FORMATION OF PSEUDORABIES VIRUS GLYCOPROTEIN E/I COMPLEX IN BACULOVIRUS RECOMBINANT SYSTEM

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Summary. – Glycoproteins E (gE) and I (gI) of Pseudorabies virus (PRV) form a non-covalently bound complex to which a number of functions have been attributed. The gE/gI complex formation was studied using a series of full-length and truncated forms of gE and gI expressed in baculovirus recombinant system. Both glycoproteins were truncated by stepwise removal of their C-terminal parts and their ability to form the complex was studied by radioimmunoprecipitation. It was found that N-terminal domains of gE and gI containing first 122 and 106 aa, respectively, were sufficient for the complex formation.

Key words: baculovirus recombinants; envelope glycoproteins; gE/gI complex; Pseudorabies virus

Introduction

PRV is a neurotropic alphaherpesvirus, which is highly pathogenic for the majority of mammals with the exception of higher primates. Similarly to other herpesviruses, the PRV genome codes for a number of envelope glycoproteins, which are required for virus attachment, entry, egress, cell-cell fusion and cell-to-cell spread (Spear, 1993; Mettenleiter, 1999, 2003). Eleven envelope glycoproteins of PRV, namely gB, gC, gD, gE, gG, gH, gI, gK, gL, gM, and gN have been identified so far.

gE and gI are glycoproteins which are present in the virus envelope and the plasma membrane of infected cell (Whealy *et al*., 1993). They are dispensable for virus replication *in vitro* but play an important role *in vivo*. gE and gI deletion mutants display strongly reduced virulence, and form smaller plaques in cell cultures and smaller primary lesions in infected animals. On the basis of these observations it is assumed that gE and gI are involved in the virus cell-to-cell spread within the host (Enquist, 1994; Card and Enquist, 1995). In the course of PRV, Herpes simplex virus 1 (HSV-1) and Varicella-zoster virus (VZV) infections, these glycoproteins also bind the Fc-part of IgG and in this way protect infected cells from the complement-mediated lysis (Johnson *et al*., 1988; Favoreel *et al*., 1997; Olson *et al*., 1997; van de Walle *et al*., 2003).

gE and gI are typical type I transmembrane glycoproteins with extracellular and cytoplasmic domains, separated by a transmembrane region. Both glycoproteins have highly conserved cysteine-rich regions in their extracellular domains. In case of gE, there are two such regions: C1 located closer towards the N-terminus, containing 4 cysteines at positions 117, 126, 131, and 141, and C2 located closer to the C-terminus, containing 6 cysteines at positions 274, 283, 292, 300, 319, and 331). gI contains one cysteine-rich region with 4 cysteines.

gE and gI form a non-covalently bound oligomeric complex, which acts as a multifunctional unit (Zuckermann *et al*., 1988). The complex has been found in all alpha-herpesviruses studied so far. It is thought that the complex is a main neurotropic and neurovirulence factor promoting transneuronal transfer. It plays a role in the efficient cell-to-cell spread in non-neuronal cells and in the anterograde spread in synaptically connected neurons (Whealy *et al*.,

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Abbreviations: AcNPV = Autographa californica nuclear polyhedrosis virus; BHV-1 = Bovine herpesvirus 1; FHV-1 = Felid herpesvirus 1; gE = glycoprotein E; gI = glycoprotein I; HSV-1 = Herpes simplex virus 1; MOI = multiplicity of infection; PRV = Pseudorabies virus; VZV = Varicella-zoster virus

1993; Enquist, 1994; Card and Enquist, 1995; Tirabassi *et al*., 1997; Mettenleiter, 2003). It is also involved in the virion maturation (Brack *et al*., 2000; Johnson and Huber, 2002). The complex formation between gE and gI is also important for maturation of these proteins (Whealy *et al.*, 1993; Tirabassi *et al*., 1997; Tirabassi and Enquist, 2000).

In this report we describe studies on the PRV gE/gI complex formation using the full-length and truncated forms of gE and gI expressed in the baculovirus system. We attempted to identify domains of gE and gI, which are sufficient for the formation of the complex.

Materials and Methods

Cells. Sf9 insect (*Spodoptera frugiperda)* cells were grown in the Grace's medium containing 3.33 g/l lactalbumine hydrolysate and 3.33 g/l Yeastolate (TNM-FH medium supplemented with 8% FBS. Swine kidney SK6 cells infected with PRV were used as a positive control in Western blot analysis.

Viruses. PRV gE and gI genes and their fragments were inserted into the Autographa californica nuclear polyhedrosis virus (AcNPV) genome under polyhedrin promoter using the Bac-to-Bac expression system (GIBCO Life Technologies). To make a series of recombinants, full-length gE and gI genes and their truncated fragments were cloned into a baculovirus transfer vector, pFastBac1, and transferred by the site-specific transposition to a bacmid containing the baculovirus genome. Recombinant bacmids were used for transfection of insect cells. In this way, baculovirus recombinants containing full-length and truncated PRV gE and gI genes, respectively, were obtained. The corresponding proteins are shown in Fig. 1. PRV NIA3 strain was used for infection of SK6 cells.

Western blot analysis. Sf9 cells grown on 12-well plates in the TNM-FH medium supplemented with 8% FBS were infected with recombinant baculoviruses at multiplicity of infection (MOI) of 10. Three days post infection the cells were harvested, washed with PBS and lysed in SDS-PAGE sample buffer. The lysates were subjected to SDS-PAGE using 12% or 15% gels (Laemmli, 1970) and the proteins were blotted onto nitrocellulose or PVDF membrane (Burnette, 1981). The blots were first incubated with rabbit anti-gI or anti-gE polyclonal sera against full-length glycoproteins. The bound antibodies were detected with secondary antibodies conjugated with horseradish peroxidase. Hydrogen peroxide and 4-chloro-1-naphtol were employed for staining.

Radiolabeling was used for detection of truncated forms of gI, which proved to be non-reactive in Western blot analysis. Sf9 cells grown on 12-well plates in the TNM-FH medium supplemented with 8% FBS and 2 μg/ml tunicamycin (for blocking N-glycosylation for easier detection of truncated forms of gI protein) were infected with the recombinant baculoviruses (MOI = 10) for 45 hrs. The infected cells were labeled with 15 μCi/ml [^{35}S] methionine and [^{35}S] cysteine (Expre ^{35}S, Du Pont-NEN)) in the Grace's medium (lacking methionine and cysteine) containing 2 μg/ml tunicamycin for 2 hrs. The labeled cells were collected, washed with PBS and lysed in 100 μl of 0.03 mol/l Tris-HCl pH 7.5 containing 0.01 mol/l magnesium acetate and 1% Nonidet P-40 for 10 mins. The lysates were centrifuged at 13,000 rpm for 20 mins and aliquots of supernatants (soluble fraction) and pellets (insoluble fraction) were subjected to SDS-PAGE. The proteins were blotted onto 0.22 μm nitrocellulose membranes and the blots were dried and autoradiographed.

Radioimmunoprecipitation. Sf9 cells grown on 12-well plates were single-infected at MOI = 10 or double-infected at MOI = 20 with the baculovirus recombinants. After 40 hrs of infection, the medium was replaced with the Grace's medium and incubated for 1 hr at 27°C. The cells were labeled with 80 μCi /ml [^{35}S] methionine and [^{35}S] cysteine in the Grace's medium for 4 hrs at 27°C, pelleted, washed with PBS and solubilized in 250 μl of lysis buffer (50 mmol/l Tris-HCl pH 7.5, 1% Nonidet P-40, 0.5% sodium deoxycholate, 100 mmol/l NaCl, and 1 mmol/l PMSF) per well. The lysates were clarified by centrifugation and the supernatants were immunoprecipitated with the rabbit anti-gE or anti-gI serum. The antigen-antibody complexes were collected with Protein

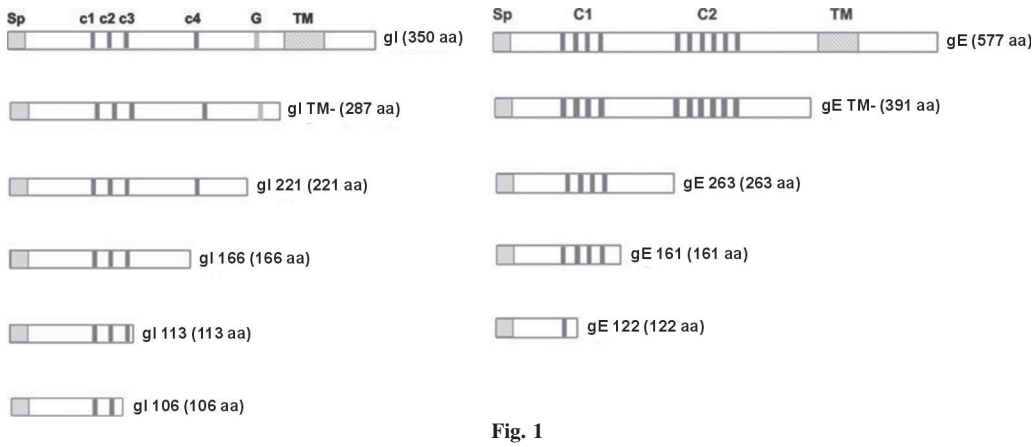

Fig. 1

Full-length and truncated forms of PRV gI and gE examined in the study

Sp = signal peptide; TM = transmembrane domain; C1, C2 = conserved cysteine domains, G = conserved GLYV motif; c1, c2, c3, c4 = conserved cysteines.

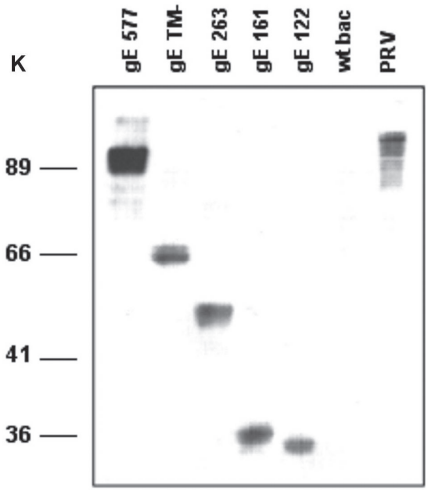

Fig. 2

Western blot analysis of different forms of PRV gE expressed in baculovirus system

Different gE fragments were detected with the anti-gE serum. A 12% gel was used in PAGE. Positive control: lysate of SK6 cells infected with PRV (lane PRV). Negative control: lysate of Sf9 cells infected with wild-type AcNPV (lane wt bac).

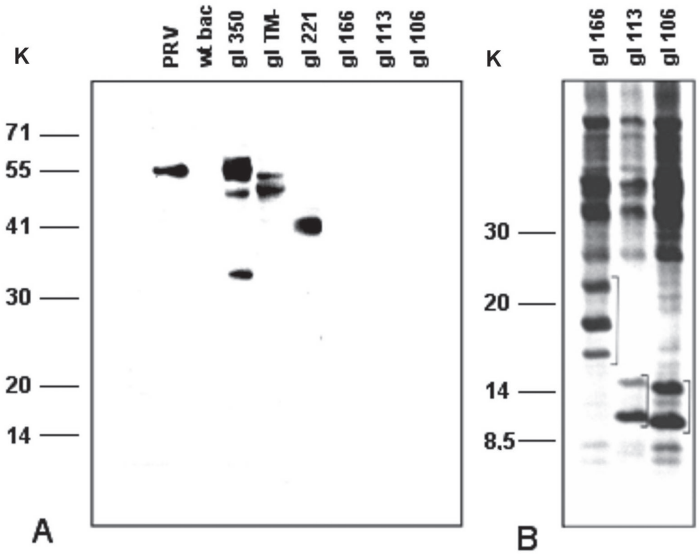

Fig. 3

Western blot analysis (A) and radiolabeling (B) of different forms of PRV gI

A. 15% gel was used in PAGE and gI fragments were detected with the anti-gI serum. Positive control: lysate of SK6 cells infected with PRV (lane PRV). Negative control: lysate of Sf9 cells infected with wild-type AcNPV (lane wt bac).

B. Radiolabeling of truncated gI forms present in insoluble fraction of cell lysates. The bands corresponding to truncated gI forms are marked with square brackets.

A-Sepharose, washed 3 times with the lysis buffer, resuspended in the SDS-PAGE sample buffer, boiled for 3 mins, and subjected to SDS-PAGE. The proteins were blotted onto 0.22 μm nitrocellulose membrane, dried and autoradiographed.

Results and Discussion

Full-length and truncated gE and gI genes and their expression in the baculovirus system

The baculovirus expression system was used for the studies of the PRV gE/gI complex. This system is currently used for expression of different viral proteins in biologically active form and enables studies of interactions of different forms of full-length and truncated proteins, which would be difficult or impossible to perform using native parental viruses. We used it successfully earlier for characterization of the BHV-1 gE/gI complex formation (Tyborowska *et al*., 2000).

To identify the domains of PRV gE and gI responsible for the complex formation, a series of baculovirus recombinants expressing the full and truncated forms of the gE and gI was constructed (Fig. 1). The truncated proteins contained progressive C-terminal deletions; the deleted regions could be involved in the process of complex formation. Western blot analysis showed that all tested gE recombinants produced detectable levels of full length or truncated gE (Fig. 2).

The expression of gI in the cells was tested by Western blot analysis using the rabbit anti-gI serum. In this case a positive reaction was seen only for the complete and longer forms of gI (gI 350, gI TM and gI 221). In contrast, shorter forms of gI (gI 166, gI 113 and gI 106) gave negative results (Fig. 3A). The lack of reactivity could be attributed to the defective folding of the truncated proteins. Therefore, the cells were infected with baculovirus recombinants expressing gI 166, gI 133 and gI 106, and radiolabeled. The results showed that also these gI fragments were expressed, but they were detectable only in the insoluble fraction of the cell lysates (Fig. 3B). In spite of the presence of tunicamycin in cultivation medium, a few bands were visible probably as the result of incomplete inhibition of N-glycosylation. Longer gI fragments (gI 350, gI TM, and gI 221) were present in both soluble and insoluble fractions (data not shown), while shorter gI fragments (gI 166, gI 113, and gI

106) were present only in insoluble fraction, suggesting their retention in endoplasmic reticulum. Similar observations have been made for gI of Felid herpesvirus 1 (FHV-1) gI, whose fragments of N-terminal 93, 113 and 152 aa were retained in endoplasmic reticulum (Mijnes *et al*., 1997). The four cysteines of FHV-1 gI have been found to form intra-molecular bridges (Mijnes *et al*., 1998). Because all four cysteines of gI are conserved among alphaherpesviruses these results suggest that such intra-molecular cysteine bridges also occur in PRV gI. Thus the removal of the last cysteine in gI 166 and gI 113 may be responsible for the lack of reaction with antibodies due to the defective folding of these fragments.

The gE/gI complex formation in the baculovirus system

To determine the domains of PRV gE involved in the complex formation with gI, the cells were double-infected with the baculovirus recombinants expressing the full-length gI (gI 350) and truncated forms of gE. The complex formation was tested by radioimmunoprecipitation with the rabbit anti-gE serum (Fig. 4). In this assay all forms of gE co-precipitated with gI. This result suggests that the cysteine regions of gE are not necessary for the complex formation.

Tirabassi *et al*. (1997) have found previously that the N-terminal part of PRV gE lacking cytoplasmic and transmembrane domains is sufficient for the gI binding. In HSV-1, the region involved in the complex formation with gI is located within the 188 N-terminal amino acids of gE (Rizvi and Raghavan, 2001). In contrast to the results with PRV gE, we have found that the shortest BHV-1 gE fragments of 109 and 136 aa containing intact cysteine-rich C1 domain did not bind to gI (Tyborowska *et al*., 2000).

To determine the gI domains involved in the complex formation with gE, the cells were double-infected with the baculovirus recombinants expressing the full-length gE (gE 577) and truncated forms of gI. Also in this case the complex formation was tested by radioimmunoprecipitation with the rabbit anti-gE serum (Fig. 5). The results showed that all tested gI fragments including those which could not be detected with the anti-gI serum alone, co-precipitated with gE, suggesting that the 106 N-terminal amino acids of gI are sufficient for binding to gE. It has been reported earlier that the cytoplasmic tails of gI glycoproteins of PRV (Tirabassi and Enquist, 2000), BHV-1 (Tyborowska *et al*., 2000) and VZV (Kimura *et al*.,

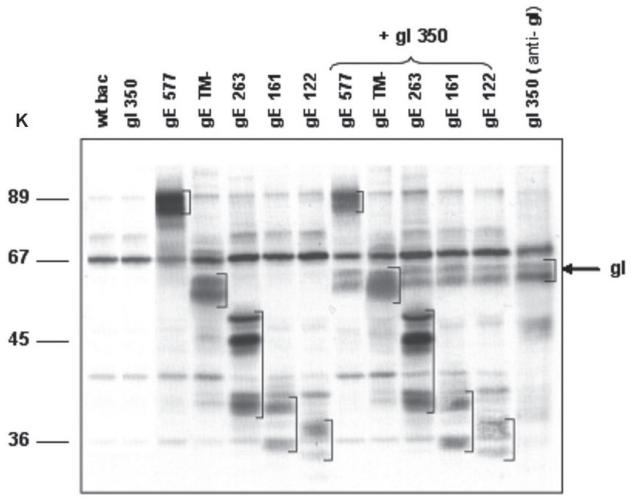

Fig. 4

Radioimmunoprecipitation of different forms of PRV gE interacting with PRV gI

Sf9 cells were single- or double-infected with the indicated baculovirus recombinants expressing full form of gI (gI 350) and truncated forms of gE, and labeled with ^{35}S. The gE/gI complexes were precipitated with the anti-gE serum. The bands corresponding to different gE forms are marked with square brackets. Positive control: gI 350 precipitated with the anti-gI serum (lanes gI 350 and gI 350 anti-gI). Negative controls: lysate of Sf9 cells infected with wild-type AcNPV (lane wt bac) and lysate of cells infected with the recombinant expressing gI 350 (lane gI 350) precipitated with the anti-gE serum.

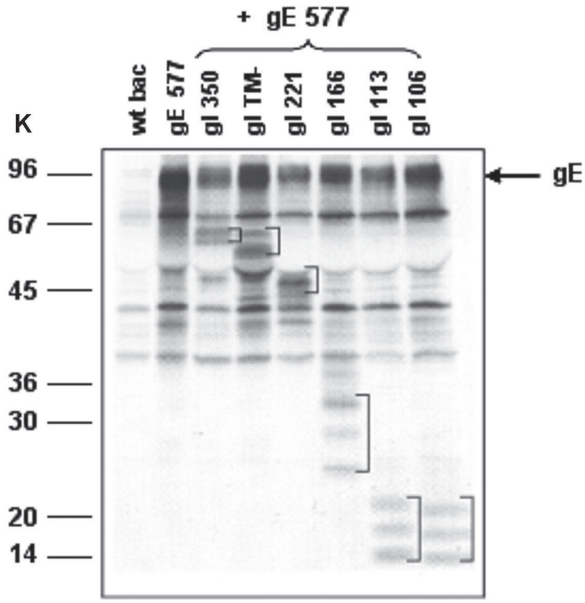

Fig. 5

Radioimmunoprecipitation of different truncated forms of PRV gI interacting with PRV gE

Sf9 cells were double-infected with baculovirus recombinants expressing full form of gE (gE 577) and truncated forms of gI, and labeled with ^{35}S. The gE/gI complexes were precipitated with the anti-gE serum. The bands corresponding to different forms of gI are marked with square brackets. Positive control: lysate of cells infected with the recombinant expressing gE 577 (lane gE 577). Negative control: lysate of cells infected with wild-type AcNPV (lane wt bac).

1997) are not required for the interaction with gE. Similarly to our observation on PRV, the 93 N-terminal amino acids of FHV-1 gI have been found to be sufficient for stable interaction with gE (Mijnes *et al*., 1997). Moreover, the mutation of two cysteines present in this fragment had no effect on the complex formation (Mijnes *et al*., 1998).

In the next experiment, the shortest form of gE containing 122 aa was tested with truncated forms of gI in formation of the complex. Even the shortest form of gI containing 106 aa was still able to form the complex (Fig. 6). This suggests that these shortest forms of gE and gI (122 and 106 aa, respectively) are sufficient for the gE-gI interaction. Apparently, formation of the complex is not dependent on the presence of sugar chains linked by N-glycosidic bond to polypeptide chains. This was proved by radioimmunoprecipitation of the gE/gI complexes formed in the presence of 2 µg/ml tunicamycin, an antibiotic that blocks N-glycosylation (data not shown). Also co-precipitation of the different forms of gI and gE (Figs. 4, 5 and 6) suggests that sugar chains are not essential for the gE-gI interaction.

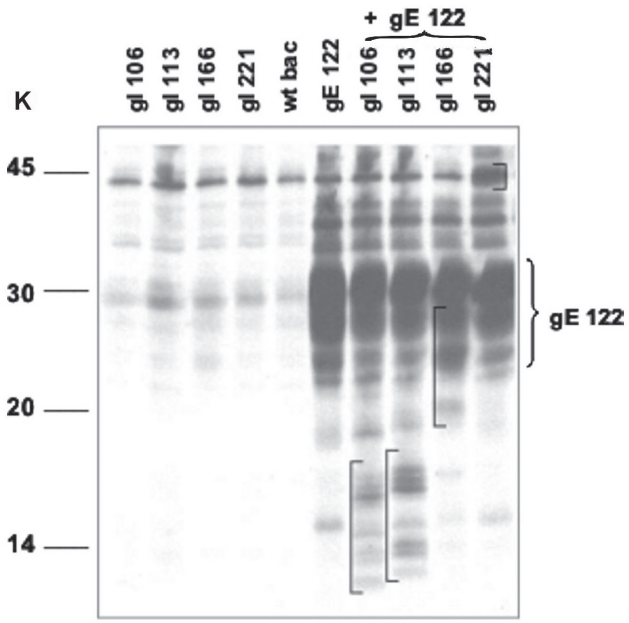

Fig. 6

Radioimmunoprecipitation of PRV gE 122 interacting with different truncated forms of PRV gI

Sf9 cells were single- or double-infected with baculovirus recombinants expressing gE 122 and truncated forms of gI and labeled with ^{35}S. The gE/gI complexes were precipitated with the anti-gE serum. The bands corresponding to different gI forms are marked with square brackets. Positive control: lysate of cells infected with recombinant gE 122 (lane gE122). Negative control: lysate of cells infected with wild-type AcNPV (lane wt bac) and lysates of cells infected with the recombinants expressing truncated forms of gI (lanes gI 106, gI 113, gI 166, and gI 221).

The results of this study identified the domains of PRV gE and gI which are crucial for the gE/gI complex formation that is a prerequisite for maturation of gE and gI. However, the interaction between gE and gI only is not sufficient for the complex gE/gI to attain all its biological properties. Cytoplasmic tails of both glycoproteins are very important for their functions. They are involved in proper maturation of both proteins, appropriate trafficking of gE/gI in polarized and neuronal cells, and virion assembly by interacting with tegument protein (Whealy *et al.*, 1993; Tirabassi *et al*., 1997, 2000; Wisner *et al.*, 2000; Johnson and Huber, 2002; Mettenleiter, 2002). Although our knowledge about gE and gI has significantly increased in the past few years, the complexity of their diverse functions requires further studies to define the mode of their interaction.

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