Chaperone Functions Common to Nonhomologous Epstein-Barr Virus gL and Varicella-Zoster Virus gL Proteins

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Herpesviruses encode the complex-forming, essential glycoproteins gH and gL. Maturation and transport of gH are dependent on coexpression of its chaperone, gL. The gL proteins of alpha herpesviruses and gamma herpesviruses do not have a significant percentage of amino acid sequence homology. Yet, as we report herein, the diverse gL glycoproteins of Epstein-Barr virus (EBV) and varicella-zoster virus (VZV) were functionally interchangeable, although membrane expression and maturation of gH were separate functions for these viruses. In VZV both functions were performed by a single protein. EBV required two separate glycoproteins, one of which can be replaced by its homologous protein from VZV, a distant relative of EBV. Collectively, these results suggested that VZV gL is a simpler form of the gL chaperone protein than EBV gL.

The envelopes of herpesviruses are relatively complex structures. Unlike some of the simpler viruses that may encode one or two glycoproteins to facilitate attachment, penetration, and egress, the herpesviruses express multiple protein species that, by their numbers and diversity, enable each member of the family to establish its own niche. For example, 11 unique glycoproteins have to date been described for herpes simplex virus (2, 26), and human cytomegalovirus may express even more (24). In the midst of this diversity, however, some common themes are emerging. In particular, two glycoproteins, gH and gL, appear to be functionally conserved. In all those viruses studied so far, gH and gL associate in a complex that is involved in penetration or fusion (8, 11–13, 16, 23, 25, 27). Glycoprotein gH is generally thought to be the major player in the fusion process. Antibodies to this molecule inhibit viral entry and cell-to-cell spread of varicella-zoster virus (VZV) (8, 14), herpes simplex virus (9, 10), cytomegalovirus (22), and human herpesvirus 6 (17). Glycoprotein gL, however, is an essential partner that is required for the transport of gH to the cell surface. This report examines in detail chaperone functions shared between Epstein-Barr virus (EBV) and VZV gL.

Glycoprotein gL, which is always smaller than its partner, gH, has no significant sequence homology across subfamilies but has been detected by the “positional” homology of its gene with the gene of the first described gL of herpes simplex virus (3, 16, 18). Its identity can be confirmed by experimental demonstration of its association with the corresponding gH. This association may be mediated either by covalent bonds, as in cytomegalovirus (13, 25) and human herpesvirus 6 (1), or by noncovalent protein-protein interactions, as in herpes simplex virus (12), VZV (7), and EBV (15). Typically gL has an N-terminal hydrophobic domain with the characteristic of a signal sequence but has no other hydrophobic domain predicted to be membrane spanning. In the absence of gH the gL proteins are either secreted, as is the case for cytomegalovirus (25) and herpes simplex virus gL (5), or expressed as type 2 membrane proteins, as is the case for EBV gL (15). The predicted se-

quence of VZV gL does not include a cleavable signal peptide but does include a hydrophobic endoplasmic reticulum-targeting sequence that may serve to anchor the protein in a membrane (7).

Curious though the lack of sequence homology between proteins that are so strikingly conserved in function may first appear, it is certainly no more curious than the ability of a relatively small number of cellular chaperones or chaperonins to facilitate folding and transport of a very large number of diverse cellular proteins. Also, structural similarity need not be mirrored by sequence identity. The 25-kDa, 137-amino-acid EBV gL homolog and the 20-kDa, 158-amino-acid VZV gL are closer to each other in molecular mass than to the gL homolog from any of the other herpesviruses. In spite of only a minimal 14% amino acid sequence identity and a 40% sequence similarity, comparison of the proteins with the BestFit program of the sequence analysis software package of the Genetics Computer Group (University of Wisconsin) indicated identical placement, in the N-terminal halves of the proteins, of two of their five cysteine residues (4). This observation stimulated an examination of whether either of these two molecules could substitute functionally for the other in a transient-transfection system.

The open reading frames encoding EBV gH (EgH), EBV gL (EgL) (17), VZV gH (VgH) (8), and VZV gL (VgL) (7) were cloned into plasmid pTM1 (20) for expression under the control of the T7 promoter. Plasmids were transfected into cells 30 min after infection with vaccinia virus that expressed the T7 polymerase (vvT7) (15). Cells were labeled biosynthetically for 18 h, at 4 h posttransfection, with 1 mCi of [³H]leucine (Amersham Corp.) in leucine-free medium, solubilized in radioimmunoprecipitation buffer, immunoprecipitated with antibody, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (15). A complex of EgH and EgL could be readily immunoprecipitated with an antipeptide antibody to EgH (anti-EgH) (21) or an antipeptide antibody to EgL (anti-EgL) (27). More surprisingly, anti-EgH could also efficiently immunoprecipitate a complex of EgH and VgL (Fig. 1, right panel). Rabbit antibody to a beta-galactosidase-VgL fusion protein (anti-VgL; also known as R60) (7), which efficiently immunoprecipitated VgL in the absence of EgH, immunoprecipitated only a small amount of the EgH-

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FIG. 1. Association of EgH and VgL. CV1 cells were labeled with [3 H]leucine for 18 h at 4 h after infection with vVT7 and transfection with combinations of plasmids encoding EgH, EgL, VgL, and mutant VgLC21G. Proteins were immunoprecipitated by anti-EgH, anti-EgL, or anti-VgL and electrophoresed in 9 to 20% polyacrylamide. Sizes are indicated in kilodaltons. Arrows indicate the positions of EgH, EgL, and VgL.

VgL complex (Fig. 1, left panel). The two proteins, however, clearly formed a stable association. Further, the relative amount of VgL immunoprecipitated by anti-VgL from cells expressing VgL alone or immunoprecipitated by anti-EgH from cells expressing both EgH and VgL suggested that a substantial amount of the coexpressed gL was complexed with EgH. To determine whether coexpressed VgL not only associated with EgH but also effected its transport to the cell surface, transfected cells were labeled extrinsically with 125 I. As previously demonstrated (15), anti-EgH failed to immunoprecipitate 125 I-labeled EgH from cells transfected only with plasmids encoding EgH (Fig. 2, left panel). However, VgL was able to substitute functionally for EgL and to chaperone EgH to the cell surface, where it became accessible to iodination.

FIG. 2. Accessibility of EgH to iodination at the cell surface. CV1 cells were labeled with 125 I at 18 to 20 h after infection with vVT7 and transfection with combinations of plasmids encoding EgH, EgL, EBV gp42, VgL, mutant VgLC79G, or mutant VgLC146. Proteins in the left panel were immunoprecipitated with anti-EgH. Proteins in the right panel were immunoprecipitated with antibody F-2-1, which reacts with EBV gp42. All proteins were electrophoresed in 12% polyacrylamide. The arrows indicate the position of EgH.

EBV is apparently unique among human herpesviruses in that it encodes a third glycoprotein, the BZLF2 gene product gp42, which interacts with the EgH-EgL complex (15). Glycoprotein gp42 carries an epitope, recognized by a monoclonal antibody called F-2-1, that is essential for infection of B lymphocytes but which may be dispensable for infection of epithelial cells, which are commonly infected by herpesviruses. It is thus an example of a herpesvirus glycoprotein that may expand the range of cells that can be infected by a particular member of the family. The tripartite complex EgH-EgL-gp42 is sodium dodecyl sulfate stable and can be immunoprecipitated by F-2-1. Coexpression of neither EgH and gp42 nor EgL and gp42 produces a complex that can be immunoprecipitated by F-2-1, but from analyses of the EgH-EgL-gp42 complex in virus-producing cells by sedimentation in sucrose (15), it appears that although coexpression of gH with gL may be important for inducing a structure that is capable of subsequent association with gp42, at least a transitory interaction occurs between EgL and gp42. It was of interest, therefore, whether VgL could substitute for EgL in the formation of an iodinated complex that included gp42 and that could be immunoprecipitated with antibody F-2-1. As previously demonstrated, antibody F-2-1 did not immunoprecipitate EgH in the absence of EgL (15), a fact which indicated that the association of proteins was not merely a nonspecific artifact of the expression system (Fig. 2, left panel). However, VgL substituted for EgL in promoting formation of a complex that was immunoprecipitated by F-2-1 and transported to the cell surface, albeit somewhat less efficiently.

Native EgH synthesized in virus-producing cells carries both endoglycosidase H-resistant and endoglycosidase H-sensitive sugars (27). We had not previously determined whether coexpression with EgL was sufficient to facilitate addition of complex sugars or whether the third partner in the complex, gp42, was required as well. We therefore examined the sensitivity to endoglycosidase H of complexes of gH and gL alone or in combination with gp42. The association of EgH with EgL and that of EgH with VgL each produced a protein that was more sensitive to endoglycosidase H than any of the complexes that included gp42. Both EgL and VgL were, however, capable of facilitating maturation of EgH.

To determine whether the ability of EgL to associate with EgH was mirrored by an association of EgL with VgH, combinations of VgH and EgL, with or without gp42, were expressed in HeLa cells. A monoclonal antibody, 258, that recognizes both the immature and the mature forms of VgH (6, 7) was able to coimmunoprecipitate immature VgH and EgL (Fig. 3, left panel). Inclusion of EBV gp42 in the transfection apparently interfered with the association. A small amount of VgH was also visualized by confocal microscopy in patches on the surfaces of cells coexpressing VgH and EgL when the cells were probed with antibody 258 (micrograph not shown). However, EgL, both in the presence and in the absence of EBV gp42, was unable to promote maturation of VgH to a form that could be recognized by a second monoclonal antibody, 206 (6, 7), that primarily recognizes mature VgH and fails to immunoprecipitate immature gH in its absence. These results were similar to previous findings in which VZV glycoproteins gE and gI were shown to facilitate the transport but not the processing of VgH (7).

Our original hypothesis was that the two cysteine residues conserved between EgL and VgL might maintain a structure that would allow one to substitute for the other. The ability to associate with EgH was therefore examined for three VgL mutants, VgLC21G, VgLC48G, and VgLC79G, in which the cysteine residues at positions 21, 48, and 79, respectively, had

FIG. 3. Association of VgH with EgL. HeLa cells were labeled with [³H]leucine for 18 h at 4 h after infection with vvT7 and transfection with combinations of plasmids encoding VgH, VgL, EgL, and gp42 or with vector alone. Proteins were immunoprecipitated with monoclonal antibody 258 to immature VgH or monoclonal antibody 206 to mature VgH. The arrows indicate the positions of VgH, VgL, and EgL.

been replaced with glycine residues and for a mutant in which the protein had been truncated at residue 145 (VgLΔC146), deleting the cysteine residues at positions 146 and 158 (7). A comparison of the amount of EgH and VgL with that of EgH and VgLC21G immunoprecipitated by anti-EgH indicated that wild-type VgL and the mutant associated equally well with EgH (Fig. 1, right panel). Each of the remaining mutants also associated with EgH in a complex that was immunoprecipitated efficiently by anti-EgH and weakly by anti-VgL (data not shown). In addition, each of the mutants not only associated with EgH but also transported it to the cell surface (Fig. 2, left panel, and data not shown). Wild-type VgL had been able not only to associate with EgH but also to facilitate further interaction with gp42 and processing, in the Golgi bodies, of the N-linked sugars on EgH. This property was also retained by each of the VgL mutants (Fig. 4 and data not shown).

The ability of mutant VgLΔC146 to associate with EgH indicated that the C-terminal 13 amino acids of VgL are not required for the interaction. This observation was consistent with the ability of the rabbit anti-EgL antibody, which was

FIG. 4. Presence of endoglycosidase H-resistant sugars on EgH. CV1 cells were labeled with [³H]leucine for 18 h at 4 h after infection with vvT7 and transfection with combinations of plasmids encoding EgH, EgL, gp42, VgL, and mutant VgLC48G. Proteins were immunoprecipitated with anti-EgH or F-2-1. After immunoprecipitation and before electrophoresis, proteins were incubated overnight with endoglycosidase H.

TABLE 1. Abilities of VgH and EgH protein combinations

Combination of proteins	Presence (+) or absence (–) of ability to facilitate:		
	Association of proteins	Transport of gH	Maturation of gH
VgH and:			
VgH or VgLC21G	+	+	+
VgLC48G or VgLC79G or VgLΔ C146	+	+	–
EgH	+	+	–
EgH and:			
EgH	+	+	–
VgH (wild type and all mutants)	+	+	–
EgH and gp42	+	+	+
VgH (wild type and all mutants) and gp42	+	+	+

raised against a peptide derived from the C-terminal 13 amino acids of EgL, to efficiently immunoprecipitate the EgH-EgL complex. In contrast, the rabbit anti-VgL antibody, which was raised against a fusion protein derived from residues 99 to 159 of VgL, had a markedly reduced ability to immunoprecipitate VgL when it was associated with EgH. This might suggest that a region of the VgL molecule between residues 99 and 146 was relevant to the association. The same observations have been made for VgH in association with EgH. Although Duus et al. (7) have interpreted this finding to mean that less gL was synthesized in the presence of gH, comparison of the immunoprecipitation of the EgH-VgH complex by anti-EgH with that by anti-VgH argues that this is not the case here. At this point it appears that any molecule that can associate with gH has the potential to secure its release from the endoplasmic reticulum, perhaps by inhibiting aggregation. Thus, a truncated form of the fibroblast growth factor receptor can transport human cytomegalovirus gH to the cell surface (25), while VZV gE or gI (7) or EgH (as shown in this report) can perform the same function for VgH. The gL homologs of the closely related human cytomegalovirus and human herpesvirus 6 can also form a complex, but it is unclear whether the gH homolog travels to the cell surface (1).

There is also a major difference between the mere ability to effect the release of gH from the endoplasmic reticulum and the ability to facilitate its authentic maturation (Table 1). Although either EgH or VgH was sufficient to release EgH from the endoplasmic reticulum into the default pathway to the cell surface, neither of the two chaperones produced a gH protein to which complex sugars were added. The generation of an EgH molecule with sugars accessible to processing enzymes required the presence of gp42 in addition to either EgH or VgH (Table 1). The work described herein has interesting parallels to experiments conducted with the same VgH cysteine mutants in the context of maturation of VgH (6). VgH in which the cysteine at position 21 was mutated to a glycine behaved as a wild type. VgH molecules having mutations involving any of the other cysteine residues were able to associate with VgH and release it from the endoplasmic reticulum but not to facilitate its complete maturation to an endoglycosidase H-resistant form. Thus, in VZV, the dual functions of (i) release from the endoplasmic reticulum into the default pathway to the cell surface and (ii) facilitation of correct folding and accessibility of sugar side chains to processing enzymes in the Golgi bodies were both found within a single protein but were segregated within distinct structural domains. EgH was able to perform the

first function but not the second. In EBV the second function, facilitation of correct folding and processing, appeared to have been delegated to the unique third glycoprotein, gp42, which had been added to the complex. This situation is another example of how individual members of the herpesvirus family have evolved with modifications to a common theme in order to adapt to the biologic niche that each inhabits. Collectively, these findings suggest that VgL is the simplest form of the gL chaperone protein, one which performs its role without ever leaving the cytoplasm (6). On the other hand, EgL, as part of the EgH-EgL-gp42 complex, travels to the cell surface, where it participates in essential interactions between the virus and its target cell. Whether other herpesviruses in which gL travels to the cell surface as part of a complex require a third, or accessory, protein needs further investigation.

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