Constitutive expression of human cytomegalovirus glycoprotein B (gpUL55) with mutagenized carboxy-terminal hydrophobic domains

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Stable transfectants were selected from human astrocytoma cells (U373) after transfection with recombinant expression vectors carrying the human cytomegalovirus (HCMV) glycoprotein B (gB; gpUL55) gene with alternative deletions of hydrophobic domain segment 1 (hdl) or segment 2 (hd2) of the carboxy-terminal potential bipartite membrane anchor domain. Comparative analysis of HCMV gB forms from cell lines gB(Mhd1) and gB(Mhd2), expressing mutagenized gB, and those from cells expressing authentic gB showed that deletion of hdl, but not that of hd2, interfered with efficient proteolytic cleavage of the gB precursor. Both mutagenized gB forms exhibited correct transport to the cell surface. Deletion of hd2, but not that of hdl, caused loss of membrane anchoring of the gB molecule, resulting in secretion of the respective gB form into the culture medium. The carboxy-terminal cleavage product of the soluble gB molecule, which migrated more slowly than its authentic counterpart, was modified by complex carbohydrate side chains and formed disulphide-linked complexes. Our observations indicate that hd2 is essential as well as sufficient for membrane anchoring of the HCMV gB molecule. For hdl, a potential fusogenic role is suggested by the conserved positional pattern of glycine residues, which is comparable to that of known fusion peptides of other viruses.

Introduction

Glycoprotein B (gB; gpUL55) of human cytomegalovirus (HCMV) is the most abundant and immunodominant envelope component, with at least two defined neutralizing epitopes (Britt et al., 1988; Rasmussen et al., 1988; Pereira et al., 1993). By analogy to its homologue, gB of herpes simplex virus (HSV), HCMV gB is thought to play an essential role in the entry of the virion into the host cell (Rasmussen et al., 1991; Gibson, 1993; Navarro et al., 1993). Furthermore, during the late infectious cycle, viral membrane glycoproteins obviously participate in and possibly direct sequential envelopmental processes in different cellular compartments, for example at the nuclear membranes and at cisternae of the early tubular endosome (Radsak et al., 1990; Tooze et al., 1993). This requires regulated and directed transport to the respective cellular target compartments. Previous work of others (Cranage et al., 1986; Spaete et al., 1990) and of our own laboratory (Reis et al., 1993) has demonstrated that the HCMV gB gene product is correctly transported and processed during eukaryotic expression in various systems. This consistent observation implies that the solitary HCMV gB polypeptide should present all the structural information necessary for its own maturational processing. Expression systems of HCMV gB can thus be used to define precisely the structural signals involved in its cellular transport and compartmentalization.

The deduced amino acid (aa) sequence for strain AD169 HCMV gB (Chee et al., 1990; Spaete et al., 1988) indicates a total of 906 aa with an amino-terminal 24 aa hydrophobic signal sequence for translocation into the rough endoplasmic reticulum (RER) and two hydrophobic aa stretches [hydrophobic domain 1 (hdl), aa 714–747; hd2, aa 751–771] that could serve as membrane spanning domains. The amino-terminal part of gB, with 19 consensus sequences for \( N \)-glycosylation, is thought to be luminal (Basgoz et al., 1992) and the hydrophilic carboxy-terminal portion of about 130 aa represents the cytoplasmic tail (Spaete et al., 1988, Cranage et al., 1986). In addition, HCMV gB exhibits the particular feature of a consensus sequence for intramolecular proteolytic cleavage at aa 459 (Spaete et al., 1988, 1990) by cellular furin (Vey et al., 1993).

It has been suggested that cellular transport and compartmentalization of membrane glycoproteins may be related to the type of membrane anchoring (Soullam & Worman, 1993; Rasile et al., 1993; Gilbert et al., 1994). A model has been suggested for the membrane...
anchoring of HCMV gB (Spaete et al., 1988) on the basis of theoretical assumptions, believed until recently for HSV gB (Pellett et al., 1985; Rasile et al., 1993), that hd1 and hd2 of HCMV gB are able to span the membrane a total of three times. In order to define experimentally the functional relevance of these domains, transfectant cell lines were established that constitutively express recombinant gB forms with appropriate deletions of hd1 and hd2. Our experiments showed (i) that hd2 was essential and sufficient to retain gB in the membrane and (ii) that deletion of hd2 promoted the secretion of gB into the culture medium.

During completion of this study, Rasile et al. (1993) reported observations for HSV gB that are in line with our data. The experiments described here were in part presented at the Spring meeting of the German Virology Society in Frankfurt, March 1994 (Reschke et al., 1994).

Methods

Cell cultures, virus propagation and titration. Monolayers of human foreskin fibroblasts (HFF) and of human astrocytoma U373 cells (kindly provided by Dr G. Jahn, Erlangen, Germany) were cultured in Eagle's MEM supplemented with 10% fetal calf serum (FCS), vitamins, non-essential amino acids, glucose, penicillin at 0.5 units/ml and gentamicin at 60 μg/ml. For propagation of strain AD169 HCMV, confluent monolayers of HFF (1.5 x 10^6 cells) were infected at a m.o.i. of approximately 0.01 and serum concentration was lowered to 2%.

For experimental infection of U373 cells, a m.o.i. of about 5 was used. To determine infectious units, coverslip cultures were infected with the virus suspensions prior to examination at 48 h post-infection (p.i.) of early antigen (EA) production by indirect immunofluorescence using a commercial monoclonal antibody (MAb; Radsk et al., 1990; Du Pont). For quantification, EA-positive nuclei were counted; the amount of virus inducing EA production in a single cell was defined as one infectious unit (Reis et al., 1993).

Construction of plasmids, site-directed mutagenesis, transfection assay and selection of stable transformants of U373 cells. The procedure for subcloning the complete coding sequence of the HCMV AD169 gB gene from a HCMV genomic bank (Fleckenstein et al., 1982) into a eukaryotic expression vector to obtain the construct pRC/CMV-gB has been described in detail previously (Reis et al., 1993). For mutagenesis, a 928 bp EcoRI-Xbal fragment of this construct containing the two coding sequences for the potential membrane anchor domains (Spaete et al., 1988; hd1 nucleotides (nt) 2140-2241, hd2 nt 2251-2313) was transferred into M13mp18 and correct insertion verified by sequencing using deazanucleotides (Spaete et al., 1988). Two alternative deletions were introduced in the gB gene insert by oligonucleotide-directed mutagenesis (Mutagenesis kit; Amersham) after transfection.

For quantitative analysis of gB expression by indirect immunofluorescence with MAb 27-156 (Spaete et al., 1990; Radsak et al., 1990) aliquots of cell extracts of comparable protein content or culture medium from labelled cells (free of insoluble material by ultracentrifugation at 100000xg for 2 h and 4 °C, mixed 1:1 with buffer) were preclotted by incubation with Protein A-Sepharose CL4B beads (Sigma) prior to incubation overnight at 4 °C with gB-specific MAb (see above). Adsorption of immunocomplexes for 1-5 h at room temperature to Protein A-Sepharose CL4B beads coated with rabbit anti-mouse IgG (Dako) was followed by seven washing cycles of the beads with PBS plus 0.1% NP40, 0.1% SDS and one washing cycle with distilled water. The washed precipitates were subjected to SDS-PAGE (Laemmli, 1970) and consecutive fixation and fluorography (Bonner & Laskey, 1974) of the dried slab gels.

Glycosidase digestion. Digestion with endoglycosidase H, peptide-N-glycosidase F (PNGase F) or O-glycosidase was performed on immunoprecipitates according to the instructions of the manufacturer (Boehringer Mannheim; Bogner et al., 1992).

Surface biotinylation and streptavidin-mediated precipitation. For surface membrane biotinylation, uninfected or infected cell cultures (1 x 10^6 cells each) were rinsed three times with cold PBS supplemented with 91 mM-CaCl_2 and 1 mM-MgCl_2 (PBS-CM) prior to incubation for 15 min at 4 °C with 10 ml PBS-CM containing 0.5 μg sulpho-NHS-biotin (sulfo-NHS-biotin; Pierce; diluted from a stock of 200 mg/ml DMSO). Subsequently cultures were again rinsed three times with PBS-CM and incubated with MEM plus 2% FCS and 10 mM-glycine to quench unreacted sulfo-NHS-biotin. The washed precipitates were subjected to SDS-PAGE (Laemmli, 1970) and consecutive fixation and fluorography (Bonner & Laskey, 1974) of the dried slab gels.

new derivatives pRC/CMV-gB(Mhd1) and pRC/CMV-gB(Mhd2). The expected deletions in the M13mp18 constructs as well as in the pRC/CMV-gB derivatives were verified by sequencing (Sanger et al., 1977).

For transfection of subconfluent U373 cells 48 h after seeding (9 cm Petri dishes), the calcium phosphate method described by Chen & Okayama (1987) was employed, using 50 μg of pRC/CMV-gB-derived DNA per dish. Two days after transfection, selection was initiated with genetin (G418) at 800 μg/ml. Resistant clones emerging after 2-3 weeks were aspirated with the tip of a micropipette (observed under a microscope) and propagated prior to examination for recombinant gB expression by indirect immunofluorescence with MAB 27-156 (Spaete et al., 1988; Radsak et al., 1990). Because selection by genetin alone did not yield populations in which 100% of the cells expressed the recombinant gB or authentic gB polypeptides, subcloning by aspiration of single cells, as described previously was performed (Reis et al., 1993). For the experiments described the stable homogeneous transformants were used between the seven and 25 passages after transfection.

Immunoassays. Visualization of gB and recombinant expression was carried out by indirect immunofluorescence with MAB 27-156 or 58-15 (Britt & Vugler, 1992) and a secondary fluorescein isothiocyanate-labelled rabbit anti-mouse IgG (Dako) on cells permeabilized by acetone fixation for 15 min at −20 °C.

Radioimmunoassays of gB and immunoprecipitation. For radioimmunoassays prior to immunoprecipitation, complete MEM on the cultures (1-5 x 10^6 cells) was replaced by labelling medium consisting of methionine-free MEM with added [35S]methionine (50-100 μCi/ml; sp. act. > 1000 Ci/mmol; Amersham) for defined intervals p.i. To chase, the radiolabelled cultures were incubated in MEM with excess unlabelled methionine for various time periods (see Results). Monolayers were washed with cold PBS, harvested by scraping, the cells sedimented and extracts prepared by solubilization of the cell pelments in immunoprecipitation buffer (20 mM-Tris-HCl pH 9, 0.3 mM-NaCl, 10% glycerol, 1 mM-CaCl_2, 0.5 mM-MgCl_2, 2 mM-EDTA, 0.5% NP40, 0.5 mM-PMSF, 100 units Trasylol/ml; 0.5 ml/5 x 10^6 cells). For immunoprecipitation (Reis et al., 1993; Bogner et al., 1992) aliquots of cell extracts of comparable protein content or culture medium from labelled cells (free of insoluble material by ultracentrifugation at 100000xg for 2 h and 4 °C, mixed 1:1 with buffer) were preclotted by incubation with Protein A-Sepharose CL4B beads (Sigma) prior to incubation overnight at 4 °C with gB-specific MAB (see above). Adsorption of immunocomplexes for 1-5 h at room temperature to Protein A-Sepharose CL4B beads coated with rabbit anti-mouse IgG (Dako) was followed by seven washing cycles of the beads with PBS plus 0.1% NP40, 0.1% SDS and one washing cycle with distilled water. The washed precipitates were subjected to SDS-PAGE (Laemmli, 1970) and consecutive fixation and fluorography (Bonner & Laskey, 1974) of the dried slab gels.
described above, cell extracts were adsorbed to streptavidin–Sepharose (Pierce) for 2 h prior to seven washing cycles in PBS plus 0.1% NP40. The extracts were then subjected to SDS–PAGE and electrotransfer to nitrocellulose (Bogner et al., 1992). HCMV gB-derived polypeptides in the blotted precipitates were again detected with MAb 27-156.

Fig. 1. (a) Schematic representation of the HCMV AD169 gB polypeptide backbone with 906 aa residues. The hydrophobic domains of the signal peptide at the amino terminus and the bipartite potential membrane anchor in the carboxy-terminal portion are not shaded. The relative position of the proteolytic cleavage site is indicated by the open triangle. The deduced aa sequence of hd1 (aa 714–747) and hd2 (aa 751–771) of the gB polypeptide is shown below. (b) Extent of the deletions of hd1 and hd2 coding regions were introduced into the eukaryotic expression construct pRC/CMV-gB described previously (Reis et al., 1993). In the case of hd1, the entire coding segment from nt 2140-2241 of the gB sequence was removed by the deletion whereas in the case of hd2 the deletion concerned nt 2251–2304 (it fell short by three aa residues at the carboxy-terminal end of hd2; Fig. 1b). The derivatives, pRC/CMV-gB(Mhd1) and pRC/CMV-gB(Mhd2) were used for transfection of human astrocytoma U373 cells and subsequent selection of stable transfectant cell lines gB(Mhd1) and gB(Mhd2) was carried out in the presence of geneticin as described in Methods. The deletions did not involve the epitope recognized by MAb 27-156 in the luminal portion of the carboxy-terminal cleavage product of gB (Hafüther et al., 1993). This MAb could thus be employed to examine recombinant gB expression in the transfectants after single-cell subcloning as reported before (Reis et al., 1993; Fig. 2a, b). AD169-infected U373 cells, 96 h p.i., were used as a control (Fig. 2c). Glycoprotein B-specific

Results

Establishment of transfectants expressing mutagenized gB forms

The two hydrophobic domains, hd1 and hd2, of the deduced aa sequence of the HCMV gB molecule are predicted to code for 34 and 21 residues, respectively (Fig. 1a). Assuming luminal orientation of the aminoterminal portion, which presents multiple potential N-glycosylation sites, and considering that, because of its length, hd1 could span the membrane twice, membrane anchoring of the gB polypeptide could conform with any of the five configurations depicted in Fig. 1 (c). Luminal orientation of the carboxy-terminal portion has been shown to be unlikely (Basgoz et al., 1992; Fig. 1c, iv and v). In order to distinguish between the remaining three possibilities (Fig. 1c, i–iii) alternative deletions of hd1- and hd2-coding regions were introduced in the eukaryotic expression construct pRC/CMV-gB described previously (Reis et al., 1993). In the case of hd1, the entire coding segment from nt 2140–2241 of the gB sequence was removed by the deletion whereas in the case of hd2 the deletion concerned nt 2251–2304 (it fell short by three aa residues at the carboxy-terminal end of hd2; Fig 1b). The derivatives, pRC/CMV-gB(Mhd1) and pRC/CMV-gB(Mhd2) were used for transfection of human astrocytoma U373 cells and subsequent selection of stable transfectant cell lines gB(Mhd1) and gB(Mhd2) was carried out in the presence of geneticin as described in Methods. The deletions did not involve the epitope recognized by MAb 27-156 in the luminal portion of the carboxy-terminal cleavage product of gB (Hafüther et al., 1993). This MAb could thus be employed to examine recombinant gB expression in the transfectants after single-cell subcloning as reported before (Reis et al., 1993; Fig. 2a, b). AD169-infected U373 cells, 96 h p.i., were used as a control (Fig. 2c). Glycoprotein B-specific

Fig. 2. HCMV gB-specific immunofluorescence of (a) gB(Mhd1), (b) gB(Mhd2) and (c) virus-infected U373 cells at 96 h p.i. Acetone-fixed coverslip cultures were subjected to indirect immunostaining using MAb 27-156.
fluorescence was detected in virtually all cells of the coverslip cultures of all transfectants. Intensity and distribution (perinuclear and cytoplasmic) of the fluorescence were comparable for gB(Mhd1) and gB(Mhd2) (Fig. 2a, b) but distinct from those of the heavily labelled infected culture exhibiting a cytopathic morphology (Fig. 2c).

**Processing of mutagenized gB forms**

In order to analyse gB-derived products, parallel cultures of the transfectants (10^6 cells each) were subjected to a 1 h pulse with [³⁵S]methionine (100 μCi/ml) and a chase for 3 or 6 h prior to preparation of extracts and immunoprecipitation with MAb 27-156 (Spaete et al., 1988). Extracts of a transfectant expressing authentic gB were included as a control (Fig. 3). Separation of the precipitates by SDS-PAGE under reducing conditions and subsequent fluorography revealed that the control gB polypeptides migrated to the same positions as the precursor molecule (approximately 150 kDa) in the pulse sample, and revealed the presence of additional proteolytic cleavage products of about 90 and 55 kDa in the chase samples (Fig. 3c). In the case of gB(Mhd1) a gB precursor molecule, migrating slightly faster than its authentic counterpart, was the main precipitate from the pulse and chase samples, and there appeared to be only slight proteolytic cleavage during the chase (Fig. 3a). From extracts of gB(Mhd2) on the other hand, a gB precursor molecule migrating slightly slower than authentic gB precursor was observed in pulse and chase samples, and significant amounts of cleavage products were precipitated from the chase samples (Fig. 3b). The apparent molecular mass of the carboxy-terminal product, 60–65 kDa, was, however, higher than that of the product (55 kDa) from the authentic gB transfectant (Fig. 3b, c).

To examine oligomerization of the mutagenized molecules caused by disulphide linkages (Britt & Vugler, 1992), immunoprecipitates from cultures radiolabelled with [³⁵S]methionine were separated under non-reducing conditions. For both gB(Mhd1) and gB(Mhd2), high molecular mass complexes of about 300 kDa, indicative of dimerization, were observed. This was also the case in virus-infected U373 cells or the gB transfectant expressing authentic gB (Fig. 4).

It should be pointed out that immunoprecipitations carried out with MAb 58-15, which recognizes a linear epitope located in the carboxy-terminal tail of the gB polypeptide (Britt & Vugler, 1992), yielded results identical to those obtained with MAb 27-156 (data not shown), indicating that the mutagenized gB forms were correctly synthesized and showed no additional (artefactual) frame-shift mutations.

**Cellular transport of mutagenized gB forms**

Correct cellular transport and processing of HCMV gB, when compared with virus-infected astrocytoma cells, have been shown previously for transfectants expressing the authentic gB molecule (Reis et al., 1993). Regarding the mutagenized gB forms, proteolytic cleavage, at least in the case of the product of gB(Mhd2), indicates correct cellular transport to the trans-Golgi network where gB is thought to be processed by cellular furin (Vey et al., 1993). In order to examine further the transport of mutagenized gB to the cell surface membrane in the transfectants, parallel cultures (1.5 x 10^6 cells each) of gB(Mhd1) and gB(Mhd2) were subjected to surface biotinylation at 4 °C prior to precipitation of cellular extracts with streptavidin-Sepharose, blotting of the precipitates and immunostaining with gB-specific MAb as described in Methods. Uninfected as well as HCMV-infected U373 cells and the transfectant expressing authentic gB (gB1) were included as controls (Fig. 5). By this approach, a gB precursor of about 150 kDa was
observed at the cell surface in all samples except uninfected U373 cells (Fig. 5). Cleavage products of about 55 kDa were recognized by MAb 27-156, which reacts with a linear epitope of the carboxy-terminal gB cleavage product, in the case of AD169-infected cells and gBI and of 60–65 kDa in the case of gB(Mhd2). In the latter instance the immunostaining also revealed possible gB-derived products readorsbbed to the cell surface membrane. (Fig. 5; see below).

**Effect of deletion of hdl or hd2 on membrane anchoring of gB**

In order to examine further whether deletion of hdl or hd2 interfered with gB membrane anchorage and thus led to secretion of gB, culture medium from transfectants labelled with [35S]methionine (50 μCi/ml) overnight was freed of cellular debris by low-speed centrifugation and subsequent ultracentrifugation (100000 g for 2 h at 4 °C). The supernatants were then immunoprecipitated with MAb 27-156 and the precipitates analysed by reducing SDS-PAGE and fluorography (Fig. 6a). Culture medium from uninfected and AD169-infected U373 cells, as well as gBI cells, served as controls. This analysis revealed a recombinant gB precipitate, consisting of a small amount of the precursor form of about 160 kDa and significant amounts of cleavage products of about 90 kDa and 60–65 kDa, with transfectant gB(Mhd2). This suggested the presence of secretory forms of gB (Fig. 6a). The media of the other cultures, including HCMV-infected U373 cells, were devoid of gB-derived products after ultracentrifugation (Fig. 6a). This result appeared to indicate that hd2 of the gB molecule is essential as well as sufficient for membrane anchoring.

With regard to the schematic models for gB membrane anchoring depicted in Fig. 1(c), our observations clearly support the second model.
The slower migration of the recombinant gB gene products from gB(Mhd2) culture medium, in particular of the precursor and the carboxy-terminal cleavage product (compare Fig. 3b and c) suggested additional modification of the secretory molecule. Previous analysis had revealed endoglycosidase H sensitivity of gB precursor and products in gB transfectants as well as in HCMV-infected U373 cells (Reis et al., 1993). Glycosidase digestion of recombinant gB immunoprecipitates from gB(Mhd2) culture medium showed partial resistance of the gB precursor and significant resistance of the gB cleavage products to endoglycosidase H (Fig. 6b). After PNGase F treatment, on the other hand, the molecular masses of the precursor and the cleavage products were reduced to the predicted sizes of the polypeptide backbones (Spaete et al., 1988), about 100 kDa for the precursor and about 50 kDa for the deglycosylated forms of the amino- and carboxy-terminal cleavage products (Fig. 6b). No additional effect was obtained by sequential PNGase F and O-glycosidase treatment (data not shown) indicating modification of the molecule by complex N-linked carbohydrates and the absence of O-glycosylation. A lack of O-glycosylation in astrocytoma cells has been pointed out previously (Kari et al., 1992).

Analysis of immunoprecipitates of the secreted gB form under non-reducing conditions clearly showed that dimers were present in gB(Mhd2) culture medium that migrated more slowly than those formed by intracellular authentic gB (Fig. 6c). Again, no evidence was obtained for soluble gB complexes in media from cultures used as controls (Fig. 6c).
**HCMV gB C-terminal hydrophobic domains**

(a) Alignment according to Kyte & Doolittle (1982), calculated with the program profilegraph by K. O. Hofmann using a window of 7 aa with respect to the proline residue preceding segment 3 (Pro; dashed line), of the hydrophobicity profile of HCMV hd1 and hd2 (aa 693–784) with the corresponding gB domains of HSV (aa 715–806), VZV (aa 665–756) and PRV (aa 739–830). The solid lines frame the glycine-rich segment shown in (b). The arrows indicate the regions of hd1 and hd2. (b) Alignment of the aa sequences of HCMV hd1 and hd2 with respect to conserved spaced glycine residues of the corresponding gB segments of HSV (Pellett et al., 1985), VZV (Keller et al., 1986) and PRV (Robbins et al., 1987). (c) Alignment of the amino-terminal fusion peptide sequences with respect to conserved spaced glycine residues of influenza A virus HA2 (Daniels et al., 1985), influenza B virus HA2 (Krystal et al., 1983), Sendai virus F1 (Morrison & Portner, 1991) and HIV-1 glycoprotein p41 (Gallaher, 1987).

**Comparative structural analysis of hd1**

The observation that hd1 is apparently not needed for membrane anchoring raised questions as to its function. Alignment of the partitioned hydrophobicity profiles for several herpesvirus gB polypeptides with respect to the proline residue in the conserved intermitting turn region preceding the most carboxy-terminal segment (Chee et al., 1990) revealed similarities as well as differences (Fig. 7a). Bipartite domains were apparent for HCMV and varicella-zoster virus (VZV; Keller et al., 1986), whereas HSV (Pellett et al., 1985) and also, to some extent, pseudorabies virus (PRV; Robbins et al., 1987) exhibited a tripartite hydrophobic domain. The most pronounced hydrophobicity was observed in the most carboxy-terminal gB segments of all species; this indicates an actual function in membrane anchoring, as was recently reported for HSV (Rasile et al., 1993) and shown here for HCMV. A further feature common to the herpesvirus gB polypeptides is the relative distribution of glycine residues. These appear to be highly conserved in the segment with intermediate hydrophobicity (HCMV hd1) preceding the anchor segment (Fig. 7b). Membrane glycoproteins of unrelated viruses, for example influenza viruses, Sendai virus and human immunodeficiency virus type 1 (HIV-1; Daniels et al., 1985; Krystal et al., 1983; Morrison & Portner, 1991; Gallaher, 1987), also exhibit glycine-rich sequences in their domains (Fig. 7c) with proved or assumed membrane fusion potential (White, 1992; Lamb, 1993). This similarity obviously does not involve the complete sequence of, for example, influenza-virus fusogenic peptides, only the strictly conserved positional pattern of glycine residues.

The hd1 domain of HCMV gB best fulfils this and other general criteria for fusogenic polypeptide segments such as a stretch of 16–26 aa of relative hydrophobicity in the vicinity of the membrane anchor (White, 1992). It is therefore suggested that hd1 and the homologous hydrophobic segments of other herpesviruses might represent a fusogenic segment of gB polypeptides which functions in the well-documented gB-mediated viral envelope–cell surface membrane fusion as well as in infected cell–cell fusion (Navarro et al., 1993).

**Discussion**

Compared with the tripartite domain of HSV gB (Pellett et al., 1985; Rasile et al., 1993), the nucleotide sequence...
of the HCMV gB gene predicts only two hydrophobic segments, hd1 and hd2, preceding a hydrophilic segment of about 130 aa in the carboxy-terminal portion of the molecule. In general support of the function of hd1 and hd2 in HCMV gB membrane anchoring it has been previously shown that eukaryotic expression of truncated gB genes lacking the entire coding regions for hydrophobic segments and the cytoplasmic tail resulted, as expected, in secretion of soluble recombinant gB polypeptides into the culture medium (Spaete et al., 1990). The experiments presented here were designed to define more precisely the putative HCMV gB membrane anchor by creating molecules with alternative deletions of the two segments in question. We have demonstrated using stable transfectants that the deletion of 18 of the 21 aa residues of hd2 yields a secreted form of HCMV gB, whereas in the case of deletion of hd1, a gB derivative was not secreted. This result is in accordance with the data recently published by Rasile et al. (1993) for HSV gB and strongly indicates that hd2, which corresponds to hydrophobic segment 3 of HSV gB, is obviously not only sufficient but also essential to anchor the gB molecule in the membrane.

Regarding intracellular processing, the results obtained here for transfectants expressing mutagenized gB indicate that deletion of hd1 or hd2 did not interfere with oligomerization of the gB molecule. Proteolytic cleavage, on the other hand, appeared to be less efficient than that of authentic gB, in particular in the case of gB(Mhd1), the most likely explanation being a conformational alteration of the molecule that protects the cleavage site. Lack of correct cellular transport of the mutant molecule as an alternative explanation seems to be excluded by our observation that the uncleaved gB molecule from gB(Mhd1) is exposed at the cell surface, as are the gB products of the other transfectants and of infected U373 cells. It is noteworthy that this observation contrasts with the situation in HCMV-infected human fibroblasts where mainly fully processed gB cleavage products and only a minor fraction of the precursor reach the cell surface (Britt & Vugler, 1992; Britt et al., 1990). The presence of the gB product of gB(Mhd2) transfectants at the cell surface might very well also reflect readsoption of the soluble molecule to cellular receptors, as gB is thought to function in viral attachment (Rasmussen et al., 1991).

Comparison of the electrophoretic mobilities of mutagenized gB forms from cell extracts with those from transfectants expressing authentic gB revealed minor differences for the precursors and a significant difference for the carboxy-terminal cleavage product of the gB form in gB(Mhd2). The enhanced migration of the precursor gB form from gB(Mhd1) might be a consequence of the deletion. In the case of the intracellular precursor product from gB(Mhd2), the unexpected slightly higher molecular mass could be due to additional N-glycosylation at Asn-801 after complete translocation of the molecule into the lumen of the RER, provided that hd1 has no function as a transfer stop signal. The slower migration of the cleavage products, which was particularly obvious for the carboxy-terminal cleavage product in gB(Mhd2), is most likely to be the result of additional modification with more complex carbohydrate side chains, as indicated by its endoglycosidase H resistance. A comparable observation has been reported for truncated soluble influenza A virus haemagglutinin (Paterson & Lamb, 1987). It is of interest to note in this context that cell-associated gB forms in the astrocytoma-derived transfectants described here (data not shown) and previously (Reis et al., 1993), as well as in virus-infected astrocytoma cells (Kari et al., 1992), acquire, at most, only partial endoglycosidase H resistance in spite of their largely unimpaired cellular transport. The anchorless gB mutant may possibly be transported at a slower rate through the Golgi compartment where complex carbohydrate side chains are added.

Our observation that only hd2 affects membrane anchoring of the HCMV gB molecule raised questions as to the possible function of hd1. It has been pointed out by others previously (Spaete et al., 1988) that the predicted conformation for hd1 (high glycine content and charged aa residues Lys-724 and Glu-742) makes it a less attractive prospect for a membrane-spanning domain. In addition, sequence alignment of hd1 with the respective segments of gB homologues of other herpesviruses and with fusion peptides of membrane glycoproteins of unrelated viruses revealed characteristic similarities involving conservation of multiple spaced glycine residues. If the hd1 segment is modelled as an α-helix, the spaced glycine residues could be positioned to one side, thus generating an amphipathic domain with the potential for membrane attachment. This might be the structural origin of the assumed fusogenic activity of the gB molecule. Furthermore, since hd2 is headed by an aa triplet, (Phe-)Gly-Ala-Phe (aa 751–754), which was tentatively described as a fusion peptide sequence (Gallaher, 1987), according to our working hypothesis it may also participate in the membrane fusion event. The fusogenic potential of the HCMV gB molecule should in any case be dependent on its adequate conformation, possibly as a consequence of interaction with other viral products, since our transfectant expressing authentic gB does not exhibit a phenotype indicative of enhanced cellular fusion (Reis et al., 1993).

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