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Assembly of G1 and G2 glycoprotein oligomers in Punta Toro virus-infected cells *

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Summary

We have studied the oligomerization of the membrane glycoproteins of Punta Toro virus (PTV), a member of the Phlebovirus genus of the family Bunyaviridae, and the effect of glycosylation on protein stability and transport. By using sucrose gradient centrifugation, the G1 and G2 glycoproteins in PTV-infected or recombinant-transfected cells were found to sediment as dimers after DSP cross-linking, suggesting that the G1 and G2 proteins are associated as dimers by non-covalent interactions. Pulse-chase and two-dimensional gel analysis indicate that dimerization occurs between newly synthesized G1 and G2 proteins, and that a small fraction of the G2 proteins is assembled into G2 homodimers. The amounts of G1 and G2 proteins were substantially decreased, while the amounts of nucleocapsid protein remained nearly unchanged, when PTV-infected cells were treated with the glycosylation inhibitor tunicamycin, indicating that the G1 and G2 proteins are unstable if glycosylation is prevented.

Punta Toro virus; Oligomerization; Glycoprotein; Tunicamycin

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Introduction

Punta Toro virus (PTV) is a member of the Phlebovirus genus of the family Bunyaviridae, with similar structural and morphological features to other members of this family (Smith and Pifat, 1982; Bishop, 1990; Matsuoka et al., 1991). A unique feature of the bunyaviruses, which are enveloped viruses with 3 single-stranded, negative sense RNA genomic segments, is their intracellular maturation by budding at smooth-surfaced membranes in the Golgi region (Murphy et al., 1973; Smith and Pifat, 1982). Two virion glycoproteins, G1 and G2, are encoded by the M genomic segment, translated from a single mRNA as a precursor glycoprotein, and possibly cotranslationally cleaved into the final protein products (Ulmanen et al., 1981; Eshita et al., 1985). The PTV glycoproteins specifically accumulate in the Golgi complex during virus infection or when expressed by recombinant vaccinia viruses (Matsuoka et al., 1988; Chen et al., 1991a, b), indicating that the bunyavirus glycoproteins possess signals for retention in the Golgi complex (Matsuoka et al., 1991).

It has been determined that the gene product order of the PTV M segment is NH2-NSM-G1-G2-COOH (Ihara et al., 1985). Distinct hydrophobic regions preceding both the G1 and G2 proteins of PTV can function as signal peptides (Matsuoka et al., 1988; Chen et al., 1991b). There is a single hydrophobic membrane anchor domain near the C-terminus of each protein, followed by charged amino acids (Ihara et al., 1985; Chen et al., 1991b), as seen for the transmembrane domains of other class I viral membrane proteins (Sabatini et al., 1982). The information for Golgi retention is located within the G1 and G2 glycoproteins, rather than in the non-structural (NSM) sequence (Matsuoka et al., 1988). In previous studies, we used a recombinant T7 transient expression system and found that Golgi retention of the G2 protein requires interaction with the G1 protein, and that the G1 and G2 glycoproteins form a G1–G2 heterodimer (Chen et al., 1991b; Chen and Compans, 1991). In this study, we have further analyzed glycoprotein oligomerization in PTV-infected cells, and compared the process with that found in recombinant-transfected cells. We have also investigated the effect of glycosylation on the stability and transport of PTV glycoproteins.

Materials and Methods

Materials

HeLa T4+ and Vero cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% newborn bovine serum. A recombinant pGEM-G, containing the PTV G1 and G2 coding sequence, and a recombinant pGEM-G(A-), which expresses the intact PTV G1 protein and external domain of the G2 protein, were constructed as described previously (Matsuoka et al., 1988; Chen et al., 1991b). Polyclonal and monoclonal antibodies against PTV G1 and G2 glycoproteins were generously supplied by Drs. J.F. Smith and D. Pifat.
Tunicamycin was purchased from BMB Corp. (Indianapolis, IN). Lipofectin was purchased from BRL Corp. (Bethesda, MD).

**PTV infection and T7 polymerase transient expression**

PTV was obtained from USAMRIID (Frederick, MD). Stocks of the virus were prepared and titrated in Vero cells (Chen et al., 1991b). A recombinant vaccinia virus containing the T7 RNA polymerase gene (VV-T7) was obtained from Dr. B. Moss (Fuerst et al., 1986). The recombinant G1 and G2 proteins were expressed by using a T7 polymerase transient expression (Fuerst et al., 1986; Chen et al., 1991b).

**Labeling of cells and precipitation of viral proteins**

Radiolabeling of viral proteins was done as described previously (Chen et al., 1991a,b). Immunoprecipitation of the PTV proteins with monoclonal or polyclonal antibodies was followed by capture with protein A-agarose CL-4B (Pharmacia Inc. Piscataway, NJ). Samples were analyzed by SDS-PAGE (Laemmli, 1970).

**Two-dimensional SDS-PAGE and densitometry**

Samples were analyzed by first dimensional SDS-PAGE under non-reducing conditions. The gels were fixed, fluorographed, dried, and then exposed on X-ray films at -70°C. The desired lanes of the gels were then cut, soaked with electrophoresis buffer containing 5% 2-mercaptoethanol at room temperature for 30 min, and placed on top of the second dimensional gel. Densitometry of autoradiograms was performed by using an Ultrascan laser densitometer (LKB Corp).

**Oligomerization assay using sucrose gradient centrifugation**

Sucrose gradient centrifugation of the PTV proteins was performed as described previously (Chen and Compans, 1991). S_{20,w} values were estimated by comparison to protein standards and use of tables published by McEwen (1967); protein standards used (Sigma Chemical Co., St. Louis, MO) were cytochrome C (12,400 daltons (Da)), carbonic anhydrase (29,000 Da), ovalbumin (45,000 Da), bovine serum albumin (66,000 Da), hexokinase (100,000 Da), yeast alcohol dehydrogenase (150,000 Da), and catalase (250,000 Da), which were centrifuged in parallel tubes. The distribution of protein standards across the gradients was analyzed by SDS-PAGE and Coomassie brilliant blue staining after fractionation.

**Chemical cross-linking of proteins**

The homobifunctional cleavable cross-linking reagent dithiobis (succinimidylpropionate) (DSP) (Pierce Corp. Rockford, IL) was used as described previously (Chen and Compans, 1991). The cross-linked samples were analyzed under non-re-
A. DSP (-)
Pulse 5 min
Chase 0 min

Pulse 10 min
Chase 120 min

B. DSP (+)
Pulse 5 min
Chase 0 min

Pulse 10 min
Chase 120 min

Fractions: 1 3 5 7 9 11 13 15 17 19 21

Kd: 116 84 58 48.5
ducing or reducing conditions, in which the DSP cross-linked complexes were cleaved.

Results

Sucrose gradient sedimentation of viral glycoproteins

To investigate the oligomerization of the PTV G1 and G2 glycoproteins, PTV infected Vero cells were pulse-labeled, and chased for the indicated times. The cell lysates were analyzed by centrifugation on 5–20% sucrose gradients. After fractionation and immunoprecipitation, the distribution of the glycoproteins across the gradient was determined by SDS-PAGE. Most of the glycoproteins in the sucrose gradient were detected in one peak, at fractions 15–17. By comparison to the sedimentation of the marker proteins, fractions 15–17 appeared to correspond to monomeric forms of G1 and G2 proteins. We used a thiol-cleavable cross-linking reagent DSP to further investigate the presence of glycoprotein oligomers. When the cell lysates were cross-linked by DSP before centrifugation, faster-sedimenting forms of the G1 and G2 proteins were recovered from the sucrose gradients in fractions 11–13 (Fig. 1). By comparison to the sedimentation of the marker proteins, it appeared that the proteins in fractions 11–13 may correspond to dimeric forms of G1 and G2. The nucleocapsid protein, with or without cross-linking, sedimented heterogenously in the sucrose gradient, which may represent its assembly into nucleocapsid structures.

To compare the glycoproteins in virus-infected cells with recombinant glycoproteins, we used a recombinant pGEM-G to express the G1 and G2 proteins in the T7 RNA polymerase transient expression system (Chen et al., 1991b). HeLa T4+ cells infected with the VV-T7 virus were transfected with the recombinant plasmid DNA, and pulse-labeled. The cell lysates with or without DSP-cross-linking were centrifuged on 5–20% sucrose gradients. Most of the glycoproteins without cross-linking were detected in one peak in the sucrose gradient, at fractions 15–17, while faster-sedimenting forms of the G1 and G2 proteins were recovered from fractions 11–13 after cross-linking (data not shown). When analyzed by SDS-PAGE under non-reducing conditions, the proteins from fractions 15–17 appeared as monomeric

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Fig. 1. Sucrose gradient sedimentation of viral G1 and G2 glycoproteins. PTV-infected cells were pulse-labeled with [35S]methionine/cysteine for the indicated times at 37°C at 18 h postinfection. The cells were then lysed and supernatants of cell lysates were incubated with DSP (250 μg/ml) at 4°C for 30 min, and quenched by glycine. The samples were then centrifuged on 5–20% sucrose gradients. After fractionation, the proteins in each fraction were immunoprecipitated with polyclonal antibody to PTV which recognizes both the G1 and G2 protein and then analyzed by SDS-PAGE under reducing conditions. Standard proteins in the sucrose gradients were centrifuged in parallel tubes. The relative sedimentation of standard proteins is shown: yeast alcohol dehydrogenase (YAD), 150 kDa, 7.6 S; bovine serum albumin (BSA), 66 kDa, 5.1 S; ovalbumin, 45 kDa, 3.7 S. Fraction 1 represents the bottom fraction.
Fig. 2. Sucrose gradient sedimentation of the G1-truncated G2 mutant. HeLa T4 + cells were infected with VV-T7 and then transfected with the recombinant pGEM-G(A-) plasmid DNA. At 12 h post-transfection cells were pulse-labeled, lysed and the supernatants of the cell lysates were centrifuged on 5–20% sucrose gradients. The distribution of the glycoproteins across the gradient was determined by SDS-PAGE under non-reducing conditions.

G1 and G2, whereas the proteins in fractions 11–13 appeared as an additional band with an estimated molecular weight of about 118 kDa and a faint additional band with an estimated molecular weight about 95 kDa, which possibly correspond to dimeric forms of the G1 and G2 proteins (not shown). When the samples were analyzed in the presence of reducing agent, the higher-order structures were resolved into the monomeric G1 and G2 proteins, indicating that the higher-order structures consist of cross-linked forms of the G1 and G2 proteins. Since the chemical cross-linking of the proteins is an inefficient reaction, we have not obtained quantitative estimates of the relative proportions of monomers and oligomers. Nevertheless, these data indicate that the G1 and G2 proteins appear to be assembled into non-covalently linked dimers, which are unstable in sucrose gradients but can be stabilized by DSP cross-linking.

We further investigated the sucrose gradient sedimentation of mutant PTV glycoproteins by using a mutant pGEM-G(A-) (Chen et al., 1991b). In contrast to the native glycoproteins, a fraction of the G1 and truncated G2 proteins without cross-linking sedimented to fractions 12–13 (Fig. 2). Consistent with the sedimentation profile, the G1 and truncated G2 proteins from fractions 15–16 appeared as monomeric forms, and proteins from fractions 12–13 appeared as dimeric forms (estimated molecular weight about 110 kDa) in non-reducing gels (Fig. 2). The dimers were dissociated into nearly equal amounts of labeled G1 and truncated G2 proteins after treatment with 2-mercaptoethanol. Thus, the G1 and anchor-minus G2 occur as stable dimers, possibly linked by covalent disulfide bonds.
Two-dimensional gel analysis and chemical cross-linking

We further investigated the oligomerization of G1 and G2 in PTV-infected cells by using chemical cross-linking and two-dimensional SDS-PAGE. It was found that

Fig. 3. Chemical cross-linking and two dimensional gel analysis of the glycoproteins. PTV-infected cells were pulse-labeled with [35S]methionine/cysteine for 10 min at 37°C at 18 h postinfection. The cells were then lysed and the supernatants of the cell lysates were cross-linked with several concentrations of DSP on ice for 30 min, quenched by glycine, and then immunoprecipitated by polyclonal antibody to PTV. (A) G1 and G2 proteins cross-linked by different concentrations of DSP and analyzed under non-reducing conditions. (B) Two-dimensional SDS-PAGE of a 10-min labeling sample with DSP cross-linking (500 μg/ml).
the G1 and G2 glycoproteins appeared as monomeric forms in non-reducing gels. However, after the cell lysates were incubated with different concentrations of DSP (cross-linking space 12 Å), two additional bands with estimated molecular weights of 118 and 95 kDa, respectively, were found in non-reducing gels (Fig. 3A). When analyzed by using a two-dimensional gel system, with non-reducing conditions in the first dimension and reducing conditions in the second dimension, the 118-kDa band was resolved into two bands with the same mobilities as monomeric G1 and G2 proteins after treatment with 2-mercaptoethanol (Fig. 3B). The 95-kDa band was dissociated into a faint band with the same mobility as the monomeric G2 protein. Furthermore, when the 95-kDa band was cut, reduced, and then analyzed by SDS-PAGE, a G2 band was observed (not shown). These results are consistent with previous results obtained using a recombinant expression vector (Chen and Compans, 1991), and indicate that the 118-kDa band is a G1–G2 heterodimer and the 95 kDa band is a G2 homodimer. Furthermore, the heterodimeric bands were resolved into nearly equal amounts of labeled G1 and G2 proteins in the 10-min pulse-labeling sample, indicating that heterodimerization occurs between the newly synthesized G1 and G2 proteins.

Effects of glycosylation on protein stability and transport

We used tunicamycin, which prevents asparagine-linked glycosylation of newly synthesized proteins by inhibiting formation of N-acetylglucosamine-lipid intermediates (Tkacz and Lampen, 1975), to determine the role of glycosylation on viral protein stability and transport. PTV-infected cells were pulse-labeled in the
presence of increasing concentrations of tunicamycin. As shown in Fig. 4, a noticeable decrease in the amount of viral glycoproteins was observed as the concentrations of tunicamycin increased. At concentrations over 1.0 μg/ml, the G1 and G2 proteins were no longer detected. However, the amount of the unglycosylated nucleocapsid protein remained nearly unchanged at all the tunicamycin concentrations tested, demonstrating that the decreased amounts of the G1 and G2 proteins are not a result of inhibition of protein synthesis. We further investigated the effect of tunicamycin on the PTV proteins by using immunofluorescence staining. In the cells treated with concentrations of tunicamycin over 1.0 μg/ml, G1- and G2-specific staining could not be detected, while the nucleocapsid protein staining was clearly observed (data not shown). A similar effect of tunicamycin on the G1 and G2 proteins when expressed by a recombinant transient expression system was observed (not shown). Thus, the amounts of the G1 and G2 proteins were substantially decreased in the presence of the tunicamycin, indicating that modification of these glycoproteins by addition of oligosaccharide chains is important for their stability.

Discussion

We have used two-dimensional SDS-PAGE, chemical cross-linking, and sucrose gradient centrifugation to analyze the oligomerization of the G1 and G2 glycoproteins in PTV-infected cells. Our results indicate that the majority of G1 and G2 proteins are assembled into non-covalently bonded G1–G2 heterodimers. The processes of heterodimerization occur between newly synthesized G1 and G2 protein soon after protein synthesis. These results are in agreement with our previous observations using recombinant expression systems (Chen and Compans, 1991), indicating that PTV glycoproteins undergo a similar oligomerization process in different expression systems and in the presence or absence of the other viral components, such as the NSm protein.

Glycosylation of proteins has been shown to be important for protein stability, and maintaining protein conformation. In the case of the vesicular stomatitis virus (VSV)-G protein, the unglycosylated form of the protein in some strains of the virus formed aggregates which were unable to exit from the ER (Rose and Doms, 1988), while the unglycosylated form in other strains was transported to the plasma membrane (Gibson et al., 1978). In this study, we observed that the amounts of PTV G1 and G2 protein were dramatically decreased when the infected cells were treated with increasing concentrations of tunicamycin. Since tunicamycin is not a single compound but is composed of several homologues, some of which inhibit glycosylation as well as protein synthesis (Mahoney and Duksin, 1979), the decreased amounts of the glycoprotein might have been due to inhibition of protein synthesis. However, this is unlikely since the amount of nucleocapsid protein remained nearly unchanged at all the concentrations of tunicamycin tested. It is also unlikely that the unglycosylated proteins exhibited a decreased solubility or conformational change which made them unrecognizable to antibodies, since both
Polyclonal and monoclonal antibodies were used to detect the proteins by immunofluorescence staining and immunoprecipitation. It has been reported that unglycosylated forms of viral glycoproteins have different stabilities within the same host cells due to degradation by endogenous cellular proteases (Schwarz et al., 1976). Thus, we believe that the unglycosylated forms of the G1 and G2 protein are unstable, and are degraded by cellular proteases. A similar effect of tunicamycin on the glycoproteins of Hantaan virus was also reported (Schmaljohn et al., 1986).

Proteins traveling in the secretory pathway require correct folding and assembly before exit from the endoplasmic reticulum (Rose and Doms, 1988). Although the mechanism for retention of resident membrane proteins is still unknown, at least two alternative mechanisms for protein retention in subcellular compartments may be employed. One mechanism is that a retention signal within the protein sequence is responsible for intracellular retention, examples of which include the C-terminal sequence of the E3/19K protein of adenoviruses, or a transmembrane domain of the E1 protein of coronaviruses (Machamer and Rose, 1987; Jackson et al., 1990). Alternatively, plasma membrane proteins may be unable to exit from the ER when they fail to correctly fold or assemble as seen in VSV-G and influenza hemagglutinin mutants (Rose and Doms, 1989). Our studies show that PTV glycoproteins are able to correctly fold and assemble into dimers, which are transported to the Golgi complex. Thus, the Golgi retention of the PTV glycoproteins is mediated by a specific retention mechanism.

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