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An α-Helical Domain within the Carboxyl Terminus of Herpes Simplex Virus Type 1 (HSV-1) glycoprotein B (gB) is Associated with Cell Fusion and Resistance to Heparin Inhibition of Cell Fusion

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Previous studies from our laboratory indicated that a 28-amino-acid carboxyl-terminal truncation of gB caused extensive virus-induced cell fusion (Baghian *et al.,* 1993, *J Virol* 67, 2396–2401). We tested the ability of additional truncations and mutations within gB to cause cell fusion in the recently established virus-free cell fusion assay (Turner *et al.,* 1998, *J. Virol. 72,* 873–875). Deletion of the carboxyl-terminal 28 amino acids of gB (gBΔ28), which removed part of the predicted α-helical structure H17b, caused extensive cell fusion. A gB truncation specified by gBΔ36, which removed the entire H17b domain, caused as much cell fusion as the gBΔ28 truncation. Similarly, gB(A874P) containing a substitution of an Ala with Pro within H17b caused cell fusion. Heparin, a gB-specific inhibitor of virus-induced cell fusion, inhibited both wild-type gB and gB(syn3)-mediated cell fusion. In contrast, fusion of cells transfected with gB(Δ28), gB(Δ36), or gB(A874P) was resistant to heparin inhibition of cell fusion. We concluded the following: (1) The predicted α-helical structure of H17b within the carboxyl terminus of gB is involved in both virus-induced and virus-free cell fusion. (2) Heparin is a specific inhibitor of gB-mediated fusion in both systems. (3) Resistance to heparin inhibition of gB-mediated cell fusion is associated with the predicted α-helical structure H17b within the carboxyl terminus of gB.

INTRODUCTION

Viral glycoproteins are key determinants in membrane fusion events throughout the virus life cycle. Herpes simplex viruses specify at least twelve glycoproteins: gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL gM, and gN, which are expressed in infected cells. These glycoproteins function in several important roles, including pH-independent virus entry via fusion of the viral envelope with cellular membranes, cell-to-cell spread, virus-induced cell fusion, and egress of infectious virion particles (reviewed in Mettenleiter, 2000; Spear, 1993). Recombinant mutant viruses that lack gB (UL27), gD (US6), gH (UL22), or gL (UL1) attach to cell surfaces through cell-surface glycosaminoglycans, but fail to penetrate, indicating these glycoproteins are required at least in part for virus-to-cell fusion (Cai *et al.*, 1988; Forrester *et al.,* 1992; Ligas and Johnson, 1988; Roop *et al.*, 1993). Either of two HSV-1 glycoproteins, gC (UL44) or gB, can mediate binding of virus to heparan sulfate proteoglycans on cellular surfaces. Accordingly, attachment is inhibited by soluble heparin, a glycosaminoglycan closely related to heparan sulfate (Gruenheid *et al.,* 1993; Herold *et al.,* 1991, 1994; Shieh *et al.,* 1992; WuDunn and Spear, 1989). Fusion between the viral envelope and cell membrane requires gB, gD, and gH/gL, and possibly the tegument protein UL25 (Addison *et al.,* 1984; Cai *et al.*, 1988; Forrester *et al.,* 1992; Ligas and Johnson, 1988; Roop *et al.*, 1993). Penetration of virus requires the interaction of gD with specific cell surface receptors (Cocchi *et al.,* 1998; Geraghty *et al.,* 1998; Montgomery *et al.,* 1996; Warner *et al.,* 1998).

Spread of infectious virus occurs either by release of virions to extracellular spaces or through virus-induced cell-to-cell fusion. Mutations, which cause extensive virus induced cell-to-cell fusion, can arise in at least four genes of the HSV genome: the UL53-gK gene, the gB gene, the UL20 gene, and the UL24 gene (Baines *et al.,* 1991; Bond and Person, 1984; Bzik *et al.,* 1984b; Cai *et al.*, 1988; Debroy *et al.*, 1985; DeLuca *et al.,* 1982; Dolter *et al.*, 1994; Engel *et al.*, 1993; Gage *et al.*, 1993; Hutchinson *et al.,* 1992; Jacobson *et al.*, 1989; Pogue-Geile and Spear, 1987; Ruyechan *et al.,* 1979; Sanders *et al.*, 1982; Weise *et al.,* 1987). However, like virus-to-cell fusion, gB, gD, gH, and gL have been shown to be necessary and sufficient to cause virus-induced cell fusion. The evidence is twofold. First, mutant viruses that do not express any one of these four glycoproteins fail to produce syncytia (Cai *et al.*, 1988; Davis-Poynter *et al.,* 1994; Ligas and Johnson, 1988; Roop *et al.*, 1993). Similarly, transient expression of HSV-1 or HSV-2 gB, gD, gH, and gL causes polykaryocyte formation in transfected Cos cells (Muggeridge, 2000; Turner *et al.,* 1998). Glycoproteins gE (US8), gI (US7), and gM (UL10), as well as the UL45 protein, may be able to enhance virus-induced cell fusion, since deletions of these genes cause inhibition of cell fusion (Davis-Poyn-

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ter *et al.,* 1994; Haanes *et al.,* 1994; Visalli and Brandt, 1991). Similarly, gC may be able to inhibit virus-induced cell fusion of at least some cell types, which may explain the lack of gC expression in many syncytial mutants (Manservigi *et al.*, 1977; Novotny *et al.*, 1996; Pertel and Spear, 1996). It is apparent there are similarities as well as significant differences between the virus-induced cell fusion and fusion of cells by transient co-expression of gB, gD, and gH/gL. Both systems require the expression of gD receptors for fusion to occur (Pertel *et al.,* 2001; Terry-Allison *et al.,* 1998); however, the transient expression system, in contrast to the virus-induced cell fusion, does not require the presence of heparan sulfate (Pertel *et al.,* 2001; Shieh and Spear, 1994). Additionally, viral proteins that exhibit the ability to influence virus-induced cell fusion do not produce similar effects in the transient fusion assay system (Klupp *et al.*, 2000; Turner *et al.,* 1998).

Glycoprotein B is highly conserved across all subfamilies of herpesviruses. It exists within cells as a homodimeric type I integral membrane protein that is N-glycosylated at multiple sites within the polypeptide. Each 904-amino-acid (aa) monomer includes a cleavable signal sequence, an extracellular domain of 696 aa, a 69-aa triple-pass transmembrane region, and a 109-aa carboxyl-terminal domain. Single amino acid substitutions within two regions of the intracellular cytoplasmic domain of gB were shown to cause syncytium formation and were designated Region I (aa positions 816 and 817) and Region II (aa positions 853, 854, and 857) (Bzik *et al.,* 1984a; Cai *et al.,* 1988; Gage *et al.*, 1993; Walev *et al.,* 1994). Deletion of the carboxyl-terminal 41 amino acids of gB did not adversely affect viral infectivity and replication; whereas, deletion of 28 amino acids caused extensive virus-induced cell fusion (Baghian *et al.,* 1993; Huff *et al.,* 1988).

We extended our previous findings by examining the role of specific carboxyl-terminal domains of gB in cell fusion in the context of viral infection as well as in a transfection-fusion system. We show that an α-helical domain within the gB carboxyl terminus appears to regulate cell fusion. Moreover, we provide evidence that heparin inhibition of gB-mediated cell fusion is also regulated by the carboxyl terminus of gB for both virus-induced cell fusion as well as fusion by co-expression of gB, gD, and gH/gL. In this regard, gB-mediated fusion functions similarly for both fusion systems.

RESULTS

The effect of gB truncations on virus-independent cell fusion

Previously, we investigated the effect of several gB truncations on virus-induced cell fusion. We found that a recombinant virus expressing a 28-amino-acid truncated gB resulted in extensive virus-induced cell fusion (Baghian *et al.,* 1993). A schematic of the predicted structure of the carboxyl terminus of gB showing the location of relevant truncations and mutations is shown in Fig. 1. The 28-amino-acid deletion of gB disrupts the predicted α-helical structure H17b composed of 16 amino acids. To assess whether these gB truncations caused similar effects in a virus-independent cell fusion assay, we transfected COS7 cells with plasmids constitutively expressing glycoproteins gD, gH, and gL in the presence of different mutated gB (Fig. 2). The extent of cell fusion was quantified by counting nuclei in 100 polykaryocytes per assay by two independent observers (Fig. 3, Table 1). As shown previously, transient expression of full-length, wild-type gB, gD, gH, and gL genes under the human cytomegalovirus immediate early promoter (CMV-IE) caused limited polykaryocyte formation (Turner *et al.,* 1998) (Fig. 2A). Similar numbers of polykaryocytes were formed when the CMV-IE driven gB gene was substituted with plasmid p9–2080 expressing gB under the adenovirus major late promoter (Ad-MLP). (Fig. 2B). Transfection controls that did not include gB failed to cause any cell fusion (data not shown). Similarly, inclusion of p9–1537 in the transfection fusion assay failed to cause any cell fusion (Fig. 2C). Plasmid p9–1537 specifies gB containing an internal, in-frame truncation spanning amino acids 130–697. This deletion causes rapid degradation of the gB product (Baghian *et al.,* 1993).

All gB mutants, which modified H17b, used in these experiments expressed high levels of gB and similar amounts of gB were detected on cell surfaces by immunofluorescence against paraformaldehyde-fixed transfected cells (Baghian *et al.,* 1993; data not shown). Transfection with p9–1511 caused more than twofold increase in cell fusion in comparison to wild-type gB (Fig. 2F, Table 1). An identical truncation of 28 amino acids in gB specified by mutant virus *amb*1511 caused extensive cell fusion (Baghian *et al.,* 1993). Interestingly, transfection of the 1511(gBΔ28) plasmid mixture resulted in fusion of the entire cell monolayer (8×10^6 cells) within 48–72 h after transfection (data not shown).

Transfections with p9–1513 or p9–1528 specifying gB truncations of 64 and 49 amino acids, respectively, both of which disrupt the predicted α-helical region H17a, caused substantially less cell fusion (approximately 50% reduction) than that of the wild-type gB gene (Figs. 2D and 2E; Table 1). Previously, we showed that gB specified by p9–1513 and p9–1528 was not as efficiently processed through the Golgi as the gB specified by p9–2080 (wild-type) or p9–1511 (Baghian *et al.,* 1993). Therefore, the inability of these two mutant forms of gB to cause cell fusion may be due to their altered processing and transport to cell surfaces.

The role of α-helical domain H17b in cell fusion

The 1511 gB truncation interrupted the predicted α-helical domain H17b, allowing only the first half of this domain to be expressed (Fig. 1) (Baghian *et al.,* 1993; Pellett *et al.,* 1985). To further assess the role of this

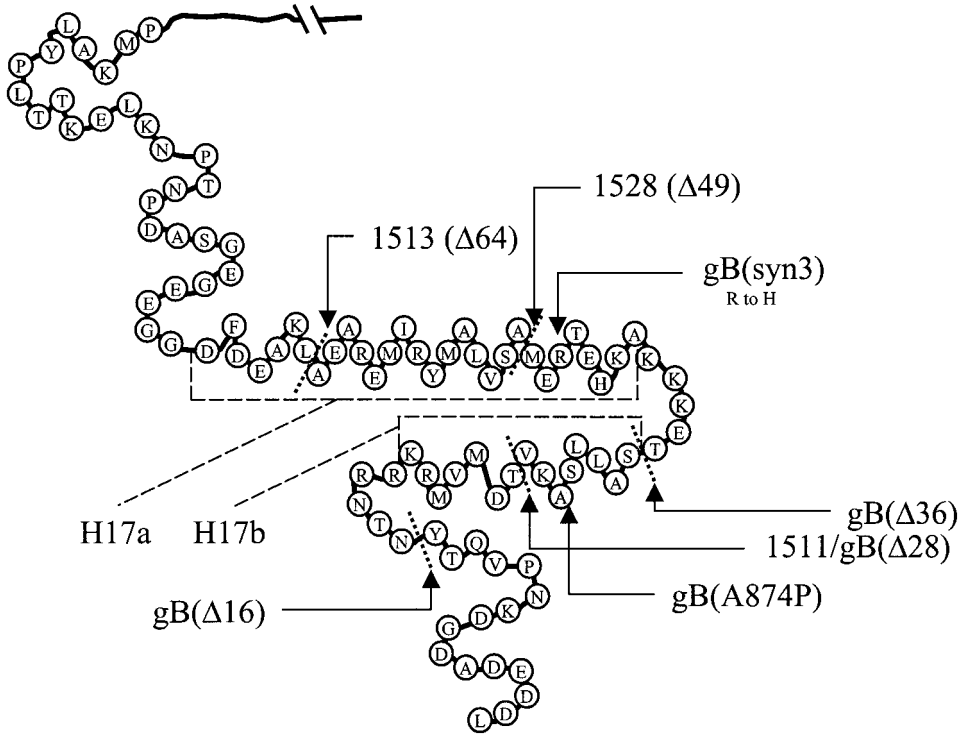

FIG. 1. Schematic diagram of the carboxyl terminus of HSV-1 gB. The predicted secondary structure of the gB carboxyl-terminal 109 amino acids is shown as described previously (Baghian *et al.,* 1993; Pellett *et al.,* 1985). The location of the gB truncations and mutations specified by plasmids p9–1513, p9–1528, p9–1511, pCMV-gB(Δ28), pCMV-gB(Δ36), pCMV-gB(A874P), pCMV-gB(Δ16), and pCMV-gB(syn3) are shown. The Δ followed by a number indicates the number of amino acids deleted from the carboxyl terminus of gB. The location of the two predicted α-helices, H17a and H17b, are shown and marked by dashed lines.

domain in the transient fusion assay, additional mutated gB genes were constructed and tested for their ability to cause cell fusion. Plasmid pCMV-gB(Δ36) specifies a gB truncated immediately before the H17b domain, while plasmid pCMV-gB(Δ16) specifies gB truncated after the H17b domain (Fig. 1). The gB(Δ16) truncation produced fusion similar to wild-type gB (Figs. 4A and 4C). In contrast, gB(Δ36) caused similar levels of cell fusion to gB(Δ28) (Figs. 4D and 4E). To evaluate the role of the predicted α-helical structure of the H17b domain in cell fusion, a single amino replacement was engineered into the H17b domain by replacing alanine 874 with proline,

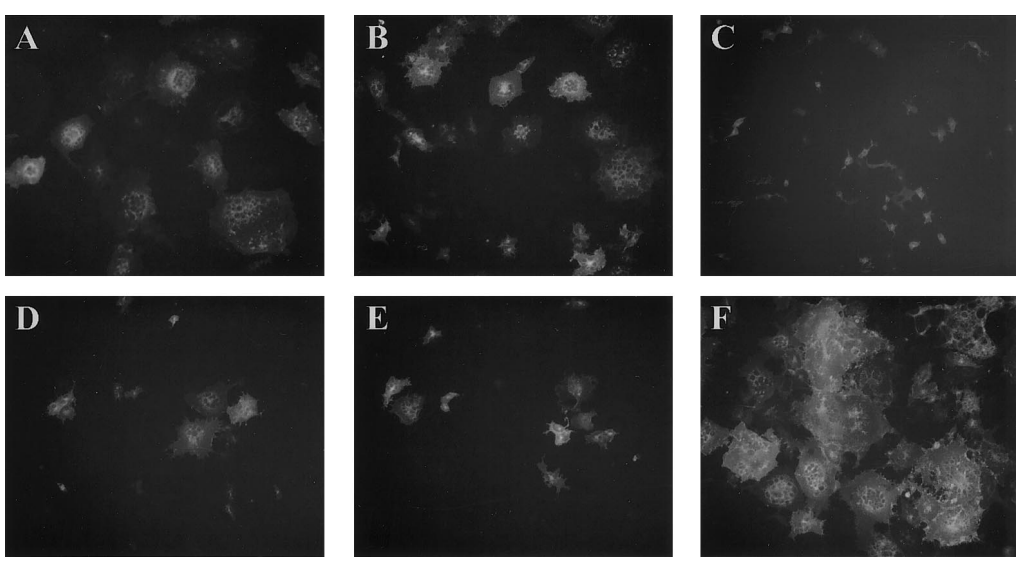

FIG. 2. Truncation of the carboxyl-terminal 28 amino acid of gB causes extensive fusion of transfected Cos cells. Cells were transfected with plasmids expressing gD, gH/gL, and plasmids pCMV-gB (A), p9–2080 (B), p9–1537 (C), p9–1513 (D), p9–1528 (E), and p9–1511 (F). Transfected cells were visualized by IFA at 36 h after transfection (65× magnification).

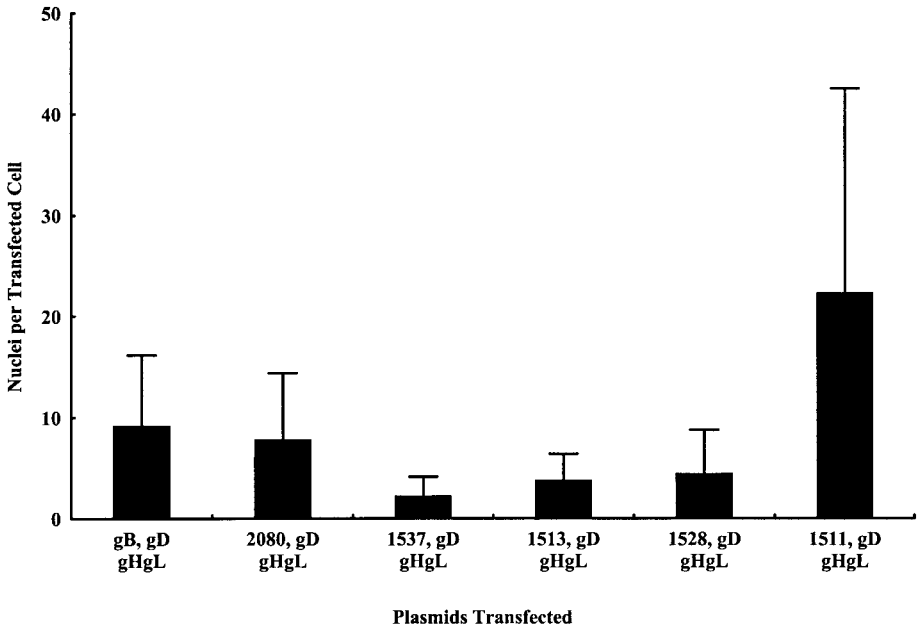

FIG. 3. Quantitation of the effect of gB truncation on fusion of transfected Cos cells. Nuclei in 100 IFA-positive cells per assay were counted by two observers and the average values and standard deviation were calculated.

causing disruption of the predicted α-helical structure (Fig. 1). Expression of gB(A874P) produced cell fusion similar to gB(Δ28) and gB(Δ36) (Figs. 4D–4F). For comparative purposes, we tested the ability of gB specified by syncytial mutant HFEM(*ts*B5), which contains a missense syncytial mutation within the H17a α-helix, to cause cell fusion. Transient expression of the gB(syn3) gene did not increase cell fusion relative to the wild-type gB, in agreement with results obtained with other HSV-1 gB syncytial mutations (Turner *et al.,* 1998) (Figs. 4A and 4B). Polykaryocyte formation was quantified by counting propidium iodide stained nuclei as explained earlier. Results are summarized in Table 2.

The effect of heparin on gB-mediated cell fusion

Heparin is a potent inhibitor of virus penetration as well as gB-mediated (syn3) virus-induced cell fusion (Gruenheid *et al.,* 1993; Herold *et al.,* 1991, 1994; Shieh *et al.,* 1992; WuDunn and Spear, 1989). Transfected cell monolayers were incubated either in the presence or absence of heparin (10 mg/ml). Cell fusion was visualized through fluorescence microscopy and the levels of observed cell fusion were quantified (Figs. 5 and 6). Incubation of transfected cell monolayers with heparin inhibited both wild-type gB and gB(syn3)-mediated cell fusion (~85% inhibition) (Figs. 4, 5A, and 5B; Table 3) as previously reported for *ts*B5 virus-induced cell fusion (Seck *et al.,* 1994). In contrast, fusion of cells expressing gB(Δ28) or gB(Δ36) was largely resistant to heparin inhibition (Figs. 4, 5D, and 5E). Cell fusion caused by gB(A874P) was inhibited by approximately 40% in heparin treated vs. untreated controls (Figs. 4 and 5F; Table 3). Heparin inhibited fusion caused by gB(Δ16) by approximately 58% (Figs. 4 and 5C; Table 3).

TABLE 1

Truncation of the Carboxyl Terminal 28 Amino Acid of gB Causes Extensive Fusion of Transfected Cos Cells

Plasmids transfected (all include gD, gHgL)	gB	2080	1537	1528	1513	1511
Total nuclei counted	1838	1561	444	888	762	4460
Average nuclei	9.19	7.81	2.22	4.44	3.81	22.30
Standard deviation	6.98	6.58	1.92	4.32	2.58	20.21
Standard error	0.49	0.47	0.14	0.31	0.18	1.43
Median	8	6	2	3	3	16
Smallest no. of nuclei	1	1	1	1	1	1
Largest no. of nuclei	51	52	17	34	15	129
% change from gB	—	−15.1%	−75.8%	−51.7%	−58.5%	142.7%

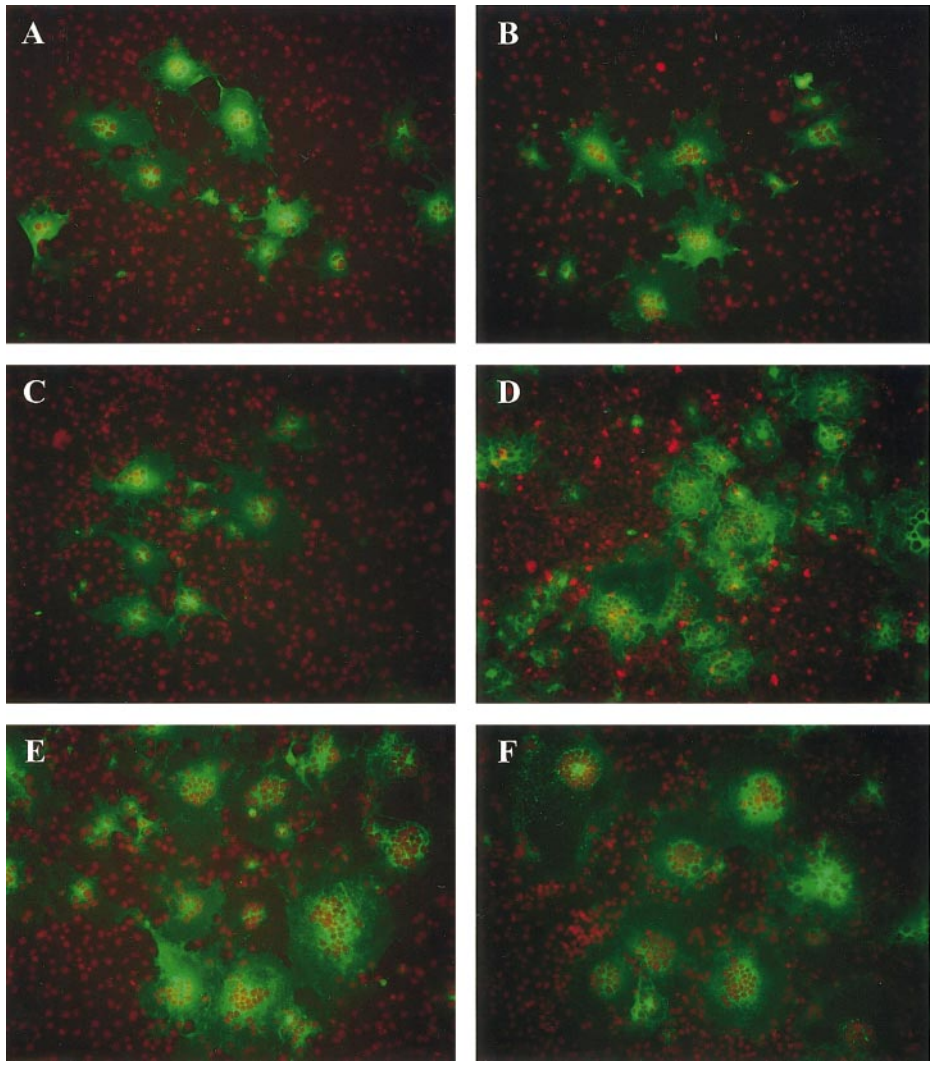

FIG. 4. gB-H17b-targeted mutations enhance fusion of transfected Cos cells. Cells transfected with plasmids expressing gD, gH/gL, and gB (A), gB(syn3) (B), gB(Δ16) (C), gB(Δ28) (D), gB (Δ36) (E), and gB (A874P) (F). Transfected cells were visualized by IFA at 36 h after transfection at 65× magnification.

The effect of heparin on virus-induced cell fusion

Heparin was unable to fully inhibit cell fusion mediated by truncated or mutated gB in the transient fusion assay. Therefore, we assayed the effect of heparin on virus-induced cell fusion caused by syncytial mutant viruses. Syncytial viruses mp(MP), HFEM(*ts*B5), and *amb*1511 were engineered to constitutively express EGFP as described previously for the construction of the KOS/EGFP

TABLE 2

gB-H17b-Targeted Mutations Enhance Fusion of Transfected Cos Cells

Plasmids transfected (all include gD, gHgL)	gB	gB (syn3)	gB (Δ16)	gB (Δ28)	gB (Δ36)	gB (A874P)
Total nuclei counted	2035	2340	2232	4146	4332	4000
Average nuclei	10.18	11.7	11.16	20.73	21.66	20
Standard deviation	6.93	8.61	7.81	15.62	17.36	14.33
Standard error	0.49	0.61	0.55	1.1	1.23	1.01
Median	9	11	10	17	18	18
Smallest no. of nuclei	1	1	1	1	1	1
Largest no. of nuclei	41	44	47	92	99	80
% Increase over wt gB	—	14.9%	9.6%	103.6%	112.8%	96.5%

TABLE 3

gB-H17b-Targeted Mutations Confer Resistance to Heparin Inhibition of Cell Fusion

Plasmids transfected (all include gD, gHgL)	gB	gB (syn3)	gB (Δ16)	gB (Δ28)	gB (Δ36)	gB (A874P)
Total nuclei counted	471	486	1059	3414	3397	2543
Average nuclei	2.36	2.43	5.3	17.07	16.99	12.72
Standard deviation	1.93	2.14	5.07	10.95	14.04	9.31
Standard error	0.13	0.15	1.1	0.77	0.99	0.66
Median	2	2	3	16	13	11
Smallest no. of nuclei	1	1	1	1	1	1
Largest no. of nuclei	10	14	26	55	91	50
% Inhibition by heparin	85.2%	86.6%	57.7%	18.6%	22.6%	38.3%

virus (Foster *et al.*, 1999). Expression of EGFP from this locus results in distinct nuclear and diffuse cytoplasmic fluorescence, facilitating visualization of polykaryocytes (Fig. 7). As previously reported (Seck *et al.,* 1994), tsB5-induced cell fusion was inhibited by heparin (Figs. 7C and 7D). In contrast, fusion caused by mp(MP), which contains the syn1 mutation in glycoprotein K (gK), was not inhibited by heparin (Figs. 7G and 7H). Similar to the

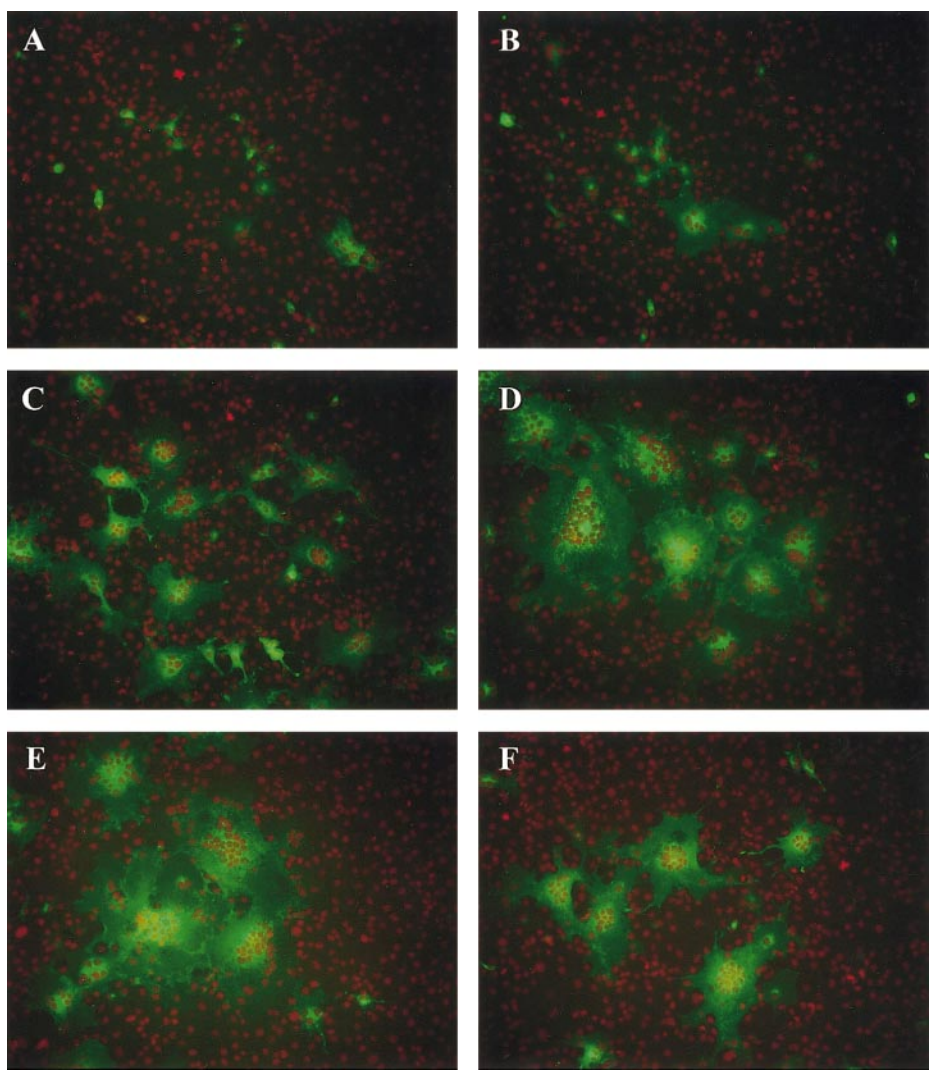

FIG. 5. gB-H17b-targeted mutations confer resistance to heparin inhibition of cell fusion. Cells transfected with plasmids expressing gD, gH/gL, and gB (A), gB(syn3) (B), gB(Δ16) (C), gB(Δ28) (D), gB (Δ36) (E), and gB (A874P) (F). Cells were incubated in the presence of heparin. Transfected cells were visualized by IFA at 36 h after transfection at 65× magnification.

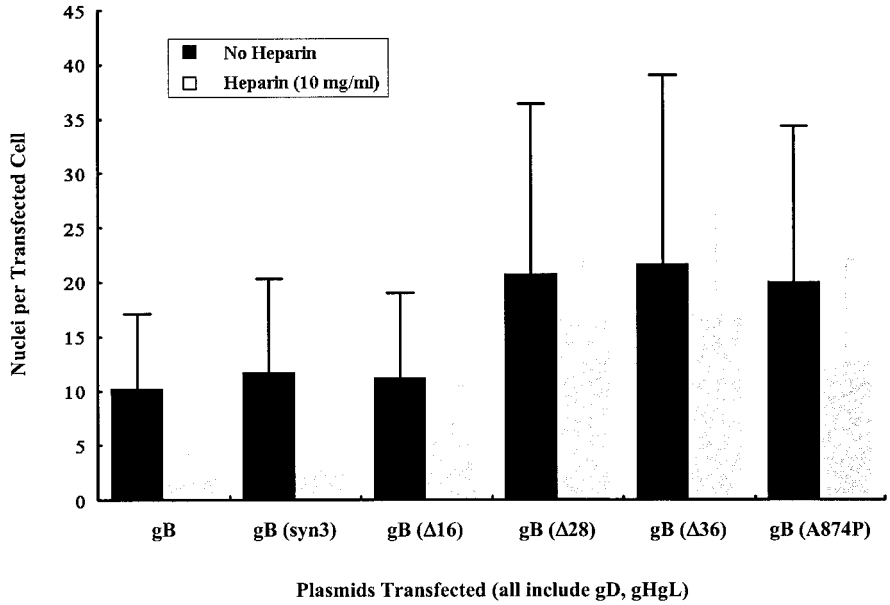

FIG. 6. Quantitative comparison of fusion of transfected cells in the presence and absence of heparin. Cell fusion represented in Fig. 4 (without heparin) and Fig. 5 (with heparin) was quantitated as described in the legend of Fig. 3.

results obtained in the transient fusion assay, cell fusion caused by mutant virus-*amb*1511/EGFP was resistant to heparin inhibition (Figs. 7E and 7F).

DISCUSSION

Studies on the role of individual viral glycoproteins in virus entry as well as virus-induced cell fusion are hampered by the fact that multiple glycoproteins and proteins are involved in both processes. In this regard, the established model system for virus-associated membrane fusion phenomena via transient co-expression of gB, gD, and gH/gL represents a reasonable model system to investigate individual glycoprotein functions. However, it is important to compare virus-induced cell fusion and virus-free fusion systems to ensure that functional aspects of glycoproteins expressed in the transfection fusion system represent glycoprotein functions manifested in the context of the viral genome. Our investigations produced the following conclusions. First, we show that the predicted α-helical domain H17b within the carboxyl terminus of gB is involved in both virus-induced and virus-free fusion systems. Second, heparin is a specific inhibitor of gB-mediated fusion in both systems. Third, resistance to heparin inhibition of gB-mediated cell fusion is associated with the predicted α-helical structure H17b. Thus, gB-mediated fusion is functionally similar for both fusion assays.

Previously, we showed that truncating gB by 28 amino acids produced wild-type levels of mature gB and caused extensive virus-induced cell fusion (Baghian *et al.,* 1993), while a truncation of 41 amino acids did not affect virus replication and failed to cause cell fusion (Huff *et al.,* 1988). We proposed that the α-helical domain H17b, which was interrupted by the 28-amino-acid truncation, must be involved in virus-induced cell fusion (Baghian *et al.,* 1993). Co-expression of the 28-amino-acid truncated gB specified by plasmid p9–1511 with gD, and gH/gL caused extensive cell fusion as evidenced by the formation of large-size polykaryocytes. The extent of the fusion occurring after cotransfection of glycoprotein genes containing the truncated gB was similar to virus-induced cell fusion caused by mutant viruses expressing the gB (syn3) or gB(Δ28). Ultimately, transfection of Cos cells led to fusion of the entire cell monolayer of up to 5×10^6 cells into a single syncytium within 48–72 h. Clearly, the 28-amino-acid gB deletion functioned similarly in both the virus-induced cell fusion and virus-free transfection fusion assay. Three conclusions can be made based on these results. First, the ability of truncated gB to cause extensive cell fusion in the transfection fusion assay provides further evidence that certain gB functions can be manifested in the absence of productive viral replication. Second, the truncated gB's ability to extensively fuse membranes requires only the presence of co-expressed gD and gH/gL glycoproteins. Third, the domains of gB that are responsible for this extensive cell fusion phenotype, in both virus-induced and virus-free transfection systems, are located in the carboxyl terminus of gB.

In agreement with our previous results, which implicated the H17b α-helical domain in virus-induced cell fusion, we found that deletion of the entire H17b domain or substitution of a single proline within the H17b helical domain caused extensive cell fusion as evidenced by the formation of large-size polykaryocytes. The ability of the two gB truncations and the single proline residue sub-

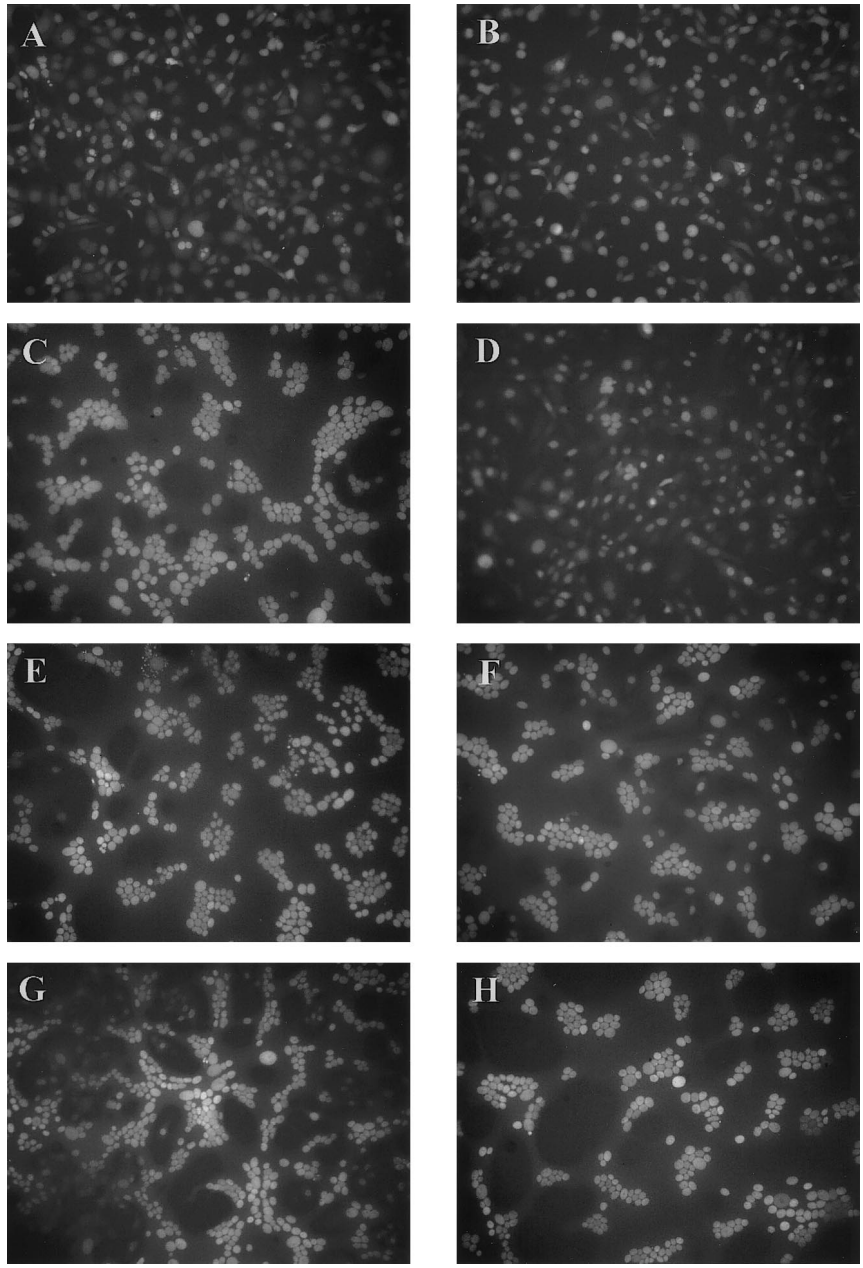

FIG. 7. Cell fusion caused by viruses specifying H17b-mutated gB is resistant to heparin inhibition. Vero cells were infected at an m.o.i. of 0.1 with KOS/EGFP (A, B), tsb5/EGFP (C, D), *amb*1511/EGFP (E, F), and MP/EGFP (G, H) in the presence (B, D, F, H) or absence (A, C, E, G) of heparin. Infected cells were visualized by fluorescence microscopy (65× magnification).

stitution to cause extensive cell fusion indicates that the predicted α-helical structure of H17b serves as a major determinant for membrane fusion. All gB mutants, which disrupted the predicted α-helical domain H17b either by truncation or the proline substitution did not affect gB synthesis or transport to cell surfaces as evidenced by immunofluorescence against transfected cells fixed with 4% paraformaldehyde (not shown). Therefore, the ability of these mutant gB forms to cause cell fusion is largely due to their specific gB mutations. H17b is located immediately adjacent to the predicted 31-amino-acid-long α-helix, H17a, within which multiple syncytial mutations have been mapped (Bzik *et al.,* 1984a; Gage *et al.*, 1993). However, syncytial mutations within H17a and H17b can be functionally differentiated with respect to their ability to cause membrane fusion in the transfection fusion system. The *syn*3 mutation within H17a failed to enhance fusion in the transfection fusion assay. In contrast, both the 36- and 28-amino-acid truncations as well as the proline substitution affecting H17b readily caused extensive cell fusion. The most plausible explanation for these results is that H17b functions as an independent fusion domain regulator. Partial or full inactivation of this domain confers the ability on gB to cause extensive cell fusion. Furthermore, fusion associated with this domain requires the presence of only gD and gH/gL. In contrast,

the H17a domain must require additional viral proteins expressed during viral infection. Truncations and mutations in the gH carboxyl terminus have been shown to inhibit cell fusion implying that the carboxyl terminus of gH is also involved in membrane fusion (Browne *et al.*, 1996; Wilson *et al.*, 1994). An alternative explanation of the role of the H17b domain in cell fusion is that the carboxyl termini of gB and gH functionally interact and this interaction affects the fusogenic abilities of both gB and gH. It is worth noting that there is a discrepancy between trasfection fusion results obtained with HSV-1 and HSV-2 glycoproteins, inasmuch as a *syn* mutation within HSV-2 gB was reported to enhance fusion in the transfection fusion system (Muggeridge, 2000). HSV-2 enters faster into cells than HSV-1 implying the presence of an enhanced mechanism for virus-to-cell fusion (DeLuca, 1981, Kousoulas, unpublished observations). Therefore, the different dynamics of HSV-1 and HSV-2 glycoprotein-mediated membrane fusion phenomena may account for the observed differences in fusion caused by syncytial mutations within HSV-1 and HSV-2 gB.

Heparin is a specific inhibitor of gB-mediated virus-induced cell fusion (Seck *et al.,* 1994). In agreement with previous reports, we confirmed that heparin specifically inhibited fusion caused by syncytial mutant HFEM (*ts*B5), while it did not inhibit fusion caused by mutants with syncytial mutations in gK. Heparin is known to predominantly bind to gC and secondarily to gB (Herold *et al.,* 1991, 1994; Laquerre *et al.,* 1998; Spear *et al.,* 1992). The binding sites of other proteins exhibiting heparin-binding activity have been mapped to dense regions of positively charged residues containing variable lengths of arginine-rich and/or lysine-rich regions (Cardin *et al.,* 1991; Marino *et al.,* 1999; Sendak and Bensadoun, 1998; Sendak *et al.,* 2000; Wong *et al.,* 1995). The primary sequence of gB contains a heparin binding site at amino acids 68–76 (KPKKNKKPK) in the amino terminus of gB. Deletion of this amino acid domain results in substantial reduction in heparin binding of gB (Laquerre *et al.,* 1998). There are additional potential heparin binding sites within gB at amino acid locations 158, 318, 494, 862, and 882 containing the consensus binding motif R/K-X-R/K. The simplest explanation for the inhibitory effect of heparin on gB-mediated cell fusion is that heparin physically blocks cell fusion. Heparin inhibited fusion in the transfection fusion system when full-copy gB, gD, and gH/gL were co-expressed or when transfections contained the gB(syn3)gene. However, heparin was unable to substantially inhibit fusion caused by truncations of gB that delete all or part of the H17b α-helix. Heparin did partially inhibit fusion caused by the single Ala to Pro substitution, which inactivates the predicted α-helical structure of the H17b domain. These differences between the truncated gB and the proline substituted gB could be due to the more severe nature of the truncations vs. the single amino acid replacement in that certain amino acids upstream or downstream of the proline substitution may confer partial susceptibility to heparin inhibition of membrane fusion. We offer the following hypotheses to explain the effect of heparin on gB-mediated cell fusion. First, truncation of gB may affect the conformation of the gB amino terminus resulting in the partial loss of heparin binding. Second, gB truncations may cause changes in the interaction of gB with cellular receptors, which render gB-mediated fusion resistant to heparin. In support of the latter hypothesis, it was shown that bovine herpesvirus gB interacted with two types of receptors on Madin Darby bovine kidney cells (MDBK), a heparan sulfate proteoglycan, and an unknown high-affinity receptor, respectively. Truncated gB retained the ability to bind heparin, but not the ability to block the interaction of gB with a high-affinity binding receptor on MDBK cells (Li *et al.,* 1996). Furthermore, deletion of the carboxyl terminus of gB resulted in the loss of one monoclonal antibody epitope located in the amino-terminal of gB, implying that the truncated gB had assumed an altered conformation, which was responsible for the loss of the binding to the high-affinity receptor (Li *et al.,* 1996). Additional evidence that perturbations of the carboxyl terminus of gB may affect the conformation of the gB extracellular portion was provided by an Ala to Val change at position 851 within the syncytial region II of HSV-1 gB, which caused delayed viral entry kinetics (Gage *et al.*, 1993).

Substantial differences between the virus-induced and transfection-induced fusion systems exist with respect to the role of heparan sulfate moieties on cell surfaces. Heparan sulfate enhanced virus-induced cell fusion, inasmuch as viruses with syncytial mutations failed to readily fuse heparan sulfate-deficient cells. In contrast, fusion caused by transient transfection of viral glycoproteins did not require the presence of heparan sulfate, although it did require the presence of cell surface entry receptors specific for gD (Pertel *et al.,* 2001; Shieh and Spear, 1994). Therefore, the ability of heparin to inhibit both virus-induced cell fusion and virus-free fusion in the transfection fusion assay must reflect direct inhibition of gB-associated functions and not heparin-competitive inhibition of gB binding to heparan sulfate proteoglycans on apposed cell surfaces. Such interactions, presumably, would strengthen interactions between plasma membranes and facilitate membrane fusion.

Heparin is known to bind to a variety of important proteins on cell surfaces and modulate the functions of these proteins predominantly through signal transduction effects to intracellular targets. However, heparin can also internalize into cells via specific or nonspecific receptors (Barzu *et al.,* 1996; Heinzelmann *et al.,* 1999; Letourneur *et al.*, 1995a,b; Watanabe *et al.*, 1995). Therefore, it is possible that endocytosed heparin may directly bind to the carboxyl terminus of gB at the predicted sites located at amino acids 862 and 882. In this scenario, resistance to heparin inhibition of cell fusion by truncated gB may be due to the lack of heparin binding sites,

particularly the site at amino acid 882, which is deleted in both gB truncations. The reduced ability of heparin to inhibit cell fusion caused by gB(A874P) and gB(Δ16) may be the result of local conformational changes, which prevent binding of heparin to upstream binding sites. Additional experiments are needed to ascertain whether heparin readily enters into gB-transfected cells and whether the carboxyl terminus of gB is able to bind heparin.

A carboxyl-terminal truncation of the PRV gB enhanced both virus-induced and virus-free cell fusion (Klupp *et al.*, 2000; Nixdorf *et al.,* 2000). However, in the virus-free transfection assay, cell fusion can occur in the absence of gD. Furthermore, a hybrid gD/gH protein composed of the extracellular portion of gD fused in-frame to a portion of gH, which contains the carboxyl terminus of gH including its membrane spanning region, functionally substituted the gH/gL heterodimer (Klupp and Mettenleiter, 1999; Klupp *et al.*, 2000). We constructed and tested a similar HSV-1 gD/gH fusion protein and found that it failed to substitute for the presence of gD or gH/gL in the virus-free fusion system (not shown). Thus, it is clear that there are similarities between PRV and HSV-1 with respect to the role of the gB carboxyl terminus in cell fusion, but also substantial differences with respect to the functions of gD and gH/gL. In this regard, the apparent disparity between the two fusion systems may reflect fundamental differences in the functions of these proteins as well as differences in their interactions with cellular receptors (Nixdorf *et al.,* 1999).

MATERIALS AND METHODS

Cells and viruses

Vero and COS7 cells were obtained from ATCC (Rockville, MD) and grown in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS). KOS/EGFP specifies the enhanced green fluorescence protein (EGFP) from a CMV-EGFP gene cassette inserted within the intergenic region between the UL53 and UL54 open reading frames (ORFs) (Foster *et al.*, 1999). To facilitate visualization of syncytia formation, recombinant viruses *amb*1511/EGFP, MP/EGFP, and tsB5/EGFP were constructed by insertion of a CMV-EGFP gene cassette as described in detail previously for the KOS/EGFP virus (Foster *et al.*, 1999). All viruses were propagated on Vero cells.

Construction of expression plasmids

Plasmid p9–2080 specifies a wild-type (wt) copy of gB; whereas, plasmids p9–1511, p9–1513, p9–1528, and p9–1537 specify carboxyl-terminally truncated gB driven by the Adenovirus Major Late Promoter (Ad MLP) and were as described previously (Baghian *et al.,* 1993). The entire open reading frames (ORFs) for gB and gD were amplified by PCR with specific primers that removed the 3′ stop codon such that the genes were inserted in frame with a V5 epitope tag when ligated to pcDNA3.1 TA TOPO to generate plasmids pCMV-gB and pCMV-gD. Truncations in gB were generated by PCR. Plasmids gB(Δ36), gB(Δ28), and gB(Δ16) express C-terminally truncated derivates of gB of 36, 28, and 16 amino acids (aa), respectively. A gB gene specifying a single codon change from alanine to proline at amino acid 874 was produced by splice-overlap extension (SOE) PCR utilizing synthetic oligonucleotides containing the point mutation. The PCR-derived gB(A874P) was ligated into pcDNA3.1 TA TOPO to generate plasmid pCMV-gB(A874P). All plasmid inserts were sequenced by Sanger dideoxy-chain termination methodology using an automated DNA sequencer (PE Biosystems, 377 Sequencer). Plasmid pcDNA3 gHgL was kindly provided by A. Minson.

Cell fusion assay

In vitro cell fusion assays were performed essentially as described previously (Klupp *et al.*, 2000; Muggeridge, 2000; Turner *et al.,* 1998). Briefly, the day prior to transfection, 35-mm 6-well plates were seeded with 8×10^4 COS7 cells. A total of 6 μg of plasmid DNA encoding the indicated glycoproteins was transfected with Superfect (Qiagen, Valencia CA) according to the manufacturer's directions. Three hours post-transfection, media was aspirated and cells were overlaid with DMEM 5% FCS. Where indicated, heparin (10 mg/ml) was included in the overlay media. Twenty-four hours post-transfection, cells were overlaid with 4×10^5 freshly trypsinized COS7 cells per well with or without the inclusion of heparin. Cells were subsequently incubated at 37°C for an additional 12 h before levels of fusion were determined.

Immunofluorescence assay and quantitation of fusion

Cells were fixed with 100% methanol for 10 min, washed twice with TBS, and incubated at room temperature for 1 h with the primary antibody anti-V5 (Invitrogen, Carlsbad, CA) diluted 1:200 in TBS-10% goat serum. Cells were then washed three times with TBS and incubated for 1 h at room temperature with FITC-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO) diluted 1:100 in TBS-10% goat serum. After incubation, excess antibody was removed by washing three times with TBS. Propidium iodide nuclear counterstain (Molecular Probes, Eugene, OR) was applied to facilitate quantitation of fusion. Fusion was examined on a Nikon Diaphot inverted fluorescent microscope. The number of nuclei in 100 transfected cells or polykaryocytes per well was determined by two independent observers. Transfected cells were defined as those cells that were IFA-positive. Average values and standard deviations were then calculated from the combined data obtained by the two observers. Percent change in levels of fusion over wild-type (wt) gB was calculated by the formula [(average nuclei mutated gB) − (average nuclei wt gB)]/(average

nuclei wt gB) × 100%. Percent inhibition of fusion by heparin was defined as {[(average nuclei of untreated assay) − 1] − [(average nuclei of heparin treated assay) − 1]}/[(average nuclei of untreated assay) − 1] × 100%.

Heparin inhibition of viral polykaryocyte formation

Subconfluent Vero cells were infected at an m.o.i. of 0.1 for 1 h and subsequently overlaid with DMEM-2% FCS, 1% methylcellulose either lacking or containing heparin (10mg/ml). The amount of heparin used in the virus-free fusion inhibition experiments was chosen on the basis of being the amount required to inhibit virus-induced cell fusion. Lower amounts of heparin (1 mg/ml instead of 10 mg/ml), effectively inhibited virus-free fusion caused by wild-type gB. Viral plaques were visualized by phase-contrast and fluorescent microscopy at 24 h post-infection.

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