Characterization of interaction of gH and gL glycoproteins of varicella-zoster virus: their processing and trafficking

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Varicella-zoster virus (VZV) glycoproteins gH and gL were examined in a recombinant vaccinia virus system. Single expression of glycoprotein gL produced two molecular forms: an 18 kDa form and a 19 kDa form differing in size by one endoglycosidase H-sensitive N-linked oligosaccharide. Coexpression of gL and gH resulted in binding of the 18 kDa gL form with the mature form of gH, while the 19 kDa gL form remained uncomplexed. The glycosylation processing of gL was not dependent on gH; however, gL was required for the conversion of precursor gH (97 kDa) to mature gH (118 kDa). Subsequent analyses indicated that gL (18 kDa) was a more completely processed gL (19 kDa). Screening of the culture media revealed that gH and gL were secreted, but only if coexpressed and complexed together. The secreted form of gL was 18 kDa while that of gH was 114 kDa. The fact that secreted gH was smaller than intracytoplasmic gH suggested a proteolytic processing event prior to secretion. The 19 kDa form of gL was never secreted. These findings support a VZV gL recycling pathway between the endoplasmic reticulum and the cis-Golgi apparatus.

Introduction

Varicella-zoster virus (VZV), like other herpesviruses, encodes a number of structural glycoproteins that enable the virus to successfully accomplish its assembly and trafficking. Two glycoproteins, gH and gL, are functionally conserved across all herpesviruses. Previous studies provide evidence that gH is responsible for cell fusion, and that mature glycosylation is essential for its fusogenic function (Forrester et al., 1992; Fuller & Lee, 1992; Peeters et al., 1992). Monoclonal antibodies against gH are able to inhibit virus entry, egress and cell-to-cell spread of VZV infection in cultured cells (Montalvo et al., 1986; Rodriguez et al., 1993). The gH protein has been found to form a complex with a gL counterpart in all three subfamilies of herpesviruses (Forghani et al., 1994; Kaye et al., 1992; Hutchinson et al., 1992; Liu et al., 1993; Klupp et al., 1994; Stokes et al., 1996). The association of gH with gL is required for correct folding, complete processing, intracellular trafficking and antigenic properties of gH. The scheme by which the gH:gL complex participates in virus entry and cell-to-cell spread has not been established yet, but the evolutionary conservation of the complex suggests that it has an essential role in the VZV infectious cycle. The gH:gL association is mediated by noncovalent protein-to-protein interactions in VZV (Duus et al., 1995), herpes simplex virus type 1 (HSV-1; Hutchinson et al., 1992) and Epstein–Barr virus (EBV; Li et al., 1995), while disulfide linkages are an important feature of the gH:gL complex in human herpesvirus-6 and human cytomegalovirus (HCMV) (Anderson et al., 1996; Kaye et al., 1992).

Unlike gH, the gL genes are not well conserved among herpesviruses. Typically, gL has an N-terminal hydrophobic domain characteristic of a signal sequence; however, gL has no other predicted membrane-spanning domains. Not only is VZV gL smaller than HSV-1 gL, but it also lacks a typical N-terminal endoplasmic reticulum (ER) signal sequence (Davison & Scott, 1986). On the other hand, VZV gL possesses a 16 residue sequence which is common with members of the ER-targeting family of proteins (Duus et al., 1995). In the absence of gH, the gL proteins are either secreted, as is the case of HCMV gL (Spaete et al., 1993) and HSV-1 gL (Dubin & Jiang, 1995), or they are found as type 2 membrane proteins, like EBV.
gL (Li et al., 1995). A model of post-translational VZV gH:gL regulation was proposed whereby the gL chaperone modulated gH expression via retrograde flow from the Golgi apparatus to the ER (Duus & Grose, 1996).

It has been reported that in certain herpesviruses, additional viral glycoproteins or cellular proteins are required for proper gH:gL interaction (L. Li et al., 1997; Wang et al., 1998). For example, glycoprotein O has been found to be associated with gH:gL in HCMV virions (Huber & Compton, 1997, 1998). Moreover, EBV has added a unique glycoprotein, gp42, for proper gH:gL complex formation (gH:gL:gp42). This specific adaptation of EBV is required for infection of B lymphocytes (Li et al., 1995). The differences in gH:gL can reflect various models of virus penetration into different cell types and a different tropism of the virus. It remains to be determined whether VZV and the other members of the alphaherpesvirus subfamily require another component in addition to the gH:gL complex. In this work, formation of the VZV gH:gL complex was analyzed in greater detail through the use of a recombinant vaccinia virus expression system.

Methods

Construction of vaccinia virus recombinants. Vaccinia virus (VV) strain Praha was used for construction of all recombinants (Kutinová et al., 1995). Fig. 1 shows the simplified design of single and double recombinants containing the VZV genes gH (ORF 37), gL (ORF 60), gB (ORF 31) and gE (ORF 68), which were inserted into the thymidine kinase (TK) or haemagglutinin (HA) genes of the VV. Expression of the extrinsic (ORF 31) and gE (ORF 68), which were inserted into the thymidine kinase recombinants containing the VZV genes gH (ORF 37), gL (ORF 60), gB, gE and gB-2, was described previously by Kutinová et al. (1996). The differences in gH:gL can reflect various models of virus penetration into different cell types and a different tropism of the virus. It remains to be determined whether VZV and the other members of the alphaherpesvirus subfamily require another component in addition to the gH:gL complex. In this work, formation of the VZV gH:gL complex was analyzed in greater detail through the use of a recombinant vaccinia virus expression system.

Radioimmunoprecipitation procedures. Overnight-cultured CV-1 cells (6 x 10⁶) were starved for 3 h in methionine/cysteine-deficient Dulbecco’s modified Eagle medium (Sigma) (labelling medium) supplemented with 10% foetal bovine serum and t-glutamine (0.58 µg/ml). Infection with VV recombinant viruses was performed at an m.o.i. of 5 p.f.u. per cell. A mixture of [35S]methionine/[35S]cysteine of 83 µCi (Pro-Mix, Amersham) in 1 ml of labelling medium was added at the same time. The cell cultures were incubated for 18 h, then harvested, washed with PBS and lysed for 30 min on ice with 0.2 ml of lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% NP-40) containing the protease inhibitor cocktail (1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 44 µg/ml PMSE, 50 µg/ml TLCK and 90 µg/ml TPK). The cell lysates were centrifuged at 19000 g for 10 min at 4°C.

Prior to harvesting the culture medium was removed from radio-labelled CV-1 cells and immediately treated with the protease inhibitor cocktail, and centrifuged gently at 300 g for 10 min at 4°C to remove dead cells and other cellular debris. The medium was then centrifuged at 100000 g for 1 h. The supernatant was removed and concentrated tenfold in Centricon-10 concentrator tubes (Amicon) at 4°C and incubated with the same volume of 2 x lysis buffer for 30 min on ice.

Both the lysates and the media were preclarified by incubation with 10 µl of human VZV-negative serum for 1 h on ice. The immune complexes were removed by 10 µl Protein A-Sepharose beads (Sigma). For radioimmunoprecipitation of VZV glycoproteins, 25 µl of either MAb or serum from the host patient was added and the samples were incubated for 1 h on ice. Precipitates were collected on Protein A-Sepharose beads. After overnight incubation, the beads were washed in lysis buffer, resuspended in Laemmli sample buffer and heated at 95°C for 10 min.

Immunoprecipitated proteins were analysed by gradient (7–12%) polyacrylamide) SDS–PAGE under reducing conditions (Laemmli sample buffer containing 0.2% mercaptoethanol) (Laemmli, 1970). Protein mobility was calibrated by unlabelled and 125I-radiolabelled molecular mass standards (Biorad, Amersham). Gels were incubated with Amplify (Amersham), dried and exposed to autoradiographic film Hyperfilm-beta max (Amersham).

Treatment with glycosidases. The immune complexes collected on Protein A-Sepharose beads were washed with lysis buffer and PBS. For endoglycosidase H (Endo H) digestion, proteins were dissociated...
Interaction between VZV gH and gL glycoproteins

**Results**

**VZV gH:gL complex synthesized by the double VV-gH+gL recombinant**

As we showed previously, coinfection of CV-1 cells with VV-gH and VV-gL recombinant viruses resulted in the formation of a gH:gL complex that carried the conformation-dependent virus-neutralizing epitope (Němečková *et al.*, 1996). To study this complex in detail, we prepared the double VV-gH+gL recombinant. It not only resulted in a higher level of gH:gL synthesis but, more importantly, guaranteed co-expression in every infected cell, which better mimicked the natural VZV infection. In lysates of CV-1 cells infected with VV-gH+gL, we found a mature gH molecule of 118 kDa and an 18 kDa form of gL, which were coprecipitated with the zoster serum (Fig. 2, lane 5). In CV-1 cells infected with VV-gH alone, the gH form of 97 kDa was detected (Fig. 2). This incompletely glycosylated precursor of gH (pre-gH) was recognized by serum from the zoster patient (lane 4) but not by V₃MAb (not shown). No specific product was immunoprecipitated from the cells infected with VV-gL alone (lane 6). Thus, coexpression of gH and gL resulted in complete gH processing and formation of the conformational epitope recognized by V₃MAb (see Fig. 4A, lanes 3 and 6). VZV glycoproteins gB and gE were similarly expressed as a control for secretion of VZV gH and gL (see later).

**Heterogeneity of VZV gL**

Since no convalescent zoster sera tested can precipitate gL selectively, we prepared the rabbit 60A antiserum raised against a predicted immunogenic peptide corresponding to the amino acid sequence spanning the residues 111–121 of the gL molecule (Davison & Scott, 1986). Two forms of gL were detected in the lysate from the VV-gL-infected CV-1 cells using this antiserum (Fig. 3, lane 3). The 18 kDa mass of the smaller protein was consistent with gL precipitated with serum from the zoster patient or V₃MAb in the gH:gL complex after infection with the double VV-gH+gL recombinant (Fig. 4C, lane 2 and Fig. 4A, lanes 3 and 6). The second gL form migrated at a slightly but perceptively higher molecular mass of 19 kDa.

When the extract of CV-1 cells infected with VV-gH+gL was precipitated by 60A antiserum, only the 19 kDa gL form was detected (Fig. 3, lane 4); no 18 kDa gL form was seen.
Fig. 2. Radioimmunoprecipitation profiles of intracellular VZV gH, gL, gE and gB. CV-1 cells were infected with wild-type VV (lane 1), VV-gB (lane 2), VV-gE+gB and VV-gL (lane 3), VV-gH (lane 4), VV-gH+gL (lane 5) or VV-gL (lane 6) and labelled with [35S]cysteine/methionine. Cell lysates were precipitated with serum from a zoster patient. Immunoprecipitates were analysed by SDS–PAGE using a gradient gel under reducing conditions. Molecular mass markers and VZV glycoprotein positions are indicated; gL is the 18 kDa form. All samples were analysed on the same gel.

Fig. 3. Immunoprecipitation of intracellular VZV gL. CV-1 cells were infected with wild-type VV (lane 1), VV-gH (lane 2), VV-gL (lane 3) or VV-gH+gL (lane 4) and labelled with [35S]cysteine/methionine. Cell lysates were precipitated with rabbit anti-gL peptide serum 60A. Immunoprecipitates were analysed by SDS–PAGE using a gradient gel under reducing conditions. Molecular mass markers and the positions of gL forms are indicated. All samples were analysed on the same gel.

Also, the 60A antiserum was not able to coprecipitate gH (Fig. 3, lanes 2 and 4). This result indicated that in the case of gH+gL coexpression, the epitope recognized by 60A serum was accessible only on the 19 kDa gL form; in other words, complex formation obscured or altered the same epitope. The 19 kDa gL form, which remained uncomplexed with gH, was not precipitated by V3MAb or zoster patient serum. The relative amount of the 19 kDa gL form precipitated by 60A antiserum was not different when expressed by the single VV-gL recombinant or coexpressed with gH by the VV-gH+gL recombinant (Fig. 3, lanes 3 and 4). Both gL forms were produced in comparable amounts. As all 18 kDa gL was bound in the gH:gL complex, it did not influence the amount of 19 kDa gL being produced.

Release of the VZV gH:gL complex into culture media

To test whether VZV glycoproteins were secreted from the CV-1 cells, we analysed the culture media of cells infected with the VV recombinants and their various coinfection mixtures (Fig. 5). In cells that coexpressed gH+gL, gH was secreted in the complex with gL (lane 4). The molecular mass of the secreted gH (precipitated by serum from a zoster patient or V3MAb) was approximately 114 kDa, which was lower by 4–5 kDa than the mature intracellular gH (118 kDa) (Fig. 4B, lane 2 and Fig. 4A, lanes 3 and 6). An intermediate 97 kDa form of gH produced by the simple VV-gH recombinant in cell lysates (Fig. 2, lane 4) was not identified in the culture medium (Fig. 5, lane 3). Results in Figs 4 and 5 show that gL was detected in the culture medium only if coprecipitated by V3MAb or serum from a zoster patient. This secreted gL form (Fig. 4A, lanes 3 and 6) had the same molecular mass of 18 kDa as that of gL coprecipitated (by V3MAb or zoster patient serum) in the complex with gH from the cell lysates (Fig. 4A, lanes 3 and 6 and Fig. 4C, lane 2). The 60A antiserum was not able to precipitate any gL molecule from the culture medium of the cells infected with the VV-gL recombinant alone (Fig. 4A, lanes 2 and 5). Thus, gL was detected in media only when associated with the secreted gH form.

VZV gE and gB were selected as examples of glycoproteins that were secreted or remained cell-associated, respectively. After precipitation with zoster patient serum the gE protein was processed to a 95–97 kDa molecule (Fig. 2, lane 3). Recombinant gB was detected by serum from a zoster patient as a doublet corresponding to 66–68 kDa (Fig. 2, lanes 2 and 3) on SDS–PAGE under reducing conditions. Analysis of the distribution of gE and gB revealed secretion of gE into media. The extracellular flow of gE may be related to the strong sorting information on the gE molecule for its targeting from the trans-Golgi network to the cell surface (Zhu et al., 1996; Olson & Grose, 1997).

Finding the VZV glycoproteins in culture medium cannot be attributed solely to the CV-1 cells damaged upon VV infection. We did not detect any β-galactosidase activity as a...
Fig. 4. Analysis of VZV gL and gH by endoglycosidases. CV-1 cells were infected with VV recombinants and labelled with [35S]cysteine/methionine. Medium removed from the labelled cells was centrifuged for 1 h at 100000 g, concentrated tenfold in Centricon-10 concentrator tubes, and immunoprecipitated with anti-peptide gL serum 60A, gH-specific human V3MAb or serum from a zoster patient. Immunoprecipitates were incubated with or without endoglycosidase H (Endo H) or peptide:N-glycosidase F (PNGase F). The samples were analysed by SDS–PAGE using a gradient gel under reducing conditions. C, material from cell lysates; M, material from culture media. Molecular mass markers and VZV glycoprotein positions are indicated: gL* and gH* represent the deglycosylated products of gL and gH respectively. (A) Wild-type VV (lanes 1 and 4), VV-gL (lanes 2 and 5), VV-gH›gL (lanes 3 and 6). (B, C) Wild-type VV (lane 1), VV-gH›gL (lane 2).

leakage marker in the culture medium of the control experiment with infection of the VV-lacZ recombinant virus alone (Fig. 6).

Analysis of glycosylation patterns of VZV gH and gL

The oligosaccharide processing of gH and gL expressed by VV-gH›gL in cell lysates and culture media was investigated by determining the sensitivity of the glycoproteins to treatment with glycosidases Endo H and PNGase F (Fig. 4). The molecular mass of the intracellular 118 kDa gH form was not reduced greatly by Endo H digestion (Fig. 4A, lane 3C). The complete removal of N-linked oligosaccharides from the 118 kDa gH form by PNGase F digestion resulted in a loss of
Fig. 5. Radioimmunoprecipitation profiles of VZV gH, gL, gE and gB from culture medium. CV-1 cells were infected with VV-gE›gL (lane 1), VV-gH›gL (lane 2), VV-gH (lane 3), VV-gH›gL (lane 4), VV-gE (lane 5), VV-gE›gL and VV-gL (lane 6), VV-gB and VV-gH›gL (lane 7), VV-gB and VV-gH (lane 8), VV-gB and VV-gL (lane 9), VV-gB (lane 10) and wild-type VV (lane 11) and labelled with [35S]cysteine/methionine. Medium removed from the labelled cells was centrifuged for 1 h at 100000 g, concentrated tenfold in Centricon-10 concentrator tubes and then subjected to immunoprecipitation with serum from a zoster patient. Immunoprecipitates were analysed by SDS–PAGE using a gradient gel under reducing conditions. Molecular mass markers and VZV glycoprotein positions are indicated; gL is the 18 kDa form.

Fig. 6. Effect of infection on leakage of β-galactosidase activity into the culture medium. The CV-1 cells were infected with VV-E/L-lacZ at an m.o.i. of 5 p.f.u. per cell. The dishes with infected cells were harvested at various time intervals. Each time-point represents the average of two dishes. Culture fluids were centrifuged at 2000 g and β-galactosidase activity in the supernatant was determined by colorimetric assay using o-nitrophenyl β-D-galactopyranoside (Sambrook & Maniatis, 1989).

molecular mass of about 40 kDa (Fig. 4A, lane 6 and Fig. 4B). This result demonstrated that the mature intracellular gH carried predominantly Endo H-resistant (complex-type N-linked) oligosaccharides, a finding consistent with a previous study demonstrating that gH is siaalted on N-linked sugars (Montalvo & Grose, 1986). The secreted 114 kDa gH form was digested by Endo H to a product which migrated at a similar mass to the intracellular form treated with Endo H. As shown in Fig. 4(A, lane 6) and Fig. 4(B, lane 2), the molecular mass of the secreted 114 kDa gH and intracellular gH after PNGase F treatment differed by 4–5 kDa. There was no evidence for a difference in sugars between the secreted form and the intracellular form of gH.

An analysis of gL expressed after VV-gH›gL infection revealed that the 18 kDa gL form precipitated from the cell lysates was digested by Endo H (Fig. 4C, lane 2 and Fig. 4A, lane 3) with a decrease in molecular mass of about 4 kDa. An analogous shift in mobility was observed after PNGase F digestion (Fig. 4A, lane 6). This finding suggested that one high-mannose/hybrid-type N-linked oligosaccharide was removed. Furthermore, the glycosidase results were the same for both intracellular and secreted 18 kDa gL (Fig. 4C, lane 2 and Fig. 4A, lanes 3 and 6), indicating that processing of gL was not changed by trafficking of the gH:gL complex to the culture media.

The deglycosylation pattern of both forms of gL detected by the 60A antiserum was tested after single VV-gL expression in the cell lysates (Fig. 4A, lanes 2 and 5). The same downward shift to approximately 14 kDa occurred for both the 18 kDa and 19 kDa gL form after digestion by either glycosidase. Thus, both forms of gL differ in processing of one high mannose or hybrid-type N-linked oligosaccharide. Since the analogous result about deglycosylation of 18 kDa gL was obtained from single VV-gL and double VV-gH›gL expression (Fig. 4A, lanes 2–3 and 5–6, respectively), we infer that the maturation of gL was not influenced by coexpression and complex formation with gH.

Discussion

The majority of the recent studies on interaction of VZV glycoproteins has been performed using the VV–T7 polymerase transfection system (Duus et al., 1995; Duus & Grose, 1996; Q. Li et al., 1997; Olson & Grose, 1998; Ye et al., 1999). We have used an expression system where VZV glycoprotein genes were inserted into recombinant VV. This approach enabled us to infect cells at a high virus multiplicity with subsequent high expression and efficient coexpression of foreign genes. We were able to detect molecular forms of VZV glycoproteins which were not previously observed in other experimental systems.

A heterogeneous molecular pattern of VZV gL was identified for CV-1 cells infected with a single VV-gL recombinant. Two gL forms, of 18 kDa and 19 kDa, were detected by the 60A antiserum recognizing amino acid residues 111–121 of the gL sequence. Analysis of the oligosaccharide moiety revealed that both forms of gL differed only in the processing of one N-linked oligosaccharide of high mannose/
hybrid-type sensitive to Endo H. It has been shown previously that gL does not change its molecular mass if expressed in the presence of monensin, which indicates that gL maturation does not require processing in the trans-Golgi system (Duus & Grose, 1996). Our data on Endo H sensitivity localized the final oligosaccharide processing step of gL within the early part of the medial-Golgi system, according to the spectrum of glycosylation reactions within the Golgi system (Kornfeld & Kornfeld, 1985).

The heterogeneity of gL forms has not been studied in detail to date. Only one band of gL was previously observed in the VV–T7 cotransfection systems with VZV glycoproteins, but two closely migrating gL species have appeared in cultured cells infected with natural VZV (Forghani et al., 1994). We postulated that the processing of gL forms reflected a difference in their trafficking status. The localization could explain a selective distribution of the gL forms in complex with gH. Since both forms differed only in a subtle nuance in their glycosylation, the two gL forms may have the same potential for interacting with gH. If it is assumed that mature gL is found in complex with mature gH, then the 18 kDa gL form should be considered as the mature form; the 19 kDa gL form would represent a premature gL containing non-trimmed oligosaccharides. Our results concerning the two gL forms are supported by diverse trafficking routes of VZV gL in the model proposed by Duus & Grose (1996), which includes the Golgi–ER recycling of gL as well as the transport of the gH:gL complex through the Golgi apparatus to the outer cell membrane.

A hypothesis on the structure of the gH:gL complex can be drawn from the fact that 60A antiserum recognized only the non-complexed 19 kDa gL molecule. The gL amino acid sequence 111–121 is exposed on the surface of the gL molecule, but is hidden in the gH:gL complex. This region is in the relative vicinity of amino acid sequence 73–100, which is highly conserved for herpesviral gL homologues (Yoshida et al., 1994) and is a candidate for the ER-targeting motif on gL responsible for its Golgi–ER recycling (Duus & Grose, 1996). We hypothesize that the ER-targeting motif on gL can be masked during complex formation with gH. Afterwards, the complex escapes the recycling, and allows gH to travel to the surface of the cell.

Our data showed that individually expressed gH was not secreted. Unlike gL of HSV-1, which is secreted from cells when expressed in the absence of gH (Dubin & Jiang, 1995), we did not detect VZV gL in the media, unless it was coexpressed with gH. The differences between HSV and VZV gL include the following points. First, HSV-1 gL was found to be membrane-associated, most likely as a result of complex formation with gH (Dubin & Jiang, 1995), whereas the VZV gL was not present on the surface of gH:gL cotransfected cells (Duus & Grose, 1996). Second, coexpression of HSV-1 gH and gL is required for normal post-translational processing and intracellular transport of both gH and gL glycoproteins. Our experiments support earlier results where VZV gL processing was not dependent on gH. Third, gL VZV is half the size of HSV-1 gL.

Finally, we propose a model to explain VZV gH secretion. This model is based on the earlier work by Dubin & Jiang (1995) in which they truncated HSV-1 gH of its transmembrane domain and cytoplasmic tail. When this truncated gH construct was cotransfected with gL, both HSV-1 gL and gH were found in the medium. Similarly, we suggest that VZV gH is proteolytically cleaved at a similar location, although the site was not located.

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### References


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