Alphaherpesvirus glycoprotein M causes the relocalization of plasma membrane proteins

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Herpesvirus glycoprotein M (gM) is a multiple-spanning integral membrane protein found within the envelope of mature herpesviruses and is conserved throughout the Herpesviridae. gM is defined as a non-essential glycoprotein in alphaherpesviruses and has been proposed as playing a role in controlling final envelopment in a late secretory-pathway compartment such as the trans-Golgi network (TGN). Additionally, gM proteins have been shown to inhibit cell–cell fusion in transfection-based assays by an as yet unclear mechanism. Here, the effect of pseudorabies virus (PRV) gM and the herpes simplex virus type 1 (HSV-1) gM/UL49A complex on the fusion events caused by the HSV-1 glycoproteins gB, gD, gH and gL was investigated. Fusion of cells expressing HSV-1 gB, gD, gH and gL was efficiently inhibited by both PRV gM and HSV-1 gM/UL49A. Furthermore, expression of PRV gM or HSV-1 gM/UL49A, which are themselves localized to the TGN, caused both gD and gH/L to be relocalized from the plasma membrane to a juxtanuclear compartment, suggesting that fusion inhibition is caused by the removal of ‘fusion’ proteins from the cell surface. The ability of gM to cause the relocalization of plasma membrane proteins was not restricted to HSV-1 glycoproteins, as other viral and non-viral proteins were also affected. These data suggest that herpesvirus gM (gM/N) can alter the membrane trafficking itineraries of a broad range of proteins and this may have multiple functions.

INTRODUCTION

During the assembly and egress of herpesviruses, two separate envelopment stages are thought to occur within host cells in the widely accepted envelopment–deenvelopment–reenvelopment model (Granzow et al., 2001; Skepper et al., 2001). A primary envelopment stage involves the budding of capsids at the inner nuclear membrane to produce enveloped particles in the perinuclear space, which subsequently fuse with the outer nuclear membrane, releasing the capsid into the cytoplasm. The secondary envelopment stage involves budding of the cytoplasmic capsids (together with tegument proteins) into the lumen of a late secretory-pathway compartment, such as the trans-Golgi network (TGN). This secondary envelopment stage is the process by which herpesviruses acquire their full complement of envelope glycoproteins (reviewed by Mettenleiter, 2002, 2003). For the assembly of herpesviruses to be successful, all components of the mature viral envelope need to be present in the final envelopment compartment. For the alphaherpesviruses such as herpes simplex virus type 1 (HSV-1), this includes at least 11 viral membrane proteins and so it is assumed that a high degree of organization is required to localize all these proteins to the correct compartment for virion incorporation. Some herpesvirus envelope glycoproteins such as the glycoprotein E/I (gE/I) complex and gB contain localization signals that allow these proteins to be targeted to the TGN independently (Alconada et al., 1999; Crump et al., 2003; Fan et al., 2002; McMillan & Johnson, 2001). However, other envelope glycoproteins such as the gH/L complex and gD of many herpesviruses do not contain any recognizable trafficking motifs and localize to the plasma membrane when expressed in cells (Hutchinson et al., 1992; McMillan & Johnson, 2001). The mechanisms by which these envelope glycoproteins may be targeted to the final envelopment compartment are currently unclear.

gM is one of the few glycoproteins to be conserved throughout the entire family Herpesviridae. Despite this conservation, gM is classed as a ‘non-essential’ protein in alphaherpesviruses because viruses with gM deletions are viable in cell culture. For members of all three herpesvirus subfamilies, gM has been shown to form a disulphide-linked complex with the gene product of UL49-5 (or homologues). In pseudorabies virus (PRV), human cytomegalovirus, Epstein–Barr virus (EBV) and human herpesvirus-8 (HHV-8), this gene product is glycosylated and is termed gN (Jons et al., 1996; Koyano et al., 2003; Lake et al., 1998; Mach et al., 2000). In contrast, the corresponding UL49-5 gene products of HSV-1 (termed UL49A), bovine herpesvirus 1 (BoHV-1) and equine herpesvirus 1 (EHV-1) do not
appear to be glycosylated and so are referred to by their gene names (UL49A or UL49-5; Adams et al., 1998; Liang et al., 1996; Rudolph et al., 2002).

Even though the gM/N complex is defined as non-essential, reports in the literature suggest potentially important roles for gM/N in viral assembly and egress. Disruption of gM coding sequences in HSV-1, PRV, EHV-1, BoHV-1 and infectious laryngotracheitis virus (ILTV) have all been reported to reduce viral titres and plaque size (Baines & Roizman, 1991; Dijkstra et al., 1996; Fuchs & Mettenleiter, 1999; Konig et al., 2002; MacLean et al., 1991, 1993; Osterrieder et al., 1996). Furthermore, disruption of gN (or the relevant homologue) coding sequences in PRV, EHV-1, varicella-zoster virus and EBV have also been reported to reduce viral titres, penetration or assembly to varying degrees (Jons et al., 1998; Lake & Hutt-Fletcher, 2000; Ross et al., 1997; Rudolph et al., 2002). Interestingly, in both PRV and EHV-1 it has been shown that, even though deletion of gM alone causes only mild defects in virus production, very severe defects in secondary envelopment are observed when gM is deleted in combination with gE and gI (Brack et al., 1999; Seyboldt et al., 2000). Taken together, these data suggest that the gM/N complex may function, at least in a redundant fashion, in the final envelopment of herpesviruses.

Included in the set of glycoproteins that need to be incorporated into the final envelope of alphaherpesviruses are the essential glycoproteins gB, gD, gH and gL. It has previously been shown that expression of these essential envelope glycoproteins from HSV-1 are necessary and sufficient to induce membrane fusion in cell-culture systems (Turner et al., 1998). Interestingly, the gM or gM/N complexes from PRV, ILTV and EHV-1 have been shown to inhibit the membrane fusion mediated by the PRV essential glycoproteins in a transfection-based assay (Klupp et al., 2000). Furthermore, gM/N complexes from HSV-1 and HHV-8 have been shown to inhibit membrane fusion caused by the HSV-1 essential glycoproteins in similar assays (Koyano et al., 2003). PRV gM demonstrated somewhat different characteristics to the gM molecules from the other herpesviruses in that PRV gM was able to inhibit membrane fusion in the absence of gN, whereas gM from EHV-1, ILTV, HSV-1 and HHV-8 all required the presence of their respective gN homologues (Klupp et al., 2000; Koyano et al., 2003). The inhibitory activity of gM on membrane fusion activity appears to have broad specificity, as PRV gM inhibits bovine respiratory syncytial virus (BRSV) F protein-induced membrane fusion, and both HSV-1 and HHV-8 gM/N complexes inhibit Moloney murine leukaemia virus (MoMLV) Env protein-induced membrane fusion (Klupp et al., 2000; Koyano et al., 2003). Furthermore, expression of BRSV F protein from recombinant BoHV-1 virions did not lead to the formation of syncytia until the gM gene was disrupted in this virus, suggesting that BoHV-1 gM expression also caused inhibition of BRSV F protein-mediated fusion (Konig et al., 2002).

We were interested in the mechanisms by which gM inhibits transfection-based membrane fusion and whether such activities of gM could correlate with a potential role of gM in the final envelopment of herpesviruses. Here we present evidence that gM/N from HSV-1 and gM from PRV cause a relocalization of several membrane proteins from the plasma membrane to the TGN, including the herpesvirus envelope proteins gD and gHgL. The mechanism by which this relocalization occurs appears to involve internalization and suggests that gM can inhibit cell–cell fusion by removing fusogenic proteins from the cell surface. These data also suggest that the gM/N complex may be involved in the correct localization of viral envelope proteins to sites of secondary envelopment.

**METHODS**

**Cells and antisera.** COS-7 and HEK-293T cells were propagated in Glasgow minimal essential medium supplemented with 10% fetal calf serum (FCS). All transfections were performed using FuGENE 6 (Roche). Monoclonal antibodies to HSV-1 gD (LP2) and gH (LP11) have been previously described (Buckmaster et al., 1984; Minson et al., 1986). Monoclonal antibody to CD8α (Ab1087) and polyclonal antisera to TGN46 (AHP500) were purchased from Abcam and Serotech, respectively. Polyclonal antisera to PRV gM (Ab183) and HSV-1 gM (Ab980) were gifts from L. W. Enquist (Princeton, USA). Polyclonal antisera to nectin-2α (R146) and the herpesvirus entry mediator (HVEM; R140) have been described previously (Terry-Allison et al., 1998; Warner et al., 1998). Polyclonal antiserum to influenza virus haemagglutinin (HA) (anti-PR8) was a gift from P. Digard (University of Cambridge, UK). Monoclonal antibody to human respiratory syncytial virus (HRSV) F protein (mAb 19) has been previously described (Taylor et al., 1992). All fluorescently labelled secondary antisera were from Molecular Probes.

**Production of rabbit polyclonal antisera to PRV gM (Ab183) and HSV gM (Ab980).** The antigen for Ab183 was a combination of two GST fusion proteins: GST–gMtail (GST fused to aa 342–393 of PRV Becker strain gM) and GST–gMlooptail (GST fused to aa 36–76 followed by three glycines and aa 342–393 of PRV gM). GST–gMtail and GST–gMlooptail were expressed in *Escherichia coli*, purified on glutathione–agarose using standard protocols, combined in a 2:1 ratio and injected into rabbits for the production of polyclonal antisera. The antigen for Ab980 was GST fused to the C-terminal 132 aa of HSV-1 strain F gM. Antigen injections and serum purification of Ab183 and Ab980 were performed by ProSci.

**Construction of expression plasmids.** Plasmids expressing HSV-1 gB, gD, gH and gL and LacZ have been described previously (Harman et al., 2002). Plasmids encoding HVEM (pBEC10) and nectin-2α (pMW20) have been described previously (Montgomery et al., 1996; Warner et al., 1998). A pcDNA3 plasmid encoding CD8α was constructed by subcloning the CD8α coding sequence from the pS84 vector (from S. Munro, Laboratory of Molecular Biology, Cambridge, UK) into the HindIII/XhoI sites in pcDNA3. A pcDNA3 plasmid encoding influenza virus strain PR8 HA was constructed by subcloning the HA coding sequence from pJZ102 (Young et al., 1983) into the HindIII site in pcDNA3. pcDNA3 plasmids encoding HRSV F protein (Long strain; pL17-F) and PRV gK (Kaplan strain) have been described previously (Bembridge et al., 2000; Klupp et al., 2000). A plasmid encoding GFP–PRV gM was generated by excising the PRV gM coding sequence from pcDNA3-PRVgM using EcoRI/XhoI and ligating into pEGFP-C3 (Clontech). A sequence containing the HSV-1 gM coding region was excised from the HSV-1 genome (strain 17; nt 23098–25190) with *Pvu*II, end...
replicated and ligated into pMV11 cut with SmaI. The entire gM coding region was excised from pMV11 by digestion with BamHI/EcoRI and ligated into pcDNA3. To construct a plasmid encoding GFP–HSV-1 gM, a BgII restriction site was introduced upstream of the gM coding region (at a site corresponding to nt 23112) by site-directed mutagenesis. The gM coding region was excised using BgII and ligated into pEGFP-C1. A sequence containing the HSV-1 UL49A coding region was excised from the HSV-1 genome (strain 17; nt position 106378–107023) with BsrBI/BamHI, end repaired and ligated into pRK19 cut with Smal. The UL49A coding region was excised using BamHI/EcoRI and ligated into pcDNA3. A pcDNA3 plasmid encoding a dominant-negative form of AP180 (AP180-C; Ford et al., 2001; Zhao et al., 2001) was constructed by excising the myc epitope-tagged AP180-C coding region and promoter region from pCMVmyc–AP180-C (a gift from B. Nichols, LMB, Cambridge, UK) with FspI/NotI and ligating into NotI-cut pcDNA3.

Transfection-based fusion assay. Fusion assays using HSV-1 gB, gD, gH and gL were performed as previously described (Harman et al., 2002). Expression plasmids encoding PRV gM, HSV-1 gM, HSV-1 UL49A or LacZ were co-transfected with the gB, gD, gH and gl expression plasmids as required. Fusion assays with HRSV F protein were performed in an identical manner but replacing expression plasmids for HSV-1 gB/D/H/L with a plasmid expressing HRSV F protein.

Fluorescence microscopy. Transfected COS-7 cells were washed with PBS at 16–24 h post-transfection and fixed with 4 % paraformaldehyde in PBS with 1 mM CaCl₂, 1 mM MgCl₂ for 20 min at room temperature. Cells were washed three times in immunofluorescence (IF) wash buffer [1 % (v/v) FCS, 0–1 % (v/v) Triton X-100 in PBS] followed by permeabilization in IF wash buffer supplemented with 0–5 % Triton X-100 for 10 min at room temperature. After additional washing, cells were incubated with appropriate primary antibodies in IF wash buffer, washed and incubated with the appropriate FITC- or Alexa Fluor 594-conjugated secondary antibodies diluted in IF wash buffer. Following additional washing, coverslips were mounted on glass slides using Slowfade (Molecular Probes). Cells were examined using a Nikon Optiphot II epi-fluorescence microscope equipped with a Bio-Rad MRC 1024 confocal laser-scanning attachment and x 100 objective. Images were processed using Adobe Photoshop software.

Antibody internalization assay. The method used to assess the internalization of cell-surface proteins was similar to that described by Roquemore & Banting (1998). Briefly, 16–24 h post-transfection, COS-7 cells were incubated with the appropriate primary antibody in PBS at 37 °C in a humidified chamber for 1 h. Cells were then washed three times in PBS and fixed with 100 % methanol at –20 °C for 5 min. Following additional washing, cells were incubated with appropriate fluorescein isothiocyanate-labelled secondary antibodies in IF wash buffer. Coverslips were mounted on glass slides and cells examined by confocal microscopy as described above. Quantification of internalization assays was performed by counting fields of cells, with those showing internalized antisera in a TGN-like pattern scored as positive and cells showing only a cell-surface signal scored as negative.

RESULTS

Inhibition of viral glycoprotein-mediated membrane fusion

It has previously been reported that the gM proteins from PRV, ILTV, EHV-1, HSV-1 and HHV-8 can inhibit membrane fusion induced by the herpesvirus proteins gB, gD, gH and gL (Klupp et al., 2000; Koyano et al., 2003). As a first step in our studies, we performed similar experiments to those described previously (Klupp et al., 2000; Koyano et al., 2003) to ensure that PRV gM and HSV-1 gM/UL49A would, as expected, inhibit cell–cell fusion mediated by HSV-1 gB/D/H/L. Effector cells expressing HSV-1 gB/D/H/L together with varying concentrations of LacZ, PRV gM, HSV-1 gM alone or HSV-1 gM plus HSV-1 UL49A were overlaid with target cells and scored for fusion by counting numbers of nuclei incorporated into syncytia. Expression of PRV gM exhibited very robust inhibition of fusion in a dose-dependent manner (Fig. 1a, columns 7–12), whereas there was no effect on fusion using varying amounts of LacZ expression plasmid (Fig. 1a, columns 1–6). Expression of HSV-1 gM also caused fusion inhibition in a dose-dependent manner in the absence of UL49A, although much greater amounts of HSV-1 gM expression plasmid were required than PRV gM (Fig. 1a, columns 13–16). Interestingly, lower amounts of HSV-1 gM-expressing plasmid (0.25 μg) very efficiently inhibited membrane fusion when HSV-1 UL49A was co-expressed (Fig. 1a, column 17). These data suggested that both PRV and HSV-1 gM could inhibit HSV-1 gB/D/H/L-mediated membrane fusion, but that co-expression of HSV-1 UL49A was required for efficient fusion inhibition by HSV-1 gM. Co-expression of the HSV-1 fusion proteins gB, gD, gH and gL with HSV-1 glycoproteins gC, gE or gN individually or in various combinations had no effect on membrane fusion (data not shown).

The membrane fusion normally stimulated by BRSV F protein or MoMLV Env protein are also inhibited by PRV gM and HSV-1 gM/UL49A, respectively (Klupp et al., 2000; Koyano et al., 2003). To extend these observations, we tested the ability of PRV gM and HSV-1 gM (with or without UL49A) to inhibit HRSV F protein-mediated fusion. In the presence of PRV gM, high levels of HSV-1 gM alone or low levels of HSV-1 gM plus HSV-1 UL49A, HRSV F protein-mediated fusion was completely abolished, demonstrating that both PRV gM and HSV-1 gM can efficiently inhibit HRVS F protein-mediated fusion. These results were consistent with previous work. The key points are: (i) that fusion inhibition by PRV or HSV-1 gM exhibits a broad specificity against a variety of viral fusion proteins; and (ii) that PRV gM operates independently of gN, whereas HSV-1 gM requires co-expression of UL49A for efficient function. Since our objective was to investigate the cellular biological basis of this inhibition of fusion, all subsequent experiments were performed with PRV gM alone or with HSV-1 gM co-expressed with UL49A.

Topology, trafficking motifs and subcellular localization of gM

Herpesvirus gM is a highly hydrophobic protein that is predicted to contain eight transmembrane domains with cytoplasmic N and C termini, a potential N-glycosylation site within the first extracellular domain and a conserved
cysteine residue within the same loop, which has been predicted to form a disulphide bond with the relevant gN homologue (Fig. 2; Dijkstra et al., 1996; Jons et al., 1998). The predicted C-terminal cytoplasmic domain of gM contains two classes of potential membrane-trafficking motifs: a tyrosine-based motif and an acidic cluster motif. Tyrosine-based motifs mediate the incorporation of membrane proteins into transport vesicles due to interaction with adaptor complexes AP-1, -2, -3 and -4 (Kirchhausen, 1999), while acidic cluster motifs are known to interact with the connector protein PACS-1, which is involved in transport from endosomes to the TGN (Crump et al., 2001; Wan et al., 1998). The presence of these trafficking motifs in all gM sequences suggests that gM could have the capacity to traffic to and from the plasma membrane. To determine the subcellular distribution of gM in transfected cells, cells expressing PRV gM, HSV-1 gM or HSV-1 gM plus UL49A were analysed by immunofluorescence. Both PRV gM and HSV-1 gM localized to a juxtanuclear compartment where they showed significant co-localization with TGN46, a marker of the TGN (Fig. 3). These data suggested that gM from both PRV and HSV-1 could localize to the TGN independently of other herpesvirus proteins and that HSV-1 gM localization was not affected by UL49A co-expression.

**Fig. 1.** Inhibition of membrane fusion by PRV gM or HSV-1 gM with or without UL49A. Plasmids expressing HSV-1 gB, gD, gH and gL (a) or HRSV F protein (b) were transfected into HEK-293T cells together with various quantities of plasmids expressing PRV gM, HSV-1 gM, HSV-1 UL49A or LacZ as shown. After 48 h, untransfected Vero cells were added and fusion was assessed after a further 24 h by counting the number of nuclei incorporated into syncytia. Results are expressed as level of fusion relative to the LacZ control and, where shown, more than one data point represents fusion levels from independent experiments with the means shown by the bars.

**Fig. 2.** Potential topology and trafficking motifs of herpesvirus gM. Topology prediction for PRV gM was performed using the HMMTOP transmembrane topology prediction server (http://www.enzim.hu/hmmtop). The approximate locations of an N-linked glycosylation site (branched structure), a cysteine residue (-S-S-), the potential tyrosine-based endocytosis motif (YXXΦ) and the acidic cluster motif that are conserved in virtually all Herpesviridae gM sequences are shown. The primary sequence of the predicted C-terminal cytoplasmic domain of PRV gM (Kaplan strain) is shown with potential tyrosine-based and acidic cluster motifs highlighted.
Effect of gM on HSV-1 gD and HSV-1 gH/L localization

Given that PRV and HSV-1 gM localized to the TGN but inhibited HSV-1 gb/D/H/L fusion at the plasma membrane, we asked whether gM affected the localization of any of these ‘fusion’ proteins. Cells expressing gD or gH/L alone or in the presence of gM were studied by immunofluorescence. When expressed alone, gD and the gH/L complex demonstrated a broad distribution with clear evidence of cell-surface expression (Fig. 4a and d). When either PRV gM or HSV-1 gM and UL49A were co-expressed, virtually all gD and gH/L was present in a juxtanuclear compartment with no observable cell-surface localization (Fig. 4b, c, e and f). Both gD and gH/L showed good co-localization with PRV and HSV-1 gM in this juxtanuclear location, suggesting that gD and gH/L are relocated to the TGN (Fig. 4, colour panels). To determine whether the alteration in gD and gH/L localization caused by gM was specific or a global effect on all plasma-membrane proteins, the effect of co-expressing gM with the cell-surface mammalian protein CD8x was tested. CD8x exhibited clear cell-surface localization, which was unchanged in the presence of gM (Fig. 4g–i).

Effect of gM on HRSV F protein, influenza HA, HVEM and nectin-2α

Given that gM inhibited the membrane fusion caused by envelope proteins from non-herpesviruses such as RSV F protein (Fig. 1b and Klupp et al., 2000), we were interested in whether gM might have an effect on the subcellular localization of other viral envelope proteins. Cells expressing HRSV F protein or influenza virus HA in the presence or absence of gM were analysed. As expected, F protein and HA demonstrated clear plasma-membrane localization when expressed alone. In the presence of PRV gM or HSV-1 gM and UL49A, both F protein and HA exhibited significant localization to a juxtanuclear compartment (Fig. 5a–f). These data suggested that gM was able to stimulate a change in the localization of a diverse range of viral fusion proteins from the plasma membrane to the TGN. It was interesting to note that the effect of gM on the localization of cell-surface membrane proteins did not appear to be restricted to viral fusion proteins. Similar experiments performed with HVEM, a TNF receptor superfamily member, also showed a change in the subcellular localization of this protein from the plasma membrane to a juxtanuclear compartment when co-expressed with gM (Fig. 5g–i). However, another mammalian cell-surface protein, nectin-2α, was unchanged by co-expression of gM, with all cells showing clear cell-surface localization and no significant localization to intracellular compartments (Fig. 5j–l).

gM-induced relocalization of gD involves clathrin-mediated endocytosis

To test whether the juxtanuclear-localized gD observed in the presence of gM could be due to endocytosis, antibody internalization assays were performed. Cells expressing...
CD8α or HSV-1 gD in the presence or absence of gM were incubated with specific monoclonal antibodies at 37°C for 1 h. Cells were then fixed and processed for immunofluorescence. Since cells were only exposed to primary antibody prior to fixation and membrane permeabilization, any internal labelling of the cells identified antigens that were present on the cell surface and internalized during the initial incubation period. CD8α demonstrated little internalization, with the majority of the signal at the cell surface in the presence or absence of gM (Fig. 6a–c). In contrast, antibody to gD exhibited a strong intracellular signal localized to a juxtanuclear compartment, but only when gM was co-expressed, eliminating the possibility that antibody binding alone was responsible for internalization (Fig. 6d–f). Internalization of gD antibody was severely inhibited by co-expressing dominant-negative AP180-C, a potent inhibitor of clathrin-mediated endocytosis (Fig. 6g–i) (Ford et al., 2001; Zhao et al., 2001). In an effort to quantify this assay, gD- or CD8α-expressing cells were counted and scored as positive if they exhibited internalized juxtanuclear fluorescent signals (as in Fig. 6d), and negative if they exhibited cell-surface-localized staining only (as in Fig. 6c). The data from several experiments were expressed as the proportion of cells showing TGN-like localized fluorescence, with the bars representing the mean of these data (Fig. 6, lower panel). Taken together, these data indicated that expression of gM induced endocytosis of gD, but not CD8, via a clathrin-dependent pathway.

**gM induces clathrin-mediated endocytosis of HSV-1 gH/L, HRSV F protein and HVEM, but not nectin-2α**

The results in the previous section indicated that gM induced the internalization of HSV-1 gD but not CD8. We therefore extended the study by examining the effect of gM expression on HSV-1 gH/L, HRSV F protein, nectin-2α and HVEM. The data in Fig. 7 showed that gM expression induced the internalization of HSV-1 gH/L, HRSV F protein and HVEM, but not nectin-2α. This is consistent with the ability of gM to inhibit cell fusion by HSV-1 gB/D/H/L or by HRSV F protein, but confirmed that there was some selectivity in the induction of endocytosis of cell-surface proteins.

**DISCUSSION**

gM and gN are conserved throughout the family *Herpesviridae* and have been shown to form a complex in members...
of all three herpesvirus subfamilies, although it is unclear whether gM and gN always exist as a complex or can have independent functions (Jons et al., 1998; Koyano et al., 2003; Lake et al., 1998; Mach et al., 2000; Rudolph et al., 2002; Wu et al., 1998). Glycosylation of the gN homologues from HSV-1, BoHV-1 and EHV-1 has not been observed, and thus these proteins are referred to by their gene names UL49A or UL49? (Adams et al., 1998; Rudolph et al., 2002; Wu et al., 1998). Although the function of the gM/N complex is poorly understood, previous reports in the literature have demonstrated that PRV gM (without gN) and the gM/N complexes from EHV-1, ILTV, HSV-1 and HHV-8 can inhibit gB/D/H/L-mediated membrane fusion in transfection-based assays (Klupp et al., 2000; Koyano et al., 2003). It should be noted that the phenomenon of fusion inhibition by gM/N in transfection assays is not thought to represent an ability of gM/N to inhibition cell fusion during infection, as mutations in gM do not lead to the formation of syncytial viruses. In this report, we have extended previous observations and shown that PRV gM and HSV-1 gM inhibit HSV-1 gB/D/H/L fusion in the absence of gN/UL49A expression. However, whereas PRV gM inhibited fusion robustly in the absence of PRV gN, HSV-1 gM fusion inhibition was far more efficient when UL49A was co-expressed. These data suggest that HSV-1 UL49A increases the activity of HSV-1 gM, with respect to fusion inhibition, but is not essential for this function. It has previously been demonstrated that efficient processing of both EHV-1 gM and HHV-8 gM requires co-expression of the corresponding gN/UL49? protein (Koyano et al., 2003; Rudolph et al., 2002). Therefore, it seemed plausible that HSV-1 gM might require UL49A expression for efficient folding and maturation. However, HSV-1 gM appeared to localize to the TGN irrespective of UL49A expression, suggesting that the absence of UL49A did not significantly disrupt gM in the early secretory pathway. PRV gM seems unusual amongst the herpesvirus gM molecules in that studies so far have shown little or no requirement for gN expression; as well as inhibiting fusion efficiently without gN, PRV gM is efficiently incorporated into virions in the absence of gN, whereas the maturation of gM and gN of other herpesviruses seems to be interdependent (Jons et al.,

Fig. 5. Effect of gM proteins on the subcellular localization of HRSV F protein, influenza virus HA, HVEM and nectin-2z. COS-7 cells were transfected with plasmids expressing F protein (a–c), HA (d–f), HVEM (g–i) or nectin-2z (j–l) together with plasmids expressing GFP alone (control), GFP–PRV gM or GFP–HSV-1 gM plus HSV-1 UL49A. At 16–24 h post-transfection, cells were fixed, permeabilized and incubated with the antisera specific for F protein, HA, HVEM or nectin-2z, followed by the appropriate Alexa Fluor 594-conjugated secondary antisera. For clarity, only Alexa Fluor 594 signals are shown.
1998; Klupp et al., 2000; Koyano et al., 2003; Lake et al., 1998; Rudolph et al., 2002). Consistent with the ability of PRV gM to function alone, we observed no significant difference in the TGN localization of PRV gM or the ability of PRV gM to stimulate the relocalization of gD in the presence or absence of a PRV gN expression plasmid (unpublished observations). Thus, so far all data on the function of PRV gM suggest that there is no role for PRV gN, although whether additional functions of PRV gM are dependent on PRV gN expression is currently unknown.

In an effort to determine the mechanisms by which gM inhibits HSV-1 gB/D/H/L-mediated membrane fusion, we examined the effects of gM on the subcellular localization of these herpesvirus glycoproteins. Consistent with their membrane fusion inhibition activity, we observed that PRV gM and HSV-1 gM plus UL49A caused both gD and gH/L to be relocalized from the cell surface to an intracellular compartment, probably the TGN. This change in the localization of gD and gH/L was at least partly due to the internalization of these membrane proteins through clathrin-mediated endocytosis. Previous reports have suggested that binding of antisera to PRV gD can stimulate the internalization of this glycoprotein in PRV-infected cells (Favoreel et al., 2000). In the present study, it seemed highly unlikely that the observed internalization of gD and gH/L monoclonal antibodies was due to a similar antibody-induced endocytosis, as internalized signals were observed only in the presence of PRV gM or HSV-1 gM/UL49A and not in control cells. The other component of the fusion apparatus, HSV-1 gB, was found to be predominantly localized to an intracellular, juxtanuclear compartment, irrespective of gM expression (unpublished observations). These observations are unsurprising given the presence of internalization motifs within the cytoplasmic domain of gB and the known localization of herpesvirus gB isoforms to the TGN (Crump et al., 2003; Fan et al., 2002). The stoichiometry of gB, gD and gH/L required at the cell surface to stimulate membrane fusion is currently unknown, but taken together these data suggest that membrane fusion inhibition by gM/N involves perturbation of this stoichiometry by the removal of gD and gH/L from the cell surface and transport to an intracellular compartment, where they are no longer available to stimulate cell–cell fusion.

We also demonstrated that PRV gM and HSV-1 gM plus UL49A were able to cause relocalization of other non-herpesvirus glycoproteins, such as HRSV F protein, from the

![Fig. 6. Effect of PRV gM on the internalization of HSV-1 gD and CD8α. COS-7 cells were transfected with plasmids expressing CD8α (a–c), gD (d–f) or gD plus AP180-C (g–i), together with plasmids expressing GFP alone (control), GFP–PRV gM or GFP–HSV-1 gM plus HSV-1 UL49A. At 16–24 h post-transfection, cells were incubated with monoclonal antibodies specific for either gD or CD8α for 1 h at 37°C. Following fixation, cells were incubated with anti-mouse Alexa Fluor 594-conjugated secondary antisera. For clarity, only Alexa Fluor 594 signals are shown. (Lower panel) All cells in at least 10 random fields of view were scored as positive for internalization if they demonstrated TGN-like staining (as in e) and negative if they demonstrated cell-surface staining only (as in d). Data are presented as the proportion of the total number of cells counted showing internalized antisera in a TGN-like location and represent the mean values from several experiments (bars) with data points from the individual experiments shown.](image-url)
cell surface to a TGN-like compartment. These observations correlated with data presented here and by others showing that PRV gM and HHV-8 and HSV-1 gM/N can inhibit fusion induced by human or bovine RSV F protein and MoMLV Env protein (Klupp et al., 2000; Koyano et al., 2003). Thus, the broad specificity exhibited by gM/N in terms of fusion inhibition is reflected in the ability of gM/N to remove a broad range of proteins from the cell surface.

The data presented in this report demonstrate that PRV gM and HSV-1 gM/UL49A are capable of altering the localization of a very broad range of membrane proteins. However, as the subcellular localization of CD8α or nectin-2α was unaffected by gM/N expression, the mechanism by which gM/N relocalizes glycoproteins is unlikely to be due to a global increase in membrane traffic events. How gM/N can stimulate changes in the subcellular localization of such a wide variety of membrane proteins is currently unclear. Given the presence of potential internalization motifs conserved within the C-terminal cytoplasmic domain of all herpesvirus gM sequences available to date, it is tempting to speculate that gM/N may physically interact with, for example, gD and cause subsequent internalization and intracellular targeting of the complex. If so, then it must be assumed that interactions would have no direct sequence requirements given that such a wide variety of proteins are affected. However, the possibility that gM could be stimulating the relocalization of such a diverse array of membrane proteins by an indirect mechanism cannot be discounted.

The ability of gM/N to cause localization of the herpesvirus envelope proteins gD and gH/L to the TGN could be part of the mechanism by which herpesviruses maintain sufficient concentrations of envelope proteins in the secondary envelopment compartment, thus allowing efficient assembly and viral egress. Such a possibility correlates well with observations showing that disruption of the gM gene in PRV and disruption of the gM or UL49-5 gene in EHV-1, together with the absence of the gE/I complex in these viruses, led to dramatic defects in secondary envelopment (Brack et al., 1999; Rudolph et al., 2002). However, the redundant requirements of alphaherpesvirus envelope proteins for correct secondary envelopment is complicated by the observation that HSV-1 lacking gD and the gE/I complex also shows significant defects in secondary envelopment and egress (Farnsworth et al., 2003). It has also been recently reported that the myristoylated tegument protein encoded by PRV UL11 is involved in secondary envelopment (Kopp et al., 2003). The different roles played by the membrane proteins gM, gE, gI, gD, UL11 and possibly other viral proteins in controlling secondary envelopment in the various alphaherpesviruses await further study.

Herpesviruses have evolved many immune evasion mechanisms, including several aimed at inhibiting antigen presentation (Yewdell & Hill, 2002). It is conceivable that one of the advantages of gM-dependent removal of herpesvirus envelope proteins from the cell surface may be to avoid the
recognition of infected cells by immune surveillance systems. Interestingly, deletion of gM in the RacH vaccine strain of EHV-1 has been shown to increase the immunogenicity of this vaccine in mice (Osterrieder et al., 2001). Whether this increase in immunogenicity is due to an increase in cell-surface levels of viral glycoproteins in infected cells remains to be determined.

In summary, we have demonstrated that PRV gM and the HSV-1 gM/UL49A complex causes relocalization of the herpesvirus envelope proteins gD and gH/L to a TGN-like compartment, at least partially through endocytosis. These data correlate well with the ability of gM/N to inhibit membrane fusion in transfection-based assays and also the proposed role of gM/N in the secondary envelopment of herpesviruses. Furthermore, the specificity of gM/N for the relocalization of membrane proteins is very broad but not ubiquitous. The mechanisms by which gM/N may stimulate these membrane-trafficking events will pose intriguing questions for further studies.

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