

Oligomerization, Transport, and Golgi Retention of Punta Toro Virus Glycoproteins

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We have investigated the oligomerization and intracellular transport of the membrane glycoproteins of Punta Toro virus, a member of the *Phlebovirus* genus of the family *Bunyaviridae*, which is assembled by budding in the Golgi complex. By using one- or two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis, chemical cross-linking, and sucrose gradient centrifugation, we found that the majority of the G1 and G2 glycoproteins are assembled into noncovalently linked G1-G2 heterodimers. At the same time, a fraction of the G2 protein, possibly produced independently of the G1 protein, is assembled into G2 homodimers. Kinetic analysis indicates that heterodimerization occurs between newly synthesized G1 and G2 within 3 min after protein synthesis, and that the G1 and G2 glycoproteins are associated as dimeric forms both during transport and after accumulation in the Golgi complex. Analysis of a G1-truncated G2 mutant, which is also targeted to the Golgi complex, showed that these molecules also assemble into dimeric forms, which are linked by disulfide bonds. Both the G1-G2 heterodimer and the G2 homodimer were found to be able to exit from the endoplasmic reticulum. Differences in transport kinetics observed for the G1 and G2 proteins may be due to the differences in the transport efficiency between the G1-G2 heterodimer and the G2 homodimer from the endoplasmic reticulum to the Golgi complex. These and previous results (S.-Y. Chen, Y. Matsuoka, and R. W. Compans, *Virology* 183:351-365, 1991) suggest that Golgi retention of the G2 homodimer occurs by association with the G1-G2 heterodimer, whereas the Golgi targeting of the G1-G2 heterodimer occurs by a specific retention mechanism.

The mechanism of transport and targeting of proteins from their sites of synthesis to their final destination has been intensively studied. Specific signals are required for targeting of proteins transported in the central vacuolar system (3) or targeted to the mitochondria (39) or the nucleus (19). Proteins traveling in the central vacuolar system require correct folding and assembly before exit from the endoplasmic reticulum (ER) and subsequent transport to the plasma membrane (32, 41). Selective retention of resident proteins against bulk flow can be accomplished by using a linear sequence as a retention signal in the case of luminal ER proteins (29, 45). Although the mechanism for retention of resident membrane proteins in intracellular compartments is still unknown, studies of several viral membrane glycoproteins and a *trans* Golgi resident membrane glycoprotein have provided evidence that their primary amino acid sequences are involved in intracellular retention (7, 16, 24, 31, 47). In contrast to such signal-mediated retention, misfolded or unassembled plasma membrane proteins are also found to be retained in the ER by forming large aggregates that are unable to enter transport vesicles (8, 9, 25, 41) or by binding to resident ER proteins like the immunoglobulin heavy-chain-binding protein (BiP) in the ER lumen, which possibly retains them in the ER (4, 32, 41).

Punta Toro virus (PTV), a member of the *Phlebovirus* genus of the family *Bunyaviridae*, has the structural and morphological features of other members of this family (2, 46). A unique feature of the bunyaviruses, which are enveloped viruses with three single-stranded, negative-sense RNA genome segments, is their intracellular maturation by budding at smooth-surfaced membranes in the Golgi region (30, 46). Two virion glycoproteins, G1 and G2, are encoded

by the M genome segment, translated from a single mRNA as a precursor glycoprotein, and cotranslationally cleaved into the final protein products (11, 21, 43, 48, 50). The glycoproteins specifically accumulate in the Golgi complex during virus infection or when expressed by recombinant vaccinia viruses (5, 22, 26, 27, 33, 51), indicating that the bunyavirus glycoproteins possess signals for retention in the Golgi complex (37).

The gene product order of the PTV M segment, NH₂-NS_M (nonstructural protein)-G1-G2-COOH, has been determined by amino acid sequence analysis (15). Distinct hydrophobic regions preceding both the G1 and G2 proteins of PTV can function as signal peptides (6, 27). There is a single hydrophobic membrane anchor domain near the C terminus of each protein, following by charged amino acids, as seen for the transmembrane domains of other class I viral membrane proteins (6, 13, 15, 38, 42). The information for Golgi retention is located within the G1 and G2 glycoproteins, rather than in the nonstructural (NS_M) sequence (27, 51). Since protein folding and assembly are important processes for transport, we have investigated the oligomerization of the PTV membrane glycoproteins and its relationship to their intracellular transport and Golgi retention. In this study, we have identified two distinct species of oligomeric glycoproteins, and we have analyzed their transport out of the ER and localization in the Golgi complex. We have also compared the oligomerization of mutant glycoprotein constructs which exhibit different transport properties.

MATERIALS AND METHODS

Materials. HeLa T4⁺ and Vero cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% newborn bovine serum. A recombinant designated pGEM-G, containing the PTV G1 and G2 coding sequence

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under control of a T7 promoter, was constructed as described previously (27). A recombinant designated pGEM-G(A-), which expresses the intact PTV G1 protein and external domain of the G2 protein, and a recombinant designated pGEM-G2, which contains the full-length PTV G2 coding sequence, were constructed as described previously (6). Polyclonal antibodies and monoclonal antibodies (MAbs) against PTV G1 and G2 glycoproteins were generously supplied by J. F. Smith and D. Pifat (USAMRIID, Frederick, Md.). 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 (5). A recombinant vaccinia virus containing the T7 RNA polymerase gene (VV-T7) was obtained from B. Moss (12). Recombinant plasmids containing PTV genes were expressed by using a T7 polymerase transient expression system as described previously (6).

Labeling of cells and precipitation of viral proteins. Transfected or infected cells were labeled with [³⁵S]methionine/cysteine (100 μ Ci/ml) in methionine/cysteine-free medium for the indicated times and then chased in Eagle's medium containing 10 mM methionine for the indicated periods. Immunoprecipitation of cell surface and intracellular proteins was carried out as described previously (6). The precipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing or nonreducing condition (23).

Two-dimensional SDS-PAGE and densitometry. For two-dimensional gel analysis, samples were analyzed by first-dimension SDS-PAGE under nonreducing conditions, and 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 finally placed on top of the second-dimension gel. Alternatively, tracks of the first-dimension gels were directly cut, incubated with Laemmli sample buffer containing 5% 2-mercaptoethanol at room temperature for 30 min, and then placed on top of the second-dimension gel. Densitometry of autoradiograms was performed by using an Ultrascan laser densitometer (LKB Corp).

Oligomerization assay using sucrose gradient centrifugation. Labeled cells were lysed as described above. The cell lysates were placed on ice and centrifuged in a microfuge for 1 min at 15,000 rpm. Samples (100 to 200 μ l) with or without dithiobis(succinimidylpropionate) (DSP) cross-linking were loaded onto 11-ml gradients of 5 to 20% (wt/vol) sucrose in buffer containing 0.1% Triton X-100, 60 mM Tris-HCl (pH 7.0), 200 mM NaCl, and 1.25 mM EDTA essentially as described by Persson et al. (35). The gradients were centrifuged in an SW41Ti rotor at 40,000 rpm for 16 h at 4°C . Fractions (0.5 ml each) were collected from the bottom, and proteins in each fraction were immunoprecipitated as described above. $s_{20,w}$ values were estimated by comparison with protein standards and use of tables published by McEwen (28); protein standards used (Sigma Chemical Co., St. Louis, Mo.) were cytochrome *c* (12,400 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. Homobifunctional cleavable cross-linking reagents DSP and ethylene glycol-bis(succinimidylsuccinate) (EGS) (Pierce Corp., Rockford, Ill.) were used. A freshly made DSP or EGS solution in dimethyl sulfoxide was added to the supernatants of cell lysates at the concentrations indicated for each experiment. After incubation for 30 min on ice, the reaction was quenched by addition of 2 mM glycine. The cross-linked samples were analyzed by sucrose gradient centrifugation following immunoprecipitation or were directly immunoprecipitated. DSP cross-linked complexes were cleaved with 5% 2-mercaptoethanol.

Endo H digestion. Immunoprecipitated samples were resuspended in 200 μ l of 0.1 M sodium acetate, pH 5.5, and then divided into two equal aliquots. One aliquot was incubated with 8 mU of endonuclease H (endo H) per ml for 16 h at 37°C and then centrifuged at $15,000 \times g$ for 5 min (21). The precipitates were resuspended in Laemmli sample buffer, boiled for 5 min, and then analyzed by SDS-PAGE.

RESULTS

Oligomerization of the G1 and G2 glycoproteins. To investigate the oligomerization of the G1 and G2 glycoproteins, we initially used recombinant plasmid pGEM-G, which contained the PTV G1 and G2 glycoprotein coding sequences under T7 promoter control for expression by the T7 RNA polymerase transient expression system (6, 12, 27). HeLa T4⁺ cells, which are relatively resistant to the cytopathic effects of vaccinia virus (27), were infected with recombinant VV-T7 encoding the bacteriophage T7 RNA polymerase, were transfected with recombinant plasmid DNA, and were radiolabeled as described in Materials and Methods. The cell lysates were analyzed by centrifugation on 5 to 20% sucrose gradients, and the distribution of the G1 and G2 glycoproteins across the gradient was determined by immunoprecipitation and SDS-PAGE. Most of the glycoproteins in the sucrose gradient were detected in one peak, at fractions 15 to 17, in either the pulse-labeled or chase samples, and only a small fraction of the proteins was detected as faster-sedimenting forms (not shown). By comparison with the sedimentation of marker proteins, fractions 15 to 17 appeared to contain monomeric forms of G1 and G2. We did not observe faster-sedimenting forms of the G1 or G2 proteins by changing the pH of the cell lysis buffer or sucrose gradient. To further investigate the presence of glycoprotein oligomers, we used the thiol-cleavable cross-linking reagent DSP. The fractions obtained from sucrose gradients from DSP cross-linked samples were analyzed in the absence or presence of reducing agent to determine whether DSP-linked G1 and G2 proteins were present. 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 to 13 (Fig. 1). Under nonreducing condition, the proteins immunoprecipitated from fractions 15 to 17 appeared as monomeric G1 and G2, whereas the proteins in fractions 11 to 13 appeared as a major band with an estimated molecular size of about 118 kDa and a faint additional band with an estimated molecular size of 95 kDa (Fig. 1). When the samples were analyzed in the presence of reducing agent, both of the higher-order structures were completely resolved into the monomeric G1 and G2 proteins, indicating that they consist of G1 and G2 proteins which are specifically cross-linked by DSP (not shown). The 118- and 95-kDa bands were observed in pulses as short as 3 min, indicating that these structures form

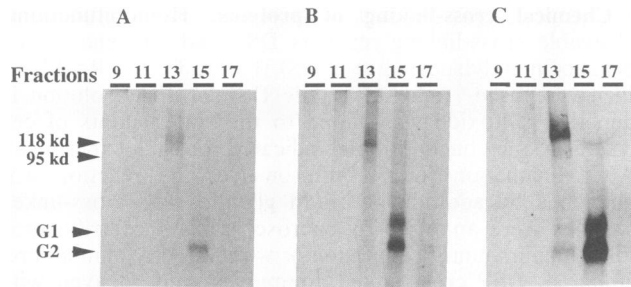


FIG. 1. Oligomerization of G1 and G2 glycoproteins. HeLa T4⁺ cells infected with VV-T7 were transfected with recombinant plasmid pGEM-G. After 12 h, cells were pulse-labeled with [³⁵S]methionine/cysteine for the indicated times at 37°C and then lysed. The supernatants of cell lysates were incubated with DSP (250 μg/ml) at 4°C for 30 min and quenched by glycine. The samples were then centrifuged on 5 to 20% sucrose gradients. After fractionation, the proteins in each fraction were immunoprecipitated with polyclonal antibody to PTV and then analyzed by SDS-PAGE under nonreducing conditions. Standard proteins in the sucrose gradients were centrifuged in parallel tubes. (A) Three-minute pulse labeling; (B) 7-min pulse labeling; (C) 10-min pulse labeling. Fraction 1 represents the bottom fraction.

immediately after or during biosynthesis. These results indicate that the G1 and G2 proteins are rapidly assembled into noncovalently linked dimers, which are unstable in sucrose gradients but can be stabilized by DSP cross-linking. Similar results were obtained when PTV-infected cells were analyzed (not shown). The mobilities of G1 and G2 monomers on reducing versus nonreducing gels were found to be dramatically different (not shown), indicating that at least one intramolecular disulfide bond is formed, which maintains a more compact structure of the proteins.

We further investigated the oligomerization of the G1 and G2 proteins by using chemical cross-linking and two-dimensional SDS-PAGE. Cells transfected with plasmid pGEM-G were pulse-labeled and chased, and the cell lysates were immunoprecipitated and analyzed by SDS-PAGE. It was found that the majority of the G1 and G2 glycoproteins remained as monomeric forms, and only a small fraction of the G1 and G2 proteins appeared as higher-molecular-weight forms in nonreducing gels, which were not easily detected (Fig. 2). However, after the cell lysates were incubated with various concentrations of DSP (cross-linking space, 1.2 nm) and then immunoprecipitated, two additional bands with estimated molecular sizes of 118 and 95 kDa, respectively, were found in nonreducing gels (Fig. 2). To define the properties of these bands, a two-dimensional gel system, with nonreducing conditions in first dimension and reducing conditions in the second dimension, was used. As shown at Fig. 3a, the 118-kDa band in the first-dimension gel was resolved into two bands with same mobilities as monomeric G1 and G2 proteins after treatment with 2-mercaptoethanol. The 95-kDa band was dissociated into a faint band with same mobility as the monomeric G2 protein. To further demonstrate that the 95-kDa band was composed of the G2 protein, the 95-kDa band was cut, treated with 2-mercaptoethanol, and reanalyzed by SDS-PAGE. The only band appearing in the gel following this treatment was a G2 band (Fig. 3b). The possibility that the G2 homodimer is formed after detergent solubilization was excluded since it was also detected when recombinant-transfected cells were cross-linked by DSP *in vivo* (not shown). These results, and results with a G1-

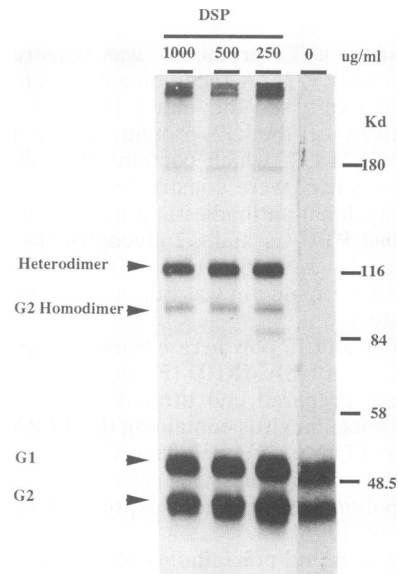


FIG. 2. Chemical cross-linking of PTV glycoproteins. HeLa T4⁺ cells infected with VV-T7 were transfected with pGEM-G. At 12 h posttransfection, the cells were pulse-labeled with [³⁵S]methionine/cysteine for 10 min at 37°C and lysed; the supernatants of the cell lysates were then cross-linked with the indicated concentrations of DSP on ice for 30 min, quenched by glycine, and then immunoprecipitated by polyclonal antibody to PTV. The immunoprecipitated proteins were analyzed under nonreducing conditions.

truncated G2 construct in which higher levels of the G2 homodimer are observed (see below), indicate that the 118-kDa band is a G1-G2 heterodimer and the 95-kDa band is a G2 homodimer. The reproducible finding of cross-linked dimers and absence of any detectable protein other than the PTV glycoproteins in the dimers indicated that the dimeric forms of the G1 and G2 proteins are specific. Furthermore, two-dimensional gels showed that the heterodimeric bands were resolved into nearly equal amounts of the labeled G1 and G2 proteins, indicating that heterodimerization occurs between the newly synthesized G1 and G2 proteins. The G1 and G2 proteins from either early or late chase samples were found as dimeric forms, indicating that the proteins remain as dimeric structures during transport and after accumulation in the Golgi complex.

Oligomerization and transport of mutant glycoproteins. Our previous study showed that a truncated anchor-minus G2 protein is secreted into the culture medium, but it is retained in the Golgi complex when coexpressed with the G1 protein (6), indicating that an interaction occurs between the G1 and truncated G2 proteins. When immunoprecipitates from the recombinant-transfected cells were analyzed under nonreducing conditions, two additional higher-molecular-weight bands with estimated sizes of about 110 and 80 kDa were observed (Fig. 4). To determine the identities of these additional bands, MAbs against the G1 and G2 glycoproteins were used. It was found that the G1 MAb precipitated the monomeric G1 and the 110-kDa band, whereas the G2 MAb precipitated the monomeric truncated G2 and the 110-kDa band (Fig. 5a). Furthermore, when analyzed by two-dimensional gels, the 110-kDa band in the first-dimension gel was completely resolved into two bands with the same mobilities as the monomeric G1 and truncated G2 proteins, whereas the 80-kDa band was dissociated into a monomeric truncated G2 band in the second-dimension reducing gels (Fig. 5b). The dimers did not appear to be an artifact of detergent

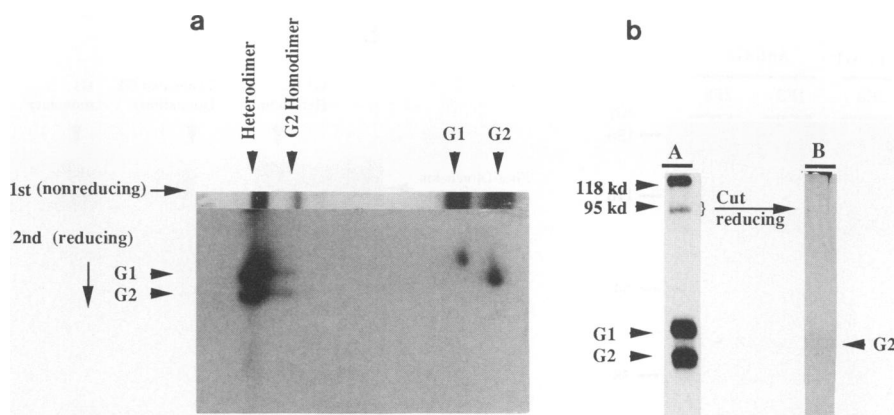


FIG. 3. Two-dimensional gel analysis of PTV glycoproteins. HeLa T4⁺ cells infected with VV-T7 were transfected with pGEM-G. At 12 h posttransfection, the cells were pulse-labeled with [³⁵S]methionine/cysteine for 10 min at 37°C and chased in the presence of excess methionine for indicated times. The cells were lysed, and the supernatants of the cell lysates were cross-linked with DSP on ice for 30 min, quenched by glycine, and then immunoprecipitated by polyclonal antibody to PTV. (a) Two-dimensional SDS-PAGE of 2-h chase sample with DSP cross-linking (500 μg/ml). (b) Analysis of the 95-kDa protein. The 95-kDa protein band appearing in a nonreducing gel (lane A) was cut, incubated with Laemmli sample buffer containing 5% 2-mercaptoethanol for 30 min, and then reanalyzed on a second SDS-polyacrylamide gel (lane B).

solubilization, since the proteins from cells lysed in the presence of iodoacetic acid behaved in the same fashion. A G2 homodimeric band from G2 MAb-immunoprecipitated samples was also observed but was visible only in overexposed films. From these results, we conclude that disulfide-bonded G1-truncated G2 heterodimers and disulfide-bonded truncated G2 homodimers are formed in cells transfected with the G1-truncated G2 construct.

Quantitative analysis by soft-laser densitometry scanning of the autoradiograms showed that about 36% of the immunoprecipitated G1 and truncated G2 molecules (mean values of 12 samples) appeared in the disulfide-bonded heterodimeric form, whereas about 8% of the immunoprecipitated G1 and truncated G2 proteins (mean values of 12 samples) were in the truncated G2 homodimeric form. Under identical labeling conditions, less than 5% of immunoprecipitated wild-type G1 and G2 proteins (mean values of 12 samples) when expressed by recombinant pGEM-G appeared as dimers in nonreducing gels. Thus, the ratio of dimers to monomers in the mutants was about 10-fold higher than that of the wild-type proteins, suggesting that the conformation of the mutant protein is different from that of the native proteins. These results also indicate that the G2 transmembrane domain and C-terminal domain are not required for dimerization of the glycoproteins.

Endo H treatment, which cleaves high-mannose N-linked oligosaccharides, was used to monitor whether the proteins are transported out of the ER and reach the medial Golgi complex, where processing reactions convert the high-mannose forms to endo H-resistant complex oligosaccharides (20). As shown in Fig. 6a, the G1-truncated G2 heterodimers as well as the truncated G2 homodimers became endo H resistant after a 6-h chase, indicating that G2 homodimers are transported out of the ER. The monomeric proteins appearing in the nonreducing gels also became endo H resistant. To clarify whether the monomeric forms of the G1 and truncated G2 proteins are derived from dimers associated by noncovalent weak interactions, we used the cross-linking reagents DSP and EGS. In the samples incubated with DSP, the G1 and truncated G2 appeared in dimeric as well as monomeric forms, whereas when cross-linked by the

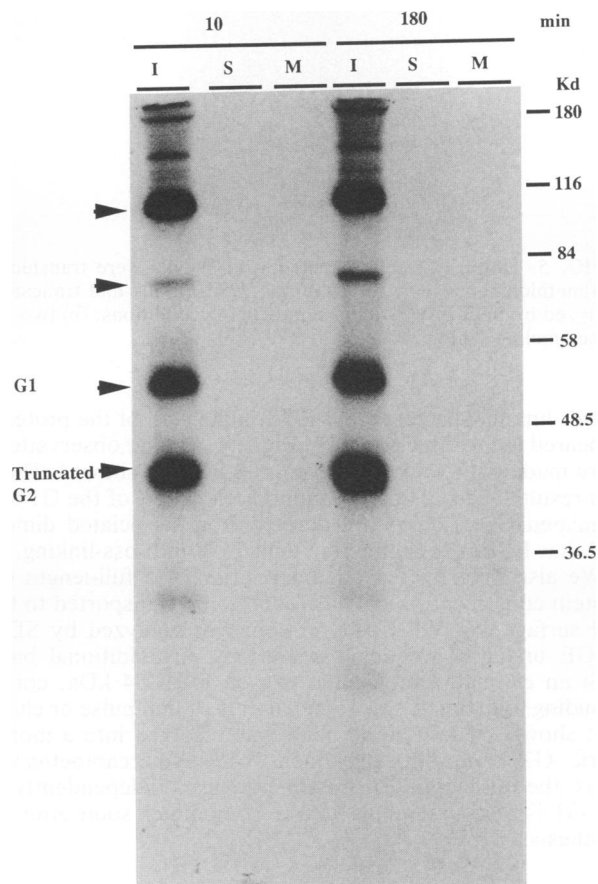


FIG. 4. Dimerization of the G1-truncated G2 mutant. HeLa T4⁺ cells infected with VV-T7 were transfected with recombinant pGEM-G(A-) plasmid DNA, pulse-labeled with [³⁵S]methionine/cysteine for 10 min at 37°C, and chased in the presence of excess methionine for the indicated times. The G1 and truncated G2 proteins were immunoprecipitated from culture medium (lanes M), cell lysates (lanes I), or cell surface (lanes S) with polyclonal antibody to PTV and analyzed by SDS-PAGE under nonreducing conditions.

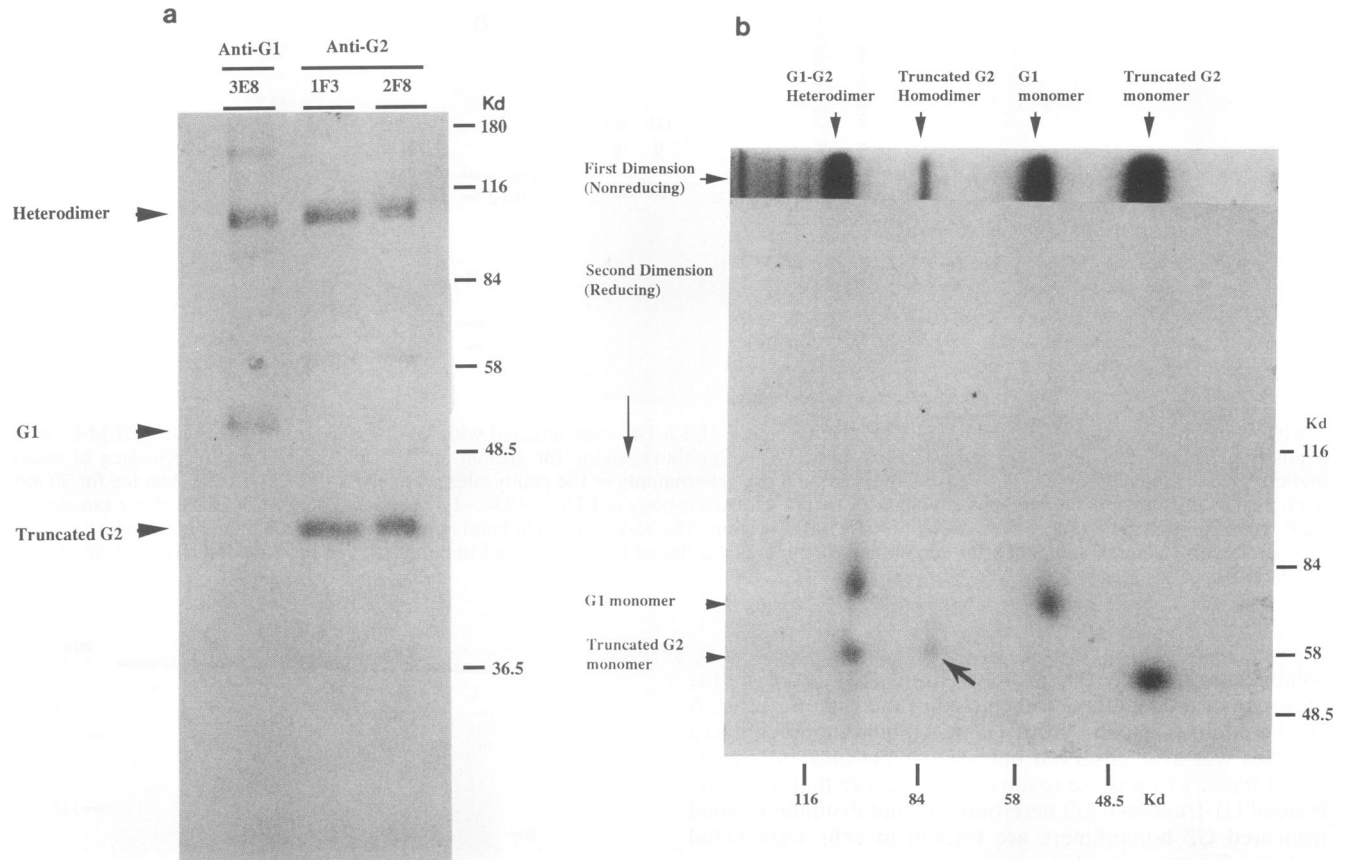


FIG. 5. HeLa T4⁺ cells infected with VV-T7 were transfected with recombinant pGEM-G(A-) plasmid DNA and pulse-labeled with [³⁵S]methionine/cysteine for 10 min at 37°C. (a) G1 and truncated G2 proteins immunoprecipitated with an MAAb to G1 or G2 protein and analyzed by SDS-PAGE under nonreducing conditions; (b) two-dimensional gel analysis of the G1 and truncated G2 proteins from a 10-min pulse-labeling sample.

longer-linking-space reagent EGS, almost all of the proteins appeared in the dimeric forms (Fig. 6b). Similar observations were made with wild-type glycoproteins (not shown). Thus, this result suggests that the monomeric forms of the G1 and truncated G2 proteins are derived from associated dimers and that DSP is less effective than EGS in cross-linking.

We also investigated oligomerization of a full-length G2 protein construct, which was found to be transported to the cell surface (6). When this protein was analyzed by SDS-PAGE under nonreducing conditions, an additional band with an estimated molecular size of about 84 kDa, corresponding to a dimer, was found after a 10-min pulse or chase (not shown). The dimeric band was resolved into a monomeric G2 band after treatment with 2-mercaptoethanol. Thus, the full-length G2 protein expressed independently of the G1 is also assembled into a homodimer soon after its synthesis.

DISCUSSION

We have used several approaches, including one- or two-dimensional SDS-PAGE, chemical cross-linking, and sucrose gradient centrifugation, to analyze the oligomerization of wild-type and mutant PTV glycoproteins. Given the consistent observations obtained by using the various approaches and expression systems, we conclude that the majority of G1 and G2 proteins are rapidly assembled into

noncovalently bonded G1-G2 heterodimers. The results indicate that heterodimerization occurs between newly synthesized G1 and G2 proteins within a 3-min labeling period, and that the glycoproteins are predominantly found as G1-G2 heterodimers either during transport (less than 45-min chase samples) or after accumulation in the Golgi complex (late chase samples).

Unexpectedly, we found that a fraction of G2, or a larger proportion of the truncated G2 mutant, forms G2 homodimers either when expressed by recombinants or during PTV infection. Further results indicate that the G2 homodimers are also transported out of the ER, since the G2 proteins appeared in the perinuclear Golgi region (5, 27), and the homodimeric forms of the mutant G2 protein became endo H resistant. Furthermore, the full-length G2 protein, which is transported to the cell surface when expressed in the absence of G1 (6), was also found to assemble into homodimers. The G1 and G2 proteins are encoded by a single open reading frame of the complementary M-segment RNA and are probably cleaved from the nascent precursor polypeptide (40, 48-50). We did not observe a G1 homodimer species. One possible explanation for failing to detect G1 homodimers could be that a G1 homodimer forms a compact structure which comigrated with the G1-G2 heterodimer; however, analysis by two-dimensional SDS-PAGE showed that nearly equal amounts of the G1 and G2 protein are present in the dimeric bands, arguing against this

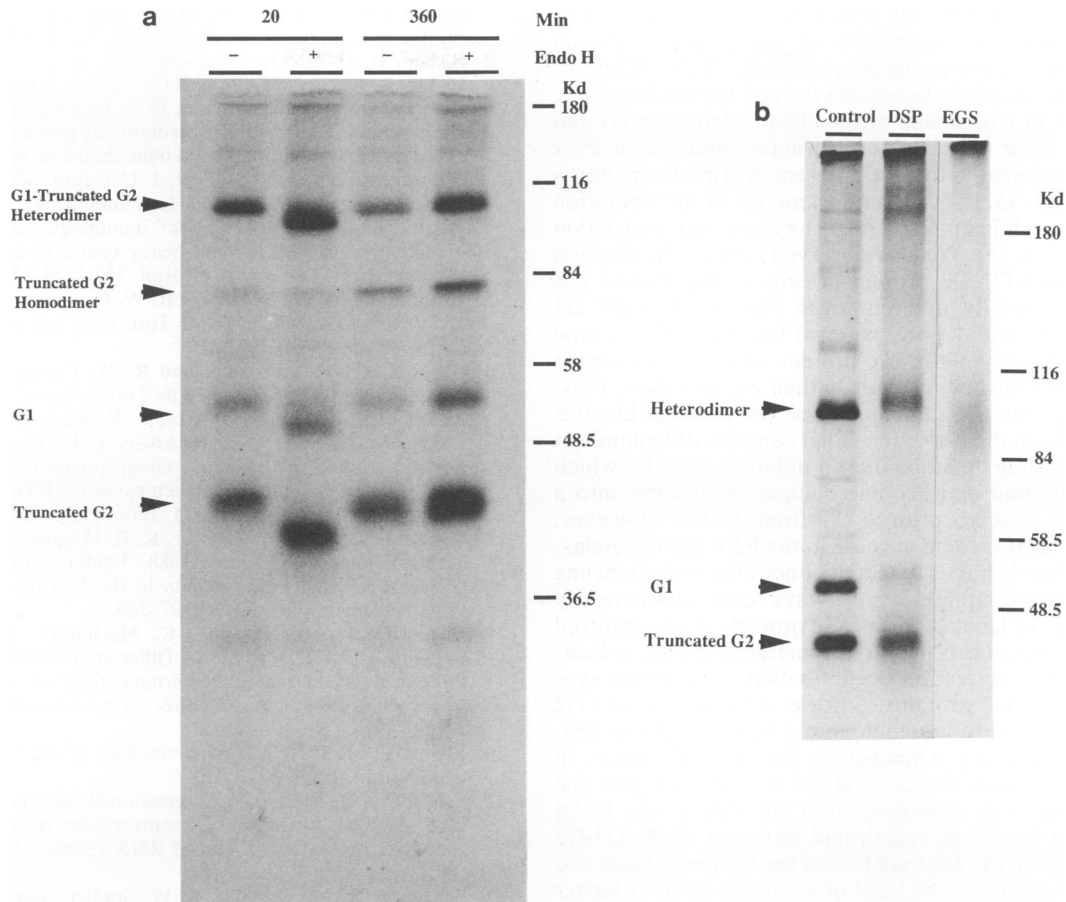


FIG. 6. Analysis of the G1-truncated G2 mutant. HeLa T4⁺ cells were infected with VV-T7 and then transfected with the recombinant plasmid DNA. At 12 h posttransfection, cells were pulse-labeled with [³⁵S]methionine/cysteine for 10 min (a) or 15 min (b) at 37°C and then chased in the presence of excess methionine for the indicated times. The cells were lysed, and the supernatants of the cell lysates were then immunoprecipitated with polyclonal antibodies to PTV. (a) The precipitated samples were aliquoted into two parts; one was treated with endo H (+), and the other was not treated (-). (b) The supernatants of the cell lysates from the 15-min pulse-labeling were cross-linked with DSP or EGS at a concentration of 500 µg/ml on ice for 30 min, quenched by glycine, and then immunoprecipitated by polyclonal antibody to PTV. All of the samples were analyzed by SDS-PAGE under nonreducing conditions.

possibility. Alternatively, a more likely explanation is suggested by recent studies of the glycoprotein biogenesis of Rift Valley fever virus (RVFV), another member of *Phlebovirus* genus. It was reported that a fraction of the second glycoprotein in the precursor polypeptide (called G1 in RVFV) is produced independently of the first glycoprotein (called G2 in RVFV) (18, 49). The mechanism for the independent production of the second glycoprotein was suggested to involve internal translation initiation (49), as observed in a number of other viral proteins (14, 17). We also observed that a larger amount of the G2 protein (the second protein in the precursor polypeptide of PTV) than the G1 protein was usually detected during either PTV or recombinant infection, possibly by a similar mechanism. Since both the G1-G2 heterodimer and G2 homodimer are able to exit from the ER, transport of the PTV glycoproteins occurs in two dimeric forms, a G1-G2 heterodimer and a G2 homodimer.

From studies on mutant glycoproteins, we have concluded the following. (i) The G2 transmembrane and C-terminal domains are not required for oligomerization and transport of the glycoproteins, since heterodimerization can occur between the G1 and an anchor-minus G2 protein, and both

the G1 and anchor-minus G2 proteins are found to be transported out of the ER. (ii) Since the amounts of disulfide-bonded dimers of the G1-truncated G2 mutant were 10-fold higher than that of wild-type glycoproteins, the ectodomain conformation of the mutant protein is different from that of native proteins, but this difference does not affect transport of the proteins or retention in the Golgi complex. (iii) The basis for Golgi retention of the G2 protein is by interaction with the G1 glycoprotein in either of two ways, one of which is assembly with the G1 protein into a G1-G2 heterodimer. The homodimeric form of the G2 protein also possibly interacts with the G1-G2 heterodimer by noncovalent weak interactions, since the anchor-minus G2 proteins in the homodimeric form are also retained in the Golgi complex when coexpressed with the G1 protein but not when expressed independently (6). (iv) The full-length G2 protein expressed in the absence of G1 is also assembled into homodimers, which are transported to the cell surface.

A common property of all bunyavirus M-segment gene products predicted from cDNA sequences studied so far is their high cysteine content and, in related viruses (i.e., Uukuniemi virus and PTV), the conservation of the positions of the cysteine and proline residues, suggesting similar

folding patterns (10, 15, 40, 43). Consistent with the high cysteine content, the mobilities of the glycoproteins on reducing versus nonreducing gels are dramatically different, suggesting that disulfide bonds are formed intramolecularly. Interestingly, in Uukuniemi virus-infected cells, the G1 and G2 proteins were found to form a heterodimer soon after synthesis but appeared to have different distribution patterns in infected cells (52) and different kinetics of incorporation into virions, modification by Golgi enzymes, and association with BiP (21, 34, 35). We also observed that the distribution pattern of the PTV G2 protein in cells (a preferential ER pattern) was initially different from that of the PTV G1 protein (a preferential Golgi pattern) but gradually became identical with that of the G1 protein after cycloheximide treatment (unpublished results). In their recent report, Persson and Petersson (34) concluded that the different kinetics of transport from the ER to the Golgi complex of Uukuniemi G1 and G2 proteins may be due to different rates by which these proteins fold and become competent to enter into a heterodimeric complex prior to exit from the ER. However, this does not appear to be the case in the PTV glycoproteins. The heterodimeric bands detected either after pulse-labeling or in longer-chase samples were always resolved into nearly equal amounts of labeled G1 and G2 proteins when analyzed by two-dimensional gels and densitometer scanning, indicating that heterodimerization occurs between the newly synthesized G1 and G2 proteins. Since a population of the G2 proteins was found to assemble into a homodimer independently, an alternative explanation for the differences in transport kinetics of the G1 and G2 protein emerges: the different kinetics of transport from the ER to the Golgi complex may be due to more rapid transport of the G1-G2 heterodimer from the ER than that of the G2 homodimer and to the observation that the level of synthesis of G2 is higher than that of G1 (18, 49). In Uukuniemi virus, the similar molecular weights of G1 and G2 (21, 34) may make it difficult to distinguish a G1-G2 heterodimer from a G2 homodimer.

On the basis of the observations presented above, we propose that the process of PTV glycoprotein oligomerization and transport is as follows. A majority of the newly synthesized G1 and G2 glycoproteins is cotranslationally cleaved into monomers and then quickly assembled into G1-G2 heterodimers. At the same time, a fraction of the G2 protein, which may be produced independently of the G1 protein, is folded and assembled into G2 homodimers. The transport of the G1-G2 heterodimers out of the ER is relatively faster than that of the G2 homodimers. Subsequently, the G1-G2 heterodimers are retained in the Golgi complex by a specific retention mechanism, whereas the G2 homodimers also accumulate in the Golgi complex by interaction with the G1-G2 heterodimers. Further studies to define the signal for Golgi retention are in progress.

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