed to gain further insight into the subcellular targeting information embedded within gM (Baines et al., 2007; Crump et al., 2004; Wills et al., 2009; Zhang et al., 2009). Our data reaffirmed that the targeting of gM to the trans-Golgi network (TGN) occurs in the absence of other viral factors, as shown in previous studies (Crump et al., 2004; Stylianou et al., 2009), and demonstrated for the first time, to the best of our knowledge, that this is independent of both the C-terminal domain including all trafficking motifs and homo-oligomerization. Variants of gM with truncated transmembrane regions were unable to target to the TGN and remained in the ER. Bacterial artificial chromosome (BAC) mutagenesis revealed that HSV-1 gM mutants compromised in transmembrane domains exhibited significantly reduced plaque diameters. In contrast, HSV-1 gM mutants lacking all C-terminal trafficking motifs (gM 1–422) showed WT-like growth properties and further C-terminal deletions up to residue 343 only moderately affected viral growth. Thus, we identified a functional core of gM comprised of residues 19–343 that is sufficient to support viral functions.

RESULTS

**gM residues 19–473, but not residues 133–473, are targeted to the TGN**

Recent evidence supports an alternative initiation codon for gM at methionine 19 (Fig. 1a) (Chouljenko et al., 2012). Other in-frame initiation codons exist further downstream at positions 133 and/or 135 (Fig. 1a) (El Kasmi & Lippe´, 2014). Whilst gM 1–473 and gM 19–473 are predicted to give rise to gM equipped with eight transmembrane-spanning domains (Fig. 1a) (Crump et al.,...
Fig. 2. Cytoplasmically exposed trafficking motifs of gM are not involved in targeting to the TGN. (a) Schematic diagram of the four Y-motifs (upper case letters) and the acidic cluster (underlined) in the cytoplasmic tail of gM. (b) HeLa cells transfection with plasmids encoding HA-tagged gM, gM 1–433, gM 1–422, gM 1–361, gM 1–342, gM 1–361-Ala, gM 1–343-Strep or TM-gM 305–473. IF was performed with anti-HA and anti-TGN antibodies. TM, transmembrane domain. (c) HA-gM and gM 1–342 were transiently expressed in HeLa cells, IF was performed with anti-HA and anti-calreticulin antibodies. (b, c) Nuclei were visualized by DAPI staining. (d) Cell lysates of 143B cells transiently expressing HA-gM or HA-gM 1–342 were treated with Endo H (+ Endo H) or incubated in the absence of the enzyme (− Endo H). Cell lysates were probed for gM (for HA-gM) or HA-tag (for HA-gM 1–342) by Western blotting.
2004), a protein initiated at codon 133 or 135 would lack the first two transmembrane regions (Fig. 1a). To investigate the role played by the N-terminal regions of gM in subcellular trafficking, full-length gM, gM 19–473 or gM 133–473 were expressed in HeLa cells and analysed at 20 h post-transfection (p.t.) using indirect immunofluorescence (IF). gM 19–473 co-localized with the TGN marker comparable to full-length gM (Fig. 1b). In contrast, gM 133–473 exhibited an ER-like distribution. Thus, whilst the first 18 residues are not essential for targeting of gM to the TGN, two hydrophobic regions present within residues 19–132 were required to release the protein variant from the ER.

**Cytoplasmically exposed trafficking motifs of gM are not involved in targeting to the TGN**

Several putative motifs have been reported in the cytoplasmic tail of gM (Fig. 2a) (Crump et al., 2004) that is exposed to the cytoplasm as demonstrated by the use of gM in the NEX-TRAP (Nuclear EXport Trapped by RAPamycin) (Raschbichler et al., 2012). To analyse whether these are required for ER exit and/or TGN targeting, the four Y-motifs and the acidic cluster were deleted sequentially. The resulting haemagglutinin (HA)-tagged mutants were expressed in HeLa cells and analysed at 20 h post-transfection (p.t.) using IF (Fig. 2b). Surprisingly, deletion of all trafficking motifs leaving ~20 residues of the cytoplasmically exposed C-terminal domain (gM 1–361) did not interfere with accumulation of gM at the TGN (Figs 2b and S1, available in the online Supplementary Material). It is worth noting that in addition to the localization at the TGN, some cytoplasmic and surface staining could be observed upon expression of gM and mutants thereof (Figs 2b and S1). We conclude that WT gM and mutants lacking the C-terminal extension are primarily targeted to the TGN, but also partially co-localize with other compartments in transfected cells, suggesting that gM is actively retained in or retrieved to the TGN. Interestingly, gM 1–342 lacking the entire C-terminal domain showed a distribution reminiscent of ER residents and rarely co-localized with the TGN marker. An alanine scan of residues 343–361 had no influence on targeting of gM 1–361 to the TGN (Fig. 2b). This suggested that rather than missing trafficking signals, gM 1–342 lacked the end of the final transmembrane domain, resulting in a misfolded protein retained by the ER quality control system (Fig. 2b).

In agreement with the above scenario, a gM mutant containing arginine 343 extended by the Streptag enabled the forward transport of the resulting protein (gM 1–343-Strep) to the TGN (Fig. 2b). A fusion protein composed of the last C-terminal transmembrane region and the cytoplasmic domain of gM 305–473 showed a phenotype resembling gM 1–342 with a distinct ER-like distribution at the nuclear perimeter (Fig. 2b), suggesting that the C-terminal tail and the associated last transmembrane domain of gM do not confer efficient TGN targeting. Taken together, we concluded that all transmembrane regions were required and sufficient to mediate targeting of gM to the TGN.

**Presence and integrity of the N-terminal transmembrane domains is required for gM glycoprotein maturation**

As with numerous other integral membrane proteins, gM synthesized in the ER is modified by the addition of high-mannose-type oligosaccharides which are converted to complex-type sugars upon arrival at the Golgi apparatus (Baines & Roizman, 1991; Zhang et al., 2009). Unlike full-length gM that located to the TGN (Figs 1b and 2b), gM 133–473, which lacked the first two transmembrane domains, and gM 1–342, which lacked the final end of the last transmembrane domain, showed an ER-like distribution (Figs 1b and 2b). This suggested that both proteins were unable to leave the ER due to partial misfolding. The co-localization of gM 1–342 with the ER marker calreticulin further substantiated this theory (Fig. 2c). For biochemical verification, we took advantage of endoglycosidase H (Endo H), an enzyme that cleaves immature but not mature oligosaccharide forms (Tarentino et al., 1973). Lysates of 143B cells expressing HA-tagged gM variants for 20 h were thus subjected to Endo H treatment and analysed by Western blot (Figs 2d and S2). In the absence of enzyme, full-length gM separated into two bands consistent with heterogeneously modified forms of gM, whilst gM 1–342 migrated as a single band (Fig. 2d). Endo H treatment revealed that the upper band of full-length gM was Endo H resistant and hence had mature sugars, whilst the lower band migrated faster, consistent with Endo H sensitivity and ER-derived immature sugars. Endo H treatment of gM 1–433, gM 1–422 and gM 1–361 also resulted in Endo H-resistant forms, whilst Endo H-sensitive forms co-existed (Fig. S2). In contrast, the single form of gM 1–342 was fully sensitive to the glycosidase and migrated at 37 kDa, i.e. the expected molecular mass of the unmodified protein (Figs 2d and S2). The gM 1–343-Strep fusion protein, however, behaved similar to full-length gM and the C-terminal truncation mutants (Fig. S2). Thus, gM 1–342 was retained in the ER but released upon addition of the Streptag extension. As expected, gM 305–473 that lacked putative ER exposed glycosylation sites was unaltered by Endo H treatment (Fig. S2). Taken together, the data showed that the presence and integrity of the transmembrane domains is required and sufficient for gM to exit the ER.

**Targeting of gM to the TGN does not involve homo-oligomerization**

Homo-oligomerization could potentially stabilize gM, thereby supporting its targeting to the TGN. In addition, homo-oligomerization may generate an ER exit motif or alternatively mask an ER retrieval signal. To determine whether gM was able to self-interact, the yeast two-hybrid system was applied. For a control, we analysed the interaction of gM with US8A, a previously reported HSV-1 gM interactor (Fossum et al., 2009). Only yeast cells expressing both gM and US8A were able to grow on medium selective for reporter gene activity (Fig. 3a). This
suggested that whilst gM was able to interact with US8A, it was unable to form homo-oligomers.

To substantiate this finding, gM and gM-ER, a gM fusion protein carrying a KKEL ER retrieval sequence at its C-terminal end, were co-expressed in mammalian cells (Fig. 3b, c). A potential homo-oligomeric complex involving gM and gM-ER would be expected to be retrieved to the ER upon arrival at the cis-Golgi. However, HA-gM was efficiently targeted to the TGN and did not significantly overlap with the co-expressed gM-ER (Fig. 3c). Similarly, HA-gM 1–361 (normally targeted to the TGN) and gM-ER exhibited distinct localizations. In contrast, co-expression of HA-gM 1–342 and c-Myc-gM-ER resulted in complete co-localization at the ER. Thus, no evidence for homo-oligomerization of full-length gM was seen in at least two distinct assays, suggesting that retention of HA-gM 1–342 at the ER was unlikely due to an inability to assemble homo-oligomers.

**BAC mutagenesis of HSV-1 gM/UL10**

To determine the functional importance of gM domains for viral replication, a series of BAC mutants was generated (Fig. 4a–i, Tables S1 and S2). Lox-UL10_mt1 contained a premature stop codon at codon 3, but retained two alternative start codons at codon 19 and 133/135 (Fig. 4b). Lox-UL10_mt2 contained an additional point mutation at position 19 leading to the exchange of a methionine for isoleucine (M19I), whilst the stop codon at position 3 was preserved (Fig. 4c). Insertion of the galK-Kan cassette into Lox-UL10_mt2 resulted in Lox-UL10_mt3, thereby completely disrupting the UL10 ORF (Fig. 4d). Several BAC mutants encoded gM variants with C-terminal truncations: Lox-UL10_mt4 (gM 1–422; Fig. 4e), Lox-UL10_mt5 (gM 1–361-Strep; Fig. 4f), Lox-UL10_mt6 (gM 1–343-Strep; Fig. 4g) and Lox-UL10_mt7 (gM 1–342; Fig. 4h). Finally, the revertant Lox-UL10_mt7 rev was generated based on Lox-UL10_mt7 (Fig. 4i). Reconstitution of virus was achieved by transfection of BAC DNA into Vero and 143B cells.

In cells infected with the parental Lox, a prominent band of ~53/63 kDa and a minor band of 47 kDa were detected by Western blot analysis using antibodies specific to the C-terminal domain of gM (Fig. 4j). Lox-UL10_mt1 encoded a less abundant protein that migrated slightly faster than the WT gM (Fig. 4j) in accordance with translational initiation at methionine 19 (Chouljenko et al., 2012). In lysates of cells infected with Lox-UL10_mt2, that had methionine 19 replaced by isoleucine, protein bands migrating at ~45 kDa were detected that, however, were
Fig. 4. BAC mutagenesis of HSV-1 gM/UL10. (a) Schematic diagram of the HSV1(17+)lox genome UL10 locus. Arrows indicate three alternative start codons at codons 1, 19 and 133/135. (b–i) A series of UL10 viral mutants was generated. Arrowheads indicate disabled start codons or introduced stop codons. (j) Expression of gM in Vero cells infected with HSV-1 mutants or the parental Lox for 12 h was monitored by Western blotting using anti-gM and anti-ICP0 antibodies.
absent in mock-infected cells (this study; El Kasmi & Lippé, 2014). In accordance with a Kozak sequence upstream of codon 133/135, translational initiation most likely occurred at either or both of these methionines (Figs 1a and 4j). No bands were detected when cells were infected with Lox-UL10_mt3, consistent with the full deletion of UL10, whilst infection with Lox-UL10_mt7 rev resulted in a protein pattern resembling the parental Lox (Fig. 4j). Due to the lack of antibodies recognizing the N-terminal part of gM, the C-terminally truncated mutants of gM were not analysed by Western blot. Thus, UL10 contained at least three alternative start sites leading to gM proteins varying in length and efficiency of expression (this study; Chouljenko et al., 2012; El Kasmi & Lippé, 2014).

A gM core composed of all transmembrane domains is sufficient to support viral functions

To determine the growth properties of the novel gM/UL10 mutants, Vero cells were infected and the area of the resulting plaques was analysed microscopically at 3 days post-infection (p.i.) (Fig. 5a, b). Lox-UL10_mt1 encoding gM 19–473 resulted in WT plaques. In contrast, Lox-UL10_mt2 encoding gM 133/135–473 generated plaques comparable to the deletion mutant Lox-UL10_mt3. Lox-UL10_mt4 (gM 1–422) that retained 78 C-terminal residues but lacked all potential trafficking motifs also resulted in WT plaques was analysed microscopically at 3 days post-infection (p.i.) (Fig. 5a, b). Lox-UL10_mt1 encoding gM 19–473 resulted in WT plaques. In contrast, Lox-UL10_mt2 encoding gM 133/135–473 generated plaques comparable to the deletion mutant Lox-UL10_mt3. Lox-UL10_mt4 (gM 1–422) that retained 78 C-terminal residues but lacked all potential trafficking motifs also resulted in WT plaques (Fig. 5a, b). Lox-UL10_mt5 (gM 1–361) that retained ~20 C-terminal residues and Lox-UL10_mt6 (gM 1–343) that lacked the complete C-terminal domain were only moderately affected in growth (Fig. 5a, b). In contrast, plaques produced by Lox-UL10_mt7 (gM 1–342) resembled those of Lox-UL10Mt2 or Lox-UL10_mt3. However, the revertant Lox-UL10_mt7 rev, which was generated by a two-step process using Lox-UL10_mt7 as a starting point, resulted in plaques comparable to the WT. Thus, whilst the first 18 residues of gM as well as the C-terminal domain were dispensable for the function of gM, gM mutants where the transmembrane regions were compromised resulted in phenotypes resembling the UL10 deletion variant (Fig. 5a, b). Taken together, residues 19–343 represented the core of gM required for normal spread of the virus.

To further evaluate the phenotype of these mutants, the replication efficiency was analysed on 143B, BHK-21 or Vero cells at m.o.i. 0.1 or 10 using HSV-1 Lox-UL10_mt1 and Lox-UL10_mt7 as an example (Fig. S3). The parental Lox and Lox-UL10_sha1 showed similar growth behaviour at both m.o.i. In contrast, Lox-UL10_mt7 expressing a non-functional gM showed slightly delayed growth at a low m.o.i. Most interestingly, infection with Lox-UL10_sha7 at a high m.o.i. resulted in a marked growth inhibition of up to 2 log. This growth defect varied depending on the cells used for infection, being marginal in 143B cells but significant in BHK-21 and Vero cells. We thus concluded that HSV-1 Lox-UL10_sha7 exhibited a growth defect that was modulated by host factors.

Characterization of gM variants during the course of infection

To follow the localization of the gM mutants during the course of infection, Vero cells were infected with the parental HSV-1 Lox or the mutants described in Fig. 4. Infected cells were analysed using antibodies specific to either gM (Fig. 6a) or Strep-tag (Fig. 6b). To monitor the infection, ICP8-specific antibodies were used (Fig. 6a, b). gM expressed by Lox or Lox-UL10_sha1 showed a clustered localization adjacent to the nucleus reminiscent of the TGN (Fig. 6a). A similar localization was observed for gM 1–361-Strep and gM 1–343-Strep expressed by Lox-UL10_sha5 and Lox-UL10_sha6, respectively (Fig. 6b). In contrast, gM 133/135–473 expressed by Lox-UL10_sha2 showed an ER-like distribution, consistent with the localization during transient transfection (Fig. 1b). No signal was observed for Lox-UL10_sha4 and Lox-UL10_sha7, consistent with the deletion of the C-terminal epitope recognized by the gM antibody. We thus concluded that the subcellular distribution of the gM mutants was comparable in transfected and infected cells, suggesting that viral proteins were unable to compensate for the defect in ER to TGN trafficking.

Lox-UL10_sha2 and Lox-UL10_sha7 encoded non-functional proteins retained in the ER. To determine whether infection with either of these viral mutants resulted in ER stress, Vero cells were mock infected, or infected with the respective virus or the parental Lox at m.o.i. 1 for 12 h. In parallel, cells were treated with thapsigargin, a drug known to induce expression of the cellular chaperone BiP (binding immunoglobulin protein), a marker for ER stress (Goodwin et al., 2011; Shukla et al., 2013), or left untreated. Whilst thapsigargin readily induced the expression of BiP, no BiP could be detected in lysates of cells infected with either virus (Fig. 6c). Thus, whilst Lox-UL10_sha2 and Lox-UL10_sha7 encoded non-functional proteins unable to support efficient viral replication, these proteins did not induce ER stress.

Expression of truncated gM variants allows for efficient localization of gD and gH at the TGN

Trafficking motifs of gM have been reported to induce the relocation of gD, gH and some host proteins from the plasma membrane to the TGN (Crump et al., 2004; Lau & Crump, 2015; Ren et al., 2012). To analyse whether the absence of the gM C-terminal trafficking motifs or a non-functional gM truncation had an influence on the TGN targeting of the viral glycoproteins gH and gD, HeLa cells were infected with virus derived from either HSV-1 Lox-UL10_sha4 encoding gM 1–422 or Lox-UL10_sha7 encoding gM 1–342 as well as the parental strain and analysed by indirect IF at 12 h.p.i. (Fig. 6d). Either gH and gD primarily located to the TGN in both mutant viruses, suggesting that localization of these viral transmembrane proteins to the TGN occurred by a mechanism independent of gM and consistent with recent reports (Lau & Crump, 2015; Ren et al., 2012).
Given the growth defect of HSV-1 Lox-UL10_mt7 (Figs 5a, b and S3), we aimed to compare this viral mutant to the parental Lox and the mutant Lox-UL10_mt1 by electron microscopy in infected BHK-21 cells (Fig. 7a). BHK-21 cells were chosen due to their most pronounced growth defect (Fig. S3). Whilst nuclear capsid formation and DNA packaging were comparable in all strains analysed, non-enveloped particles were over-represented in the cytoplasm.

**Fig. 5.** A gM core composed of all transmembrane domains is sufficient to support viral functions. (a) Plaque areas generated by infection of Vero cells with HSV-1 Lox or Lox-UL10 mutants were analysed microscopically at 3 days p.i. (b) Plaque areas were measured using Adobe Photoshop or Zeiss Axiovision software. Statistical analyses of the plaque areas of all mutants are presented, average values represent a minimum of 9 measured plaques. The respective bar corresponds to the standard deviation.
upon infection with HSV-1 Lox-UL10_mt7 (Fig. 7b).
Moreover, compared with the parental Lox and the mutant Lox-UL10_mt1, Lox-UL10_mt7 resulted in a pronounced lack of surface virions. In summary, our data supported a functional role of gM at the TGN, conceivably to recruit cellular and/or viral components to the site of secondary envelopment.

**DISCUSSION**

During HSV-1 infection, gM is targeted to distinct membrane compartments (Baines & Roizman, 1993; Baines et al., 2007; Crump et al., 2004; Lau & Crump, 2015; Loret et al., 2008; Maringer et al., 2012; Stylianou et al., 2009; Wills et al., 2009; Zhang et al., 2009) and regulated
(a) Mock vs Lox

Lox-UL10_mt1

Lox-UL10_mt7

(b) Capsid per cell (%)

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* P < 0.05
** P < 0.01
*** P < 0.001

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targeting of gM to these membranes is presumably important for its proper viral function(s). We found that gM reaches the TGN independent of other viral proteins and of homo-oligomerization, and that targeting information is present within the eight transmembrane regions of gM.

Our current knowledge of the biosynthesis of complex multi-membrane-spanning proteins is rather limited (Martínez-Gil et al., 2011). Folding and interaction of α-helical membrane domains is proposed to occur in a two-step process: (i) membrane incorporation of single transmembrane domains and (ii) formation of higher-order transmembrane structures. We show here that an N-terminal domain consisting of residues 19–343 was sufficient for trafficking of gM to the TGN and for efficient viral propagation. Thus, the first 18 residues and the complete C-terminal domain are dispensable for proper membrane insertion and folding. gM 133/135–473 and gM 1–342, where the two first or the last transmembrane domains, respectively, were compromised, were retained in the ER and non-functional. We therefore conclude that all eight transmembrane domains of gM are required for correct membrane insertion and bundling into a core unit that is released from the ER and important for viral function. Furthermore, this study shows that HSV-1 infection provides a valuable functional system to gain insight into the biogenesis of complex transmembrane proteins.

Er retention of the N- and C-terminal deletions gM 133–473 and gM 1–342 could be mediated by the ER quality control system. Interestingly, viral mutants expressing these gM variants do not induce expression of BiP, a chaperone known to compensate ER stress. Furthermore, our data suggest that homo-oligomerization of gM is unlikely to occur in the ER and consequently does not play a role in ER exit. Alternatively, assembly of all transmembrane domains may constitute an ER exit signal or mask an ER retrieval motif, possibilities that await further analysis.

Recent data provided evidence for a second start site at position 19 of UL10 that results in a gM variant that is fully functional. However, in the absence of methionine 1 and 19, an additional even shorter gM variant was still expressed, although it was less abundant. Consistent with the size of the protein detected in Lox-UL10_mt2 and a well-conserved Kozak sequence preceding codons 133 and 135, one or both of these codons are likely used for translational initiation. Whether proteins varying in N-terminal length co-exist during HSV-1 WT infection is currently unknown, but interesting considering the complex situation observed for varicella-zoster virus gM (Sadaoka et al., 2010) and human cytomegalovirus (HCMV) UL136 (Caviness et al., 2014). Insertion of a galK-Kan cassette downstream of codons 133/135 into the backbone of Lox-UL10_mt2 resulted in Lox-UL10_mt3, a mutant carrying a full deletion of UL10. Virus derived from Lox-UL10_mt3 resembled the previously published mutants summarized in Table S2 (Baines & Roizman, 1991; Browne et al., 2004; Lau & Crump, 2015; Legee et al., 2009; MacLean et al., 1991, 1993; Ren et al., 2012). All but one of these mutants that carried a short C-terminal deletion (Browne et al., 2004) showed a five- to 10-fold reduction in replication efficiency. Thus, although not essential, gM/UL10 is important for efficient viral replication.

We found that Lox-UL10_mt4 encoding gM 1–422, but lacking all trafficking motifs, showed WT-like growth behaviour, and sequential deletion of additional C-terminal sequences correlated well with a gradual and moderate loss of function. In contrast, Lox-UL10_mt2 (gM 133/135–473), which lacked the first transmembrane domains, and Lox-UL10_mt7 (gM 1–342), which lacked a positively charged residue following the last transmembrane domain, resembled the full deletion variant Lox-UL10(mt3). Consistently, cells infected with virus derived from Lox-UL10(mt7 revealed an increase in naked cytoplasmic virosomes concomitant with a defect in secondary envelopment, in line with results from a UL10 deletion variant (Legee et al., 2009). Importantly, mutants Lox-UL10(mt5 and Lox-UL10(mt6 that were generated in parallel to Lox-UL10(mt7 and merely deviated in short C-terminal extensions resulted in a gain of function. Furthermore, the revertant Lox-UL10(mt7 rev regained full viability, thus indicating the integrity of the BAC backbone. Together, this reiterates that the eight transmembrane regions of gM form a core structure that supports vital functions of gM in an otherwise WT background. We thus conclude that the core formed by all eight transmembrane domains of gM is important for secondary envelopment of capsids, most likely by recruiting viral and cellular factors.
Previous data support a role of gM in endocytic retrieval of viral glycoproteins from the cell surface to maintain optimal levels for assembly of infectious virions (Crump et al., 2004; Lau & Crump, 2015; Ren et al., 2012). The cytoplasmic tail of gM harbours several potential trafficking sequences, including four Y-motifs and one acidic cluster. These kind of motifs, found within many viral transmembrane proteins, were previously shown to play a role in endocytosis (Favoreel, 2006). We show here that in transfected cells, full-length gM and all mutants that contained an intact core (residues 19–343), but lacked the trafficking motifs, showed a prominent localization at the TGN and some additional localization in other compartments. In the viral context, gM and respective mutants also localized to the TGN. Furthermore, gD and gH were efficiently targeted to the TGN upon viral infection irrespective of a functional gM. Finally, viral mutants lacking the endocytic motifs support viral growth similar to the WT. We therefore conclude that the endocytic information harboured within the tail of gM seems to be redundant with motifs present in the core of gM and/or in other viral proteins. This is in line with recent studies that show that the role of gM in endocytic retrieval of viral glycoproteins that are essential for viral entry is redundant in the viral context (Lau & Crump, 2015; Ren et al., 2012).

Our finding that the transmembrane domains of gM form a unit for TGN targeting could point to inner-membrane interactions between gM and other transmembrane proteins. In this way, hetero-oligomeric assemblies could be established that recruit viral envelope proteins together with interacting tegument proteins to sites of virion envelopment. Naturally, cytoplasmic and luminal loops could further assist in this process. With this study, we have generated a series of novel gM/UL10 mutants suited to identify protein interactors of this highly interesting protein. Optimal candidates for viral envelope proteins are gE/gI and UL11, two proteins that cooperate with gM/UL10 in cytoplastic virion envelopment (Brack et al., 2000; Chouljenko et al., 2012; Kim et al., 2013; Lege et al., 2009; Maringer et al., 2012). gN is yet another interactor of an N-terminal part of gM (El Kasmi & Lippé, 2014; Fossum et al., 2009; Fuchs & Mettenleiter, 2005; Koyano et al., 2003; Mach et al., 2000, 2005; Wu et al., 1998). These options are currently being evaluated.

A contribution of cellular factors to the phenotype of Lox-UL10_m7 is supported by its cell-type dependency. Cellular proteins may directly or indirectly interact with gM during secondary envelopment. Indeed, MS analysis has identified numerous host proteins packed into mature extracellular virions (Loret et al., 2008), supported by data from HCMV (Krzyzaniak et al., 2009). Thus, our novel mutants are valuable tools to identify and analyse the contribution of cellular proteins to secondary envelopment.

METHODS

Cells and viruses. HeLa (ATCC CCL-2), 143B, a human osteosarcoma cell line (ATCC CRL-8303), BHK-21 (ATCC CCL-10) and Vero cells (ATCC CRL-1587) were cultured in Dulbecco’s modified Eagle’s medium containing 10% FCS. The strain HSV1(17+)lox (provided by B. Sodeik, Hannover Medical School, Hannover, Germany) was used for all experiments and as PCR template. Plasmid transfection was performed using Effectene Transfection Reagent (Qiagen), whilst BAC transfection was exclusively performed using Lipofectamine 2000 (Invitrogen). General cloning, BAC cloning, Endo H treatment and electron microscopy methods are detailed in Supplementary Materials. Primers and plasmids are described in Table S1.

Indirect IF analysis. Indirect IF was essentially performed as described previously (Schmidt et al., 2010). Mouse monoclonal anti-HA antibody (Santa Cruz), anti-ICP8 (provided by R. Heilbronn, Charité Universitätsmedizin Berlin CCM, Berlin, Germany) and LP11 anti-gH (Buckmaster et al., 1984) as well as rabbit polyclonal antibodies anti-TGN46 (AbD Serotec), R45 anti-gd (Cohen et al., 1978), anti-gM (provided by L. Enquist, Department of Molecular Biology, Princeton University, Princeton, New Jersey, USA; Princeton Neuroscience Institute, Princeton University, Princeton, New Jersey, USA and H. Browne, Division of Virology, Department of Pathology, University of Cambridge, Cambridge, UK) (Crump et al., 2004), anticalretucin (Sigma), anti-Strep (IBA) and anti-c-Myc (Cell Signalling) were used. All fluorescently labelled secondary antibodies were from Invitrogen. Cells were examined using a Leica confocal microscope and images were processed using Adobe Photoshop.

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