Glycoproteins M and N of human herpesvirus 8 form a complex and inhibit cell fusion

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Glycoproteins M (gM) and N (gN) are well conserved across the herpesvirus family and their involvement in virus penetration and egress is well described, especially for alphaherpesviruses. Because there was no previous study on the homologues of human herpesvirus 8 glycoproteins M (gM8) and N (gN8), we analysed their biochemical and functional characteristics. We found that: (i) gM8 aggregated following heat treatment; (ii) gM8 was a virion component; (iii) gM8 and gN8 were N-glycosylated; (iv) gM8 formed a specific complex with gN8; and (v) gN8 was required for functional processing of gM8. Co-expression of gM8 and gN8 inhibited cell fusion induced either by a combination of herpes simplex virus type 1 glycoproteins or by Molony murine leukaemia virus envelope protein. These results indicate that, in addition to the similar biochemical properties, the fusion inhibition reported previously only for alphaherpesviruses is a function conserved in the gammaherpesvirus subfamily.

Herpesviruses encode several glycoproteins; some are unique to a particular subfamily or virus, while others, including glycoprotein B (gB), gH, gL, gM and gN, are conserved throughout the herpesvirus family. These glycoproteins are likely to play essential and similar roles during the infectious cycle. However, their functional similarities cannot be assumed based only on the conservation of sequences and biochemical characteristics. An instructive example in this regard is gB, which was not detectable in the virions of some gammaherpesviruses (Gong & Kieff, 1990; Stewart et al., 1994). Although gMs and gNs of most herpesviruses share several biochemical characteristics in addition to their sequence homologies (Baines & Roizman, 1993; Dijkstra et al., 1996; Jöns et al., 1998; Lake et al., 1998; Mach et al., 2000; MacLean et al., 1993; Osterrieder et al., 1996; Pilling et al., 1994; Rudolph et al., 2002; Wu et al., 1998), differences have been noted in their fundamental properties, specifically, the degree of dispensability for growth in cell culture and effects on penetration and/or egress (Adams et al., 1998; Baines & Roizman, 1991; Dijkstra et al., 1996; Hobom et al., 2000; Jöns et al., 1998; König et al., 2002; Lake & Hutt-Fletcher, 2000; MacLean et al., 1991; Osterrieder et al., 1996; Rudolph & Osterrieder, 2002; Rudolph et al., 2002; Tischer et al., 2002). Because the properties of these glycoproteins have mainly been studied in alphaherpesviruses, additional studies are required to clarify whether any function found in alphaherpesviruses is applicable to other subfamilies. Neither the gM nor the gN homologue of human herpesvirus 8 (HHV-8) has yet been studied in detail. Therefore, in this study, we analysed their biochemical and functional properties.

Expression of HHV-8 gM (gM8) (ORF39) was analysed by immunoblotting of cell lysates prepared from BCBL-1 cells, a primary effusion lymphoma cell line containing latent HHV-8 (Renne et al., 1996). The lytic infection was induced by treatment of the cells with 20 ng 12-O-tetradecanoylphorbol 13-acetate (TPA) ml⁻¹. Anti-gM8 serum was prepared by immunization of rabbits with a gM8 oligopeptide (KDSTPAPRTQYQSD) conjugated with keyhole limpet haemocyanin, as described previously (Inoue et al., 2000). Two proteins with molecular masses of 46 and 80 kDa (‘46K’ and ‘80K’ proteins, respectively) were detected in TPA-treated but not in untreated BCBL-1 cells (Fig. 1A). To confirm that these were gM8 products, we constructed pcDNA-gM8 by PCR amplification with primers 5'-ccgatctATCAGATTGGCCTCCGCCGCAG-3' (GenBank U75698, nt 60233–60210) and 5'-ccgctcagCTAAATGATATCATTTGCGTTTCG-3' (nt 58973–58997), followed by cloning into pcDNA3 (Invitrogen). Two identical 46K and 80K proteins were detected in 293T cells transfected with pcDNA-gM8 (Fig. 1B), indicating that these are gM8 products. We also constructed pCMV-gM8F, which expresses gM8 with a FLAG tag, by PCR with the same forward primer and a reverse primer, 5'-ccgctcagATGATATCATTTGCGTTTCG-3', followed by cloning into pCMV-tag4 (Stratagene). Anti-FLAG and anti-gM8 antibodies reacted with the same 46K and 80K proteins in 293T cells transfected with pCMV-gM8F (data not shown), which confirmed the specificity of the anti-gM8 antibody and the integrity of the plasmids. Culturing the cells in the presence of tunicamycin (TM) for 48 h decreased the

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Fig. 1. Expression of gM8. (A) Cell lysates were prepared from untreated (lane 1), TPA-treated (lane 2) and TPA plus 1 μg tunicamycin (TM) ml⁻¹-treated (lane 3) BCBL-1 cells, separated on an SDS-polyacrylamide gel containing 3 M urea and analysed by immunoblotting with anti-gM8 serum. Open and closed arrows indicate the glycosylated and unglycosylated forms of gM8, respectively. (B) 293T cells were transfected without added DNA (lane 4) and with pcDNA-gM8 (lanes 5 and 6) and then cultured in the absence (lanes 4 and 5) or presence (lane 6) of TM. Lysates of these cells were analysed as described above. Symbols are as in (A). (C) Cell lysates prepared from 293T cells transfected with pcDNA-gM8 were incubated at 4 °C for 1 h (lane 7), at 37 °C for 30 min (lane 8) or at 56 °C for 5 min (lane 9) and analysed as described above. The asterisk indicates aggregated gM products. (D) IVTT reactions programmed with pcDNA-gM8 (lane 10) and without added DNA (lane 11) were incubated in the presence of [³⁵S]methionine and -cysteine and the products were detected by fluorography as described previously (Inoue et al., 1994). The arrow indicates gM8 expressed in vitro. (E) Lysates of TPA-treated BCBL-1 cells (3 x 10⁶ cells per lane) (lane 12) and 1 μl of the purified virion sample (lane 13) were analysed as described in (A). The amount of the virion loaded on the well was equivalent to 2-5 ml of the original supernatant, because the virion sample was obtained from 1 l of culture supernatant of TPA-treated BCBL-1 cells, purified by two gradients and suspended in 0-4 ml. Open arrowheads and arrows indicate the gM8 products in virions and in the cell lysate, respectively.
molecular mass of the gM8 products to 39 and 71 kDa (Fig. 1A, B), suggesting N-glycosylation of gM8.

gM8 aggregated easily on incubation at moderate to high temperatures (Fig. 1C), a consistent biochemical characteristic of gMs from other herpesviruses. Even incubation at 4 °C for 1 h decreased 46k protein and increased 80k protein and other aggregated products. 46k protein was barely detectable after incubation at 37 °C for 30 min. The amount of 80k protein was similar after incubation at 37 °C for 3 h. From these observations, we assumed that the 80k protein was a dimeric form of gM8. A single band with a molecular mass of 39 kDa (39k protein) was expressed from pcDNA-gM8 in a coupled in vitro transcription–translation (IVTT) reaction (Fig. 1D) and aggregated after incubation at moderate temperatures, indicating that the 39k protein is a precursor of the other products.

Next, we examined whether gM8 is a virion component. Virion samples were prepared from culture supernatant of TPA-treated BCBL-1 cells by passage through 0.45 μm membranes and centrifugal precipitation, followed by two sucrose gradient centrifugations. The virion-associated gM8 products were 48 kDa and 86 kDa proteins, slightly larger than those expressed in TPA-induced BCBL-1 cells (Fig. 1E).

HHV-8 virions and the cell extracts prepared from 293T cells transfected with pcDNA-gM8 were enzymatically deglycosylated with peptide: N-glycosidase F (PNGaseF) and with endoglycosidase H (EndoH). Only the 80k protein and its digested products were analyzable because the incubation at 37 °C decreased the amount of 46k protein (Fig. 1C). Although gM8 in the virions was EndoH-resistant, gM8 in the transfected 293T cells was EndoH-sensitive (Fig. 2A), suggesting that transiently expressed gM8 is posttranslationally processed differently from the form present in virions.

Since a complex formation between gM and gN has been demonstrated in other herpesviruses, we examined whether gM8 and gN8 form a complex. The gN8 (ORF53) gene was amplified by PCR with primers 5'-cggattgcccacc-ATGACAGCGTCCACGGTGGC-3' (nt 77665–77646) and 5'-ccctcgagATGCATGGACCACCTGTCCACAAA-3' (77335–77359) and cloned into pA3M, a pcDNA3-derived plasmid that allows addition of three Myc tags (~4.5 kDa) at the C terminus (Fries et al., 1999). Myc-tagged gN8 (gN8–Myc) was detected as a 30 kDa protein in 293T cells transfected with pA3M-gN8 by immunoblotting with anti-gN8 antibody, which was prepared by immunization of rabbits with pcDNA-gM8 and pA3M-K1, which expresses HHV-8 K1 glycoprotein with the same tags (K. Fries, F. R. Stamey & P. E. Pellett, unpublished). Immunoprecipitation of this extract did not co-precipitate gM8 (data not shown). In addition, gN8–Myc was immunoprecipitated with anti-FLAG antibody conjugated with agarose beads (Sigma) only from the extract of the cells co-transfected with gM8–Myc, indicating complex formation between gM8 and gN8–Myc (Fig. 2C). To confirm the specificity of the immunoprecipitation, 293T cells were transfected with pcDNA-gM8 and pA3M-K1, which expresses HHV-8 K1 glycoprotein with the same tags (K. Fries, F. R. Stamey & P. E. Pellett, unpublished). Immunoprecipitation of this extract did not co-precipitate gM8 (data not shown).

In addition, gN8–Myc was immunoprecipitated with anti-Myc tag antibody conjugated with beads (Santa Cruz Biotechnology) for 2 h at 4 °C. gM8 was immunoprecipitated only from the extract of the cells co-transfected with gN8–Myc, indicating complex formation between gM8 and gN8–Myc (Fig. 2C). Therefore, our results indicated that gM8 forms a complex with gN8 as demonstrated in other herpesviruses. However, further study will be required to determine whether this complex is formed by direct gM8/gN8 interaction or by indirect interaction mediated through a cellular factor. gM8 in 293T cells co-transfected with the gM8 and gN8–Myc plasmids was detected as a broader band with a slightly slower migration than when expressed alone (Fig. 2C). Also gM8 co-precipitated with gN8–Myc was larger than gM8 expressed alone and it was the same as that in virion samples (Fig. 2A, C). It is possible that gN8 co-expression alters the post-translational modifications of gM8 and that only gM8 forming a complex with gN8 obtained the additional modifications, resulting in the migration difference. To examine this hypothesis, extracts of 293T cells expressing gM8 alone or gM8/gN8 together were digested with glycosidases. The PNGaseF digestion generated products with an identical size in both cases (Fig. 2A, E). However, gM8 co-expressed with gN8, but not gM8 expressed alone, was EndoH-resistant (Fig. 2E), indicating that gN8 is required for the additional modification of gM8. Because EndoH resistance indicates that a glycoprotein has moved from the endoplasmic reticulum to the trans-Golgi network (TGN) (Dunphy & Rothman, 1985), our results suggest that: (i) authentic trafficking and post-translational processing of gM8 depend on gN8 expression; and (ii) HHV-8 virions acquire gM8 from the TGN.

Inhibition of cell fusion was reported previously as one of the gM functions (Klupp et al., 2000). Because the inhibition was demonstrated only for alphaherpesviruses, namely pseudorabies virus (PRV) and equine herpesvirus 1, we examined whether this is the common function of gM among the herpesvirus subfamilies. To compare the inhibition between alpha- and gammaherpesviruses, we amplified herpes simplex virus type 1 (HSV-1) gM (gM1) and gN (gN1) genes by PCR with primer sets 5'-cggattgcccacc-ATGGGACGCCGGCAGCCAG-3' and 5'-gctctagatacACGAAGGCGGCGGAGT-3', and 5'-ggctctagatacACGAAGGCGGCGGAGT-3', and 5'gaattc-ggctctagatacACGAAGGCGGCGGAGT-3', and 5'-ccctcgagATGCATGGACCACCTGTCCACAAA-3' (77335–77359) and cloned into pcDNA3, pSMH-gB, -gD, -gH and -gL, which encode HSV-1 gB, gD, gH and -gL, respectively, were gifts of H. Browne (University of Cambridge, UK) (Turner et al., 1998) and each HSV-1 glycoprotein gene was recombined into
Fig. 2. Processing and complex formation. (A) Cell extracts prepared from 293T cells transfected with pcDNA-gM8 (lanes 1, 2, 5 and 6) and HHV-8 virions (lanes 3, 4, 7 and 8) were treated with PNGaseF (lanes 2 and 4) and EndoH (lanes 6 and 8), separated on an SDS-polyacrylamide gel containing 3 M urea and analysed by immunoblotting with anti-gM8 serum. The closed circle indicates gM8 deglycosylated with PNGaseF. Open and closed squares indicate glycosylated and EndoH-digested gM8 detected in the transfected 293T cells, respectively. The open arrowhead indicates gM8 in virions. The asterisk indicates aggregated gM products.

(B) Cell extracts prepared from 293T cells transfected with pA3M-gN8 were treated without added enzyme (lane 1), with EndoH (lane 2) or with PNGaseF (lane 3), separated on a 4–12 % Bis/Tris gel with MES SDS buffer (Invitrogen) and analysed by immunoblotting with anti-gN8 serum.

(C) 293T cells transfected without added DNA (lane 1), with pA3M-gN8 (lane 2), with pcDNA-gM8 (lanes 3 and 5) and with pcDNA-gM8 and pA3M-gN8 (lanes 4 and 6). Their extracts were reacted with anti-c-Myc monoclonal antibody conjugated with beads. Proteins bound to the beads (lanes 1–4) and the extracts prior to the reaction (lanes 5 and 6) were analysed with anti-gM8 antibody as described for (A). The closed arrowhead and the asterisk indicate the immunoprecipitated proteins and aggregated gM products, respectively.

(D) 293T cells transfected with pA3M-gN8 (lanes 1 and 4), with pCMV-gM8F and pA3M-gN8 (lane 2) and with pCMV-gM8F (lane 3). Their extracts were reacted with anti-FLAG monoclonal antibody conjugated with agarose beads. Proteins bound to the beads (lanes 1–3) and the extract prior to the reaction (lane 4) were analysed with anti-gN8 serum as described for (B). (E) The extracts prepared from 293T cells transfected with pcDNA-gM8 and pA3M-gN8 together (lanes 1, 2 and 3) and with pcDNA-gM8 alone (lanes 4 and 5) were incubated without added enzyme (lanes 1 and 4), with PNGaseF (lane 3) and with EndoH (lanes 2 and 5). gM8 products were detected by immunoblotting with anti-gM8 serum as described in (A). The open arrowhead and closed square indicate glycosylated and deglycosylated forms of gM8, respectively.
pcDNA3. 293T-T7RP was established by transfection of 293T cells with a plasmid encoding the T7 RNA polymerase (T7RP) gene (a gift of B. Moss, NIH, USA) (Elroy-Stein & Moss, 1990) and a plasmid encoding hygromycin B phosphotransferase. A clone expressing T7RP at the highest level was selected from hygromycin B-resistant clones. Forty-eight hours after transfection of 293T-T7RP cells with plasmid(s) encoding viral glycoproteins, the cells were mixed with an equal number of 293T cells that had been transfected with pOS8, a plasmid containing the lacZ gene under control of the T7 promoter (a gift of B. Moss) (Wyatt et al., 1995). Fusion events were measured 24 h after mixing the cells as T7RP-dependent β-galactosidase expression, using a chemiluminescent assay (Luminescent β-galactosidase detection kit II; Clontech). As shown in Fig. 3(A), co-expression of gM1 and gN1 inhibited cell fusion by 60%. Co-expression of gM8 and gN8 also decreased cell fusion, although more weakly than HSV-1 gM/gN. gM1 or gM8 alone did not inhibit fusion significantly (Fig. 3A and data not shown). Similar results were obtained by X-Gal staining of fused cells. The fusion inhibition was not due to toxicity of these proteins, because the β-galactosidase expression level in 293T cells transfected with pCMVβ, a plasmid containing lacZ under the control of the cytomegalovirus (CMV) promoter, and the pcDNA-gM and -gN constructs was the same as in the cells transfected with pCMVβ and pcDNA3 vector (data not shown). In our preliminary experiments, human CMV gM/gN strongly inhibited the fusion induced by HSV-1 glycoproteins. Therefore, the fusion inhibition is likely to be one of the conserved gM/gN characteristics across the herpesvirus family.

Finally, we examined whether gM and gN inhibit cell fusion mediated by an envelope glycoprotein (Env) of ecotropic Molony murine leukemia virus (Mo-MuLV). Because Mo-MuLV Env lacking the carboxyl 16 amino acid R-peptide has a strong fusion activity (Melikyan et al., 2000), a DNA fragment encoding the truncated Env was generated by PCR amplification of a plasmid encoding the full-length gene (a gift of D. W. Russell, University of Washington) with primers 5′-cccaagcttCTTATATGGGGCACC-3′ and 5′-attgctacaAGAGGCTGGACCACTGATA-3′ and was cloned into pcDNA3, resulting in pEcoMLVenvAR. 293T-T7RP cells were transfected with pEcoMLVenvAR and the gM and gN constructs, cultured for 48 h and then overlaid with an equal number of NIH3T3 cells that had been transfected with pOS8 and cultured for 24 h. A combination of gM and gN from either HSV-1 or HHV-8 partially inhibited this fusion (Fig. 3B).

PRV gM inhibited fusion induced by the F protein of bovine respiratory syncytial virus (BRSV) (Klupp et al., 2000) and in the presence of BRSV F protein expression, bovine herpesvirus 1 (BHV-1) lacking gM formed larger syncytia than did wild-type BHV-1 (König et al., 2002). These and our results suggest that there is a common mechanism of fusion inhibition by the gM/gN complex among some enveloped viruses. Potential mechanisms include inhibition of authentic glycoprotein processing and modification of viral glycoprotein distribution on the plasma membrane. Integration of highly hydrophobic gM proteins into membranes has been hypothesized to reduce their fluidity and consequently their ability to fuse (Klupp et al., 2000). Interestingly, a gM mutant of BHV-1 was more susceptible to antibody-mediated neutralization (König et al., 2002), suggesting differences in the topological distribution of glycoproteins on the viral envelope. To explain the apparently diverse defects in the Epstein–Barr virus gN...
mutant, Lake & Hutt-Fletcher (2000) hypothesized that gM plays a role in association and dissociation of capsids with membranes for budding through the inner nuclear membrane during egress and for penetration through the plasma membrane during infection. Brack et al. (1999) hypothesized that PRV gM is required for directing intracytoplasmic capsids to the TGN. Taken together with the fusion inhibition, it is possible that the gM/gN complex plays a role in virus entry and egress through modulation of glycoprotein trafficking and/or membrane fusion. Further studies to identify cellular proteins that interact with gM/gN may clarify the exact mechanisms.

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