

# Formation and Intracellular Transport of a Heterodimeric Viral Spike Protein Complex

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**Abstract.** We have analyzed the heterodimerization and intracellular transport from the ER to the Golgi complex (GC) of two membrane glycoproteins of a bunyavirus (Uukuniemi virus) that matures by a budding process in the GC. The glycoproteins G1 and G2, which form the viral spikes, are cotranslationally cleaved in the ER from a 110,000-D precursor. Newly synthesized G1 was transported to the GC and incorporated into virus particles about 30–45 min faster than newly synthesized G2. Analysis of the kinetics of intrachain disulfide bond formation showed that G1 acquired its mature form within 10 min, while completion of disulfide bond formation of G2 required a considerably longer time (up to 60 min). During the maturation process, G2 was transiently associated with

the IgG heavy chain binding protein for a longer time than G1. Protein disulfide isomerase also coprecipitated with antibodies against G1 and G2.

In virus particles, G1 and G2 were present exclusively as heterodimers. Immunoprecipitation with monoclonal antibodies showed that heterodimerization occurred rapidly, probably in the ER, between newly made G1 and mature, dimerization competent G2.

Taken together, our results show that these two viral glycoproteins have different maturation kinetics in the ER. We conclude that the apparent different kinetics of ER to GC transport of G1 and G2 is due to the different rates by which these proteins fold and become competent to enter into heterodimeric complexes prior to exit from the ER.

SECRETORY and membrane proteins are synthesized and acquire asparagine-linked glycans in the ER. In most cases, they then embark onto the exocytic transport pathway to the plasma membrane (PM)<sup>1</sup> via the Golgi complex (GC) (17, 23, 24, 42). Our understanding of the early steps involved in this exocytic pathway, i.e., disulfide bond formation, folding and oligomerization of membrane proteins has improved substantially during the last few years. It now appears clear that an elaborate quality control of protein folding and oligomerization exists in the ER that prevents un- or misfolded and unassembled proteins from leaving this compartment (19, 24, 37, 44, 46). The maturation process may involve the catalytic action of several proteins such as the IgG heavy chain binding protein (BiP or GRP78) (37, 46), protein disulfide isomerase (PDI) (11, 12), glycosylation site binding protein (15), and perhaps yet other chaperonin-like proteins. It has been known for a long time that various secretory and membrane proteins have very different transport kinetics from the ER to the GC (10, 13, 30, 34). An apparent possibility is that protein folding and oligomerization is the rate-limiting step. In agreement with this, it has been found that different proteins fold and assemble into oligomers with vastly different kinetics. Oligomerization seems to

be an important prerequisite for exit of membrane proteins from the ER compartment (19, 44).

Studies on the folding and oligomerization of cellular proteins, such as procollagens (22), the T cell receptor (24, 33), glycoprotein hormones (48), class I and class II MHC antigens, and immunoglobulins (for references, see 19), have revealed the intricate steps involved in protein folding and oligomerization in the ER. Many of the details involved in the early steps have been elucidated using two viral model systems, the hemagglutinin of influenza virus (2, 5, 6, 16) and the G protein of vesicular stomatitis virus (VSV) (7, 8, 25, 31). These model proteins have many features in common, such as the kinetics of folding, disulfide bond formation, and trimerization, which in both cases take ~7–10 min (6–8, 16). Properly folded and trimerized proteins are then rapidly transported to the GC. Misfolded proteins, on the other hand, tend to aggregate and are retained in the ER (19). In only a few cases has it been directly shown that proteins are transiently associated with BiP during the folding process (1, 9, 31, 35, 47). Although the exact role of BiP is still under debate, it may assist in the folding process, prevent aggregation and prevent un- or misfolded proteins from exiting the ER (18, 19, 37).

We are using a different viral model system, the glycoproteins of bunyaviruses and in particular those of Uukuniemi virus, to study the above processes. All bunyaviruses contain two glycoproteins, G1 and G2, that form the surface projec-

1. *Abbreviations used in this paper:* BiP, IgG heavy chain binding protein; GC, Golgi complex; PDI, protein disulfide isomerase; PM, plasma membrane.

tions, or spikes (41). In the case of Uukuniemi virus, G1 ( $M_r$  70,000) and G2 ( $M_r$  65,000) form an icosahedral surface lattice comprising an estimated 720 molecules of each species per particle (51). They are encoded as a 110,000-D precursor (p110) by the medium-sized RNA segment of the tripartite genome (40, 43, 50). p110 is cotranslationally cleaved in the ER approximately in the middle. This cleavage is probably carried out by the signal peptidase, as there is an internal signal sequence for the downstream G2 in p110 (43; unpublished results). Both G1 and G2 have 4 N-linked glycans and 26 cysteine residues in their ectodomains (43). In virus particles, G1 contains only endoglycosidase H (endo H)-resistant glycans, while the glycans in G2 are mainly endo H sensitive (26, 38). The  $t_{1/2}$  for acquisition of endo H-resistant glycans in G1 is  $\sim 45$  min (26).

A characteristic feature of all bunyaviruses is that they mature by budding into the Golgi cisternae (41). Mature virus particles are then transported in large vesicles to the PM and released from the cell after fusion of the vesicles with the PM. Our aim is to elucidate the mechanisms that determine the maturation in the GC rather than at the PM. Previous work has shown that G1 and G2 accumulate in the GC and are not transported further to the PM (14, 27–29). During the course of our studies on the biosynthesis of G1 and G2, results were obtained suggesting that G1 is transported faster than G2 to the site of virus budding in the GC (26). This suggested that G1 and G2 might be transported from the ER to the GC independently of each other. Here we show that this is not the case. Instead, G1 and G2 dimerize probably already in the ER and are therefore dependent on each other for transport to the GC. The apparent difference in transport kinetics is due to the fact that G1 folds much faster than G2. Thus, newly synthesized G1 is able to dimerize only with a G2 that was synthesized  $\sim 20$ – $45$  min earlier. Newly formed G1 and G2 made from the same p110 precursor cannot therefore dimerize with each other. We also show that both BiP and PDI can be coprecipitated with newly synthesized G1 and G2, suggesting that these ER proteins play a role in the folding process.

## Materials and Methods

### Chemicals

Cell culture medium and fetal calf serum were purchased from Gibco Ltd., Middlesex, England. Pansorbin (10% suspension) was obtained from Calbiochem-Behring Corp., San Diego, and L- $^{35}$ S]methionine ( $>1,000$  Ci/mmol) was from Amersham International, Buckinghamshire, England. N-Ethylmaleimide was obtained from Sigma Chemical Co., St. Louis, MO.

### Cells and Virus

The origin and cultivation of BHK-21/clone 13 cells, as well as the origin of the prototype strain S23 of Uukuniemi virus, have been described previously (39).

### Metabolic Labeling of Infected Cells

BHK-21 cells, grown on 6-cm dishes ( $\sim 2.5 \times 10^6$  cells/dish), were infected with Uukuniemi virus at a multiplicity of  $\sim 20$  PFU/cell. At 17-h postinfection, the cells were incubated for 60 min in methionine-free medium and then pulse-labeled with  $^{35}$ S]methionine (50–100 mCi/ml) for 2 or 10 min, as indicated in the text, followed by chase periods at which a 100-fold excess of the normal amount of methionine was added. In some experiments, cells were chased in the presence of cycloheximide (at a final concentration of 50  $\mu$ g/ml) or pretreated with the drug for 3 h before the

radioactive pulse. After the chase the dishes were put on ice and the cell monolayer was rinsed twice with ice-cold PBS. The cells were then lysed in 200 or 300 ml/dish of solubilization buffer (10 mg/ml Triton X-100, 5 mM EDTA, 150 mM NaCl, 100 IU/ml Trasylol in 50 mM Tris-HCl [pH 8.0]). The lysed cells were centrifuged for 5 min at 15,000 g, and the supernatants were frozen in liquid nitrogen. In experiments where disulphide bond formation of viral proteins was studied, the infected, labeled, and chased cells were rinsed with ice-cold PBS and incubated for 10 min with 100  $\mu$ l of 20 mM N-ethylmaleimide in PBS. After incubation, the cells were lysed with 100  $\mu$ l of two-fold concentrated solubilizing buffer and treated as described above.

### Isolation of Extracellular Virus Particles

Infected cells were pulse labeled and chased as described above and medium from the cells were collected. Virus particles were then isolated in a two-step procedure as described by Kuismanen (26).

### Subcellular Fractionation

Cells were infected, labeled with  $^{35}$ S]methionine, chased as described above, and subsequently homogenized as reported earlier (13). The homogenate was centrifuged to yield a postmitochondrial supernatant that in turn, was centrifuged in a sucrose gradient as described earlier (52) to separate the ER and the GC. The gradient was fractionated into 10 fractions, and 4.5 ml of PBS was added to each fraction, followed by centrifugation at 150,000 g for 60 min. The supernatants were aspirated, and 200  $\mu$ l of solubilizing buffer was added to the pellets. After thorough mixing, samples were frozen in liquid nitrogen and kept at  $-70^\circ\text{C}$ . NADPH cytochrome c reductase, which is a marker for ER-derived membranes, was assayed for as described by Omura and Takesue (36), and galactosyl transferase, which is a marker for *trans*-GC, was assayed for as described by Brew et al. (4).

### Sedimentation of Viral Proteins in Sucrose Gradients

Virions solubilized with 1% Triton X-100 in PBS were analyzed by centrifugation in sucrose gradients (5–20% [wt/wt] sucrose in 40 mM MES, 60 mM Tris-HCl [pH 7.4], 200 mM NaCl, 1.25 mM EDTA, 0.1% Triton X-100) essentially as described by Doms et al. (7). The samples were centrifuged for 16 h at 40,000 rpm and  $4^\circ\text{C}$  in an SW50.1 rotor (Beckman Instruments Inc., Palo Alto, CA). Gradients were fractionated from the bottom into 25 fractions and each was used in immunoprecipitation analyses with polyclonal antibodies against G1 + G2, and monoclonal antibodies against G1 and G2 (the latter recognize the complex between the two proteins). The total amount of radioactivity was measured directly by liquid scintillation counting. Rabbit IgG, bovine serum albumin, and ovalbumin (100  $\mu$ g of each), centrifuged as above, served as sedimentation markers. The sedimentation values for these proteins are 7.1S, 4.7S, and 3.7S, respectively.

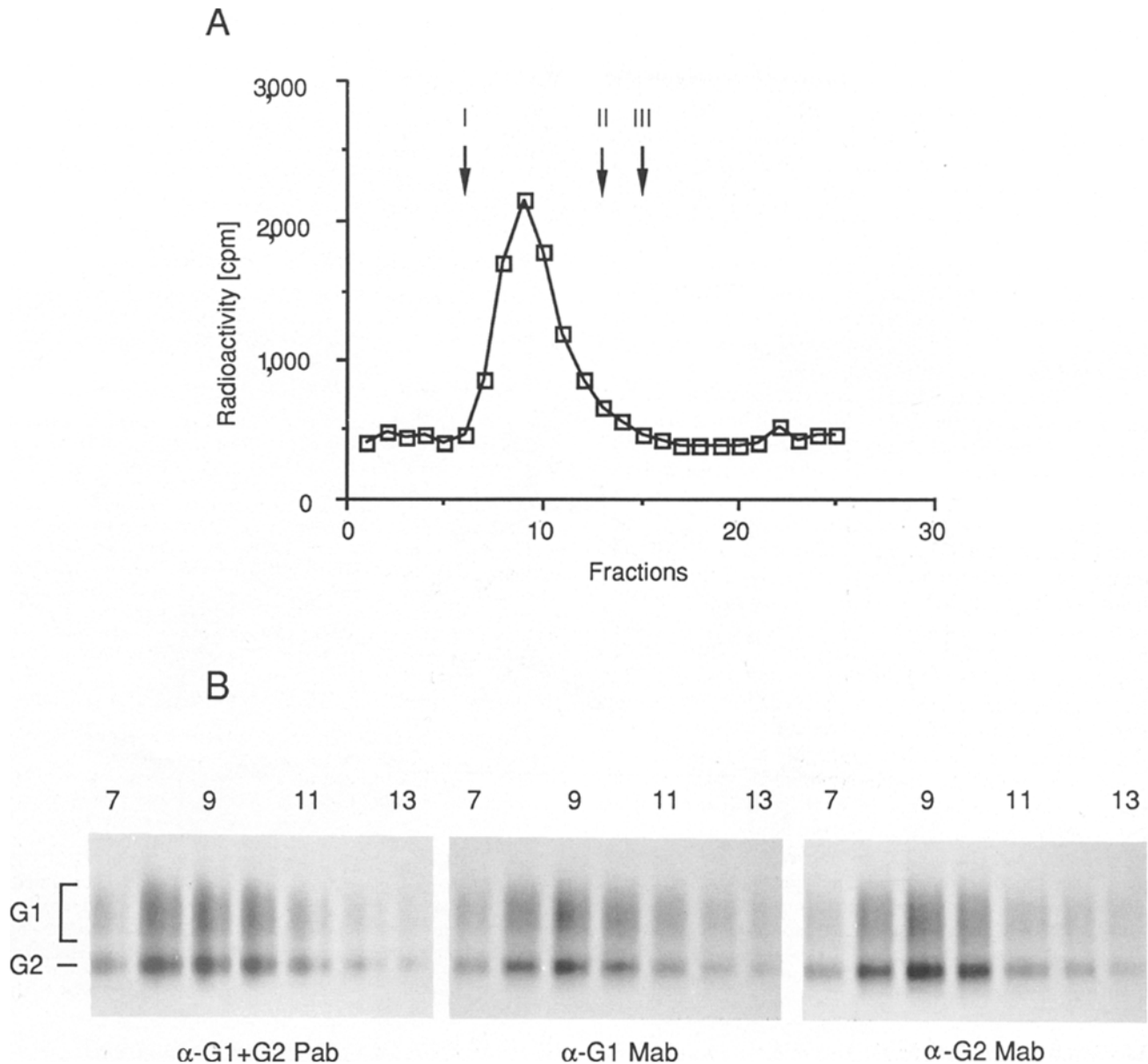
### Antisera

The preparation and characterization of a polyclonal antiserum against both G1 and G2 (G1 + G2) have been described by Kuismanen et al. (27), one set of monoclonal antibodies to G1 and G2, used in analysis of the virion glycoproteins (Fig. 1 B) has been described previously (28).

Preparation of another set of G1- and G2-specific monoclonals, used to immunoprecipitate the complex between the proteins (Fig. 6), was carried out using standard procedures. The preparation of the polyclonal antiserum to G1 and to G2 has been described by Wikström et al. (53); monoclonal antibody to BiP (immunoglobulin heavy chain binding protein) was kindly provided by Dr. D. Bole (Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI), and Dr. L. Hendershot (University of Alabama at Birmingham, AL); and polyclonal antibodies to PDI (protein disulfide isomerase) were kindly provided by Dr. R. Myllylä (University of Oulu, Finland).

### Immunoprecipitation

Aliquots of solubilized cells were incubated with preimmune sera or non-relevant ascites fluid in the presence of 10% Pansorbin (suspension of formaldehyde-fixed and heat-inactivated *Staphylococcus aureus* cells) for 60 min on a rotating device at  $6^\circ\text{C}$ , to reduce nonspecific binding during immunoprecipitation. The amount of nonrelevant antibodies and Pansorbin was the same as that for the components during immunoprecipitation. After clearing the samples from the bacteria, relevant antibodies were added in excess to the samples, which were incubated for 3 h at  $6^\circ\text{C}$ . Pansorbin



**Figure 1.** Analysis of the state of oligomerization of G1 and G2 in virions. (A) Purified [ $^{35}\text{S}$ ]methionine-labeled Uukuniemi virus particles were treated with 1% Triton X-100 followed by fractionation on a 5–20% sucrose gradient. The relative sedimentation of the labeled proteins was compared with that of three different proteins, rabbit IgG (I), bovine serum albumin (II), and ovalbumin (III), which have sedimentation values of 7.1S, 4.7S, and 3.7S, respectively. (B) Autoradiograms from immunoprecipitation analyses of the peak fractions (7–13) performed using the indicated antibodies.

(10%) was added in relation to the amount of antibodies used (10% Pansorbin binds  $\sim 2$  mg IgG/ml suspension) and the samples were mixed for 60 min at 6°C. The bacteria were then pelleted, washed once with 500 ml NET-buffer (10 mg/ml NP-40, 400 mM NaCl, 5 mM EDTA, 0.2 mg/ml  $\text{NaN}_3$ , 100 IU/ml Trasylol in 50 mM Tris-HCl [pH 8.0]) and then with 500 ml 10 mM Tris-HCl (pH 8.0). Finally, the cells were resuspended in 35  $\mu\text{l}$  sample buffer (83 mM Tris-HCl [pH 8.8], 100 g/liter sucrose, 30 g/liter SDS, 0.1 g/liter bromophenol blue, 4 mM EDTA), and heated at 95°C for 3 min. When samples were to be reduced, 8 mM dithiothreitol was included in the sample buffer. After cooling, the bacteria were removed by centrifugation and 5 ml 0.5 M iodoacetamide was added to samples that had been reduced. The supernatants were analyzed by SDS-PAGE.

### SDS-PAGE

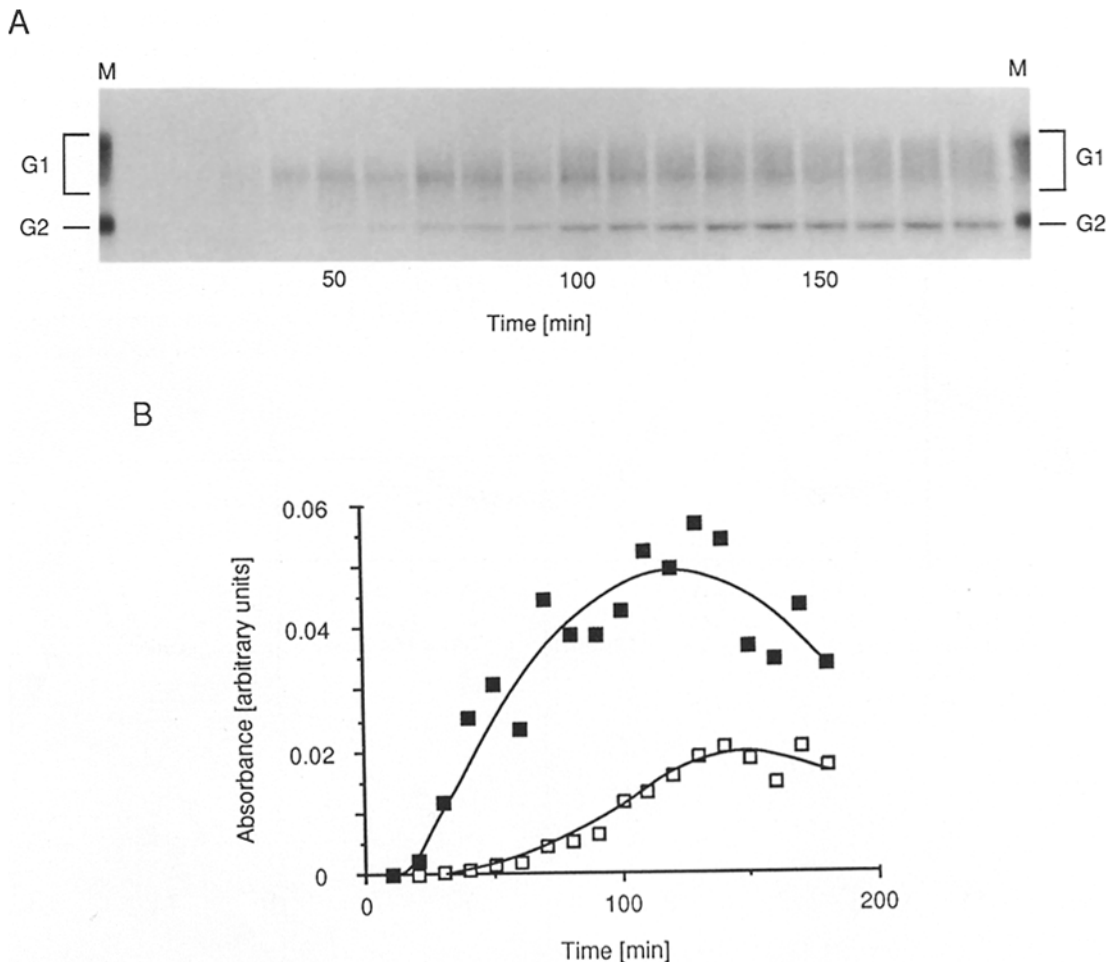
Slab gels (200  $\times$  200  $\times$  1.0 mm) with 10–15% polyacrylamide gel gradients were prepared essentially as described by Maizel (32). After electrophore-

sis, the gels were treated with EN $^3$ HANCE according to the manufacturer's recommendations (DuPont Co., Wilmington, DE), soaked in water, dried, and put on Kodak X-omat AR films. The relative amounts of radioactivity in the bands were determined by densitometric scanning of the films using Gelscan XL (LKB-Pharmacia).

### Results

#### *G1 and G2 Are Present as Heterodimers in Virus Particles*

G1 and G2 are found in approximately equimolar amounts in virions (26, 51). For most viruses, it has been shown that viral spike proteins form oligomeric structures (dimers, trimers, or tetramers) (19). To analyze the possibility that G1



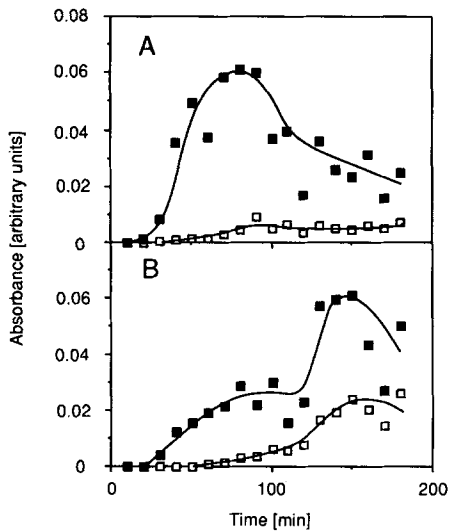
**Figure 2.** Kinetics of incorporation of newly synthesized G1 and G2 into virus particles. (A) Uukuniemi virus-infected BHK-21 cells were pulse labeled with [<sup>35</sup>S]methionine for 10 min and chased for 180 min. Every 10 min of the chase, the medium was collected and replaced by new medium. Virus particles were isolated from the collected media by centrifugation and analyzed by SDS-PAGE under nonreducing conditions. Markers (M) for G1 and G2 are shown. (B) The protein bands were quantitated by densitometric scanning of the autoradiogram and the amounts of G1 (■) and G2 (□) plotted as a function of time.

and G2 also form complexes, Uukuniemi virus particles homogeneously labeled with [<sup>35</sup>S]methionine, were solubilized with Triton X-100 and fractionated on a 5–20% sucrose gradient in the presence of the detergent. A single peak of radioactivity containing the viral glycoproteins was recovered sedimenting at 6.0S relative to the marker proteins IgG, BSA and ovalbumin (Fig. 1 A). In a separate experiment, the same S value was obtained using the monomeric and trimeric forms of the VSV G membrane protein as markers (7) (data not shown). The observed S value is consistent with a dimeric structure of G1 and G2. The ribonucleoproteins sedimented to the bottom of the gradient under the conditions used here. The glycoproteins in fractions 7–13 were immunoprecipitated with either a polyclonal G1/G2 antiserum, or with G1- and G2-specific monoclonal antibodies (27, 29) and analyzed by SDS-PAGE. With each of the monoclonal antibodies, both glycoproteins were precipitated in the same ratio as with the polyclonal antiserum. Thus, G1 and G2 are present as heterodimers in virions. We have so far been unable to obtain evidence for the presence of higher order oligomers by using several different crosslinkers (Persson, R., unpublished results).

### *G1 Is Incorporated Faster Than G2 into Virions*

Previous preliminary results indicated that newly synthesized G1 was incorporated into virus particles somewhat faster than G2 (26). In addition, by using pulse labeling followed by the addition of monensin (that blocks further virus formation) 45 min later, we found only labeled G1 in extracellular virus particles, whereas G2 was unlabeled (29). Since virus particles are formed in the GC, the kinetics of incorporation of G1 and G2 into virions can be taken as an indirect measurement for the rate of transport of the glycoproteins from the ER to the GC.

To confirm and extend our previous results, infected BHK-21 cells were pulse labeled for 10 min with [<sup>35</sup>S]methionine followed by chase periods of up to 3 h. Extracellular virus was collected at 10-min intervals, the virus concentrated, and the kinetics of incorporation of G1 and G2 analyzed by SDS-PAGE (Fig. 2 A). The relative intensities of the glycoprotein bands were determined by densitometric scanning of the autoradiogram (Fig. 2 B). As shown in Fig. 2, A and B, labeled G1 was incorporated into virions ~30–45 min faster than G2. The  $t_{1/2}$  of the incorporation of G1 and G2 into ex-



**Figure 3.** Effect of cycloheximide on the incorporation of newly synthesized G1 and G2 into virus particles. Experiments were performed as described in the legend to Fig. 1. (A) The cells were either chased in the presence of cycloheximide (50 µg/ml) or (B) pretreated with the drug for 3 h before the pulse. The amounts of radioactive G1 (■) and G2 (□) in the isolated virus particles, from medium taken at the indicated times, were plotted as a function of time.

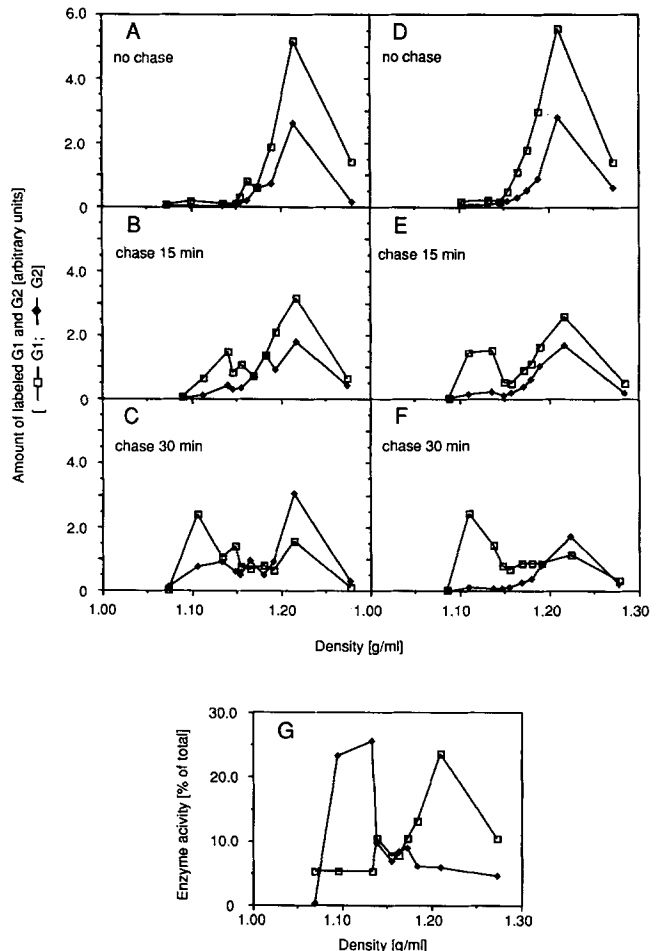
tracellular virus was estimated to be ~45 and 90 min, respectively. The ratio of labeled G1 to G2 was about 10 in virus particles collected at 60–70 min after the pulse. It thereafter gradually declined to ~3 at 90–100 min and 2 at 150–160 min. The ratio of methionine residues present in G1 and G2 is 1.7 as determined from the deduced amino acid sequences (43). A ratio close to this value was observed shortly after the pulse in intracellular glycoproteins immunoprecipitated with a polyclonal G1/G2 antiserum.

In the above experiment, protein synthesis was allowed to continue throughout the chase. If cycloheximide was added immediately after the pulse to inhibit further protein synthesis, the difference in kinetics of incorporation of G1 and G2 increased even further. Under these conditions, the G1/G2 ratio was ~6 at 90–100 min, 5 at 150–160 min, and 3 at 180–190 min after the pulse. As shown in Fig 3 A, very little labeled G2 was found in virions, whereas labeled G1 was readily incorporated. This indicated that for efficient transport of G2 to occur from the ER to the GC, continuous synthesis of glycoproteins was required. To further elucidate this, infected cells were pretreated for 3 h with cycloheximide to chase out as much of the glycoproteins as possible from the ER. Protein synthesis was then allowed to recover for 40 min in the absence of cycloheximide (21) before a 10-min radioactive pulse and chase periods of up to 3 h. Extracellular virus was again analyzed as above. The incorporation of G1 into particles now showed a biphasic pattern. One smaller peak was observed at 80–100 min, and a larger one at 120–160 min. In contrast, very little labeled G2 was incorporated into particles during the first 100 min. The peak of G2 coincided with the second G1 peak. Based on these results we postulated the following scenario. G1 and G2 are dependent on each other for transport to the GC and could perhaps dimerize already in the ER. If so, then newly

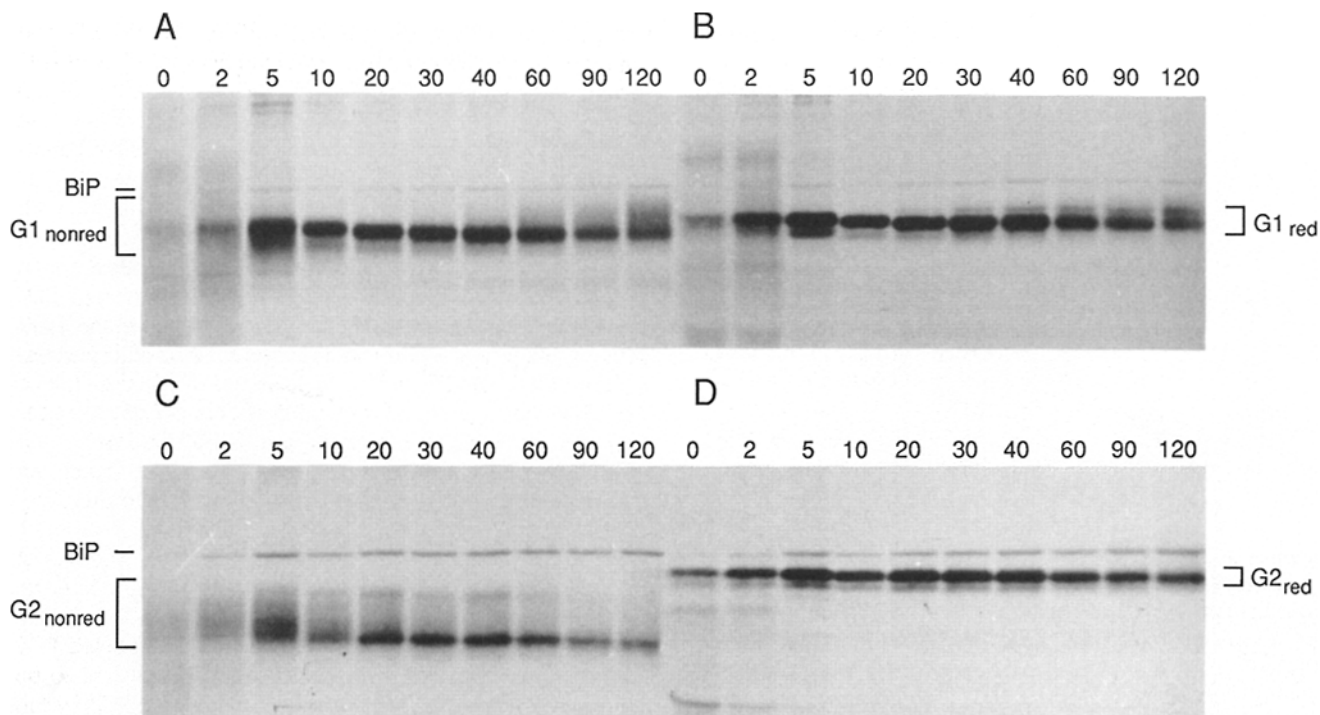
synthesized G1 (labeled) might complex with G2 made before the pulse (unlabeled), whereafter the dimer would be transported to the GC. In a pulse-chase experiment this would be scored as an apparent faster transport of G1. Our next experiments were designed to test this hypothesis.

### **Analysis of the Transport of G1 and G2 by Subcellular Fractionation**

To obtain a more direct measurement of the kinetics of transport of G1 and G2 to the GC, we pulse-labeled infected cells for 10 min, chased for 0, 15, and 30 min either in the presence or absence of cycloheximide and then fractionated the



**Figure 4.** Intracellular transport of proteins G1 and G2. Virus-infected BHK-21 cells were pulse labeled for 10 min with [<sup>35</sup>S]methionine and either harvested directly after the pulse (A and D), or chased for 15 min (B and E), or 30 min (C and F). The chase periods were performed either in the absence (A–C) or the presence (D–F) of cycloheximide (50 µg/ml). The cells were then homogenized, subjected to equilibrium density centrifugation in sucrose gradients, and fractionated. After fractionation, the membranes were pelleted and solubilized and the amount of G1 and G2 in each fraction was determined by immunoprecipitation and SDS-PAGE followed by densitometric scanning of the protein bands. G demonstrates the distribution of NADPH-cytochrome c reductase activity (□) (36) (a marker for ER-derived membranes) and the activity of galactosyl transferase (◆) (4) (a marker for *trans*-Golgi compartment) run in a parallel sucrose gradient.



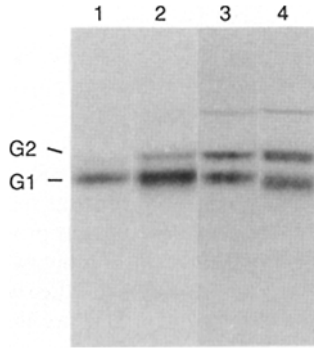
**Figure 5.** Formation of disulphide bonds in G1 and G2. Virus-infected cells were pulse-labeled with [<sup>35</sup>S]methionine for 2 min and chased for 0, 2, 5, 10, 20, 30, 40, 60, 90, and 120 min. After the chase, the cells were incubated on ice with 20 mM N-ethylmaleimide for 10 min and subsequently detergent solubilized. The cell lysates were immunoprecipitated with antiserum specific to either G1 (A and B) or G2 (C and D) and analyzed by SDS-PAGE under nonreducing (A and C) and reducing (B and D) conditions.

ER and GC membranes on sucrose gradients. The membranes in each fraction were then pelleted and immunoprecipitated with antiserum against G1 or G2. The precipitated proteins were analyzed by SDS-PAGE and the G1 and G2 bands quantitated by densitometric scanning. As shown in Fig. 4 (G), the ER and Golgi membranes were well separated as determined by using a Golgi enzyme (Galactosyl transferase) and an ER enzyme (NADPH cytochrome c reductase) as markers. Both in the absence (Fig. 4, A-C) and the presence (Fig. 4, D-F) of cycloheximide, G1 and G2 were found in the ER fraction ( $P = 1.17$ - $1.28$  g/ml) immediately after the pulse. In the absence of cycloheximide, ~35% of G1 was found in the Golgi fraction ( $P = 1.07$ - $1.16$  g/ml) already at 15 min (Fig. 4 B) and more pronounced at 30 min (~60%) (C). At 15 min, <20% of G2 had reached the GC. By 30 min, this figure increased to only about 30%. In the presence of cycloheximide (Fig. 4, D-F), the results were even clearer. During the chase, G1 was transferred to the GC efficiently and with the same kinetics as in the absence of the drug, whereas hardly any G2 was recovered in the Golgi fraction. In particular this latter result is in good agreement with the results presented in Fig. 3 A showing that during the first 80 min almost no labeled G2 was incorporated into virions.

#### **Disulfide Bonds Are Formed Faster in G1 Than in G2**

One explanation for the above results could be that G1 matures into a dimerization competent form faster than G2. Disulfide bond formation is an important step in stabilizing the folded conformation of proteins (11, 12). To analyze whether disulfide bonds were formed with different kinetics

in G1 and G2 we used an analytical procedure recently applied to study the folding of prolactin (20) influenza virus HA (3) and VSV G proteins (31). Infected cells were pulsed for only 2 min with [<sup>35</sup>S]methionine followed by chases ranging from 2 to 120 min. Before solubilization, cells were treated on ice with N-ethylmaleimide to prevent free sulfhydryl groups from forming disulfide bonds after disruption of the cells. The proteins were then immunoprecipitated and analyzed by SDS-PAGE under nonreducing (Fig. 5, A and C) and reducing conditions (Fig. 5, B and D). Immediately after the pulse, the G1 antiserum precipitated heterogeneous products migrating both slower and faster than the mature G1 marker (Fig. 5 A). The latter products probably represent unfinished, nascent polypeptide chains, whereas the former are likely to represent completely synthesized chains that have not yet formed the correct disulfide bonds. Such molecules are presumed to have a more extended conformation and thus slower mobility than molecules with all disulfide bonds formed correctly and a more compact structure (20, 31). Already after a 5-min chase, most G1 had acquired a mobility similar to that of G1 found in virions. A gradual small increase in the mobility of G1 was observed during the first 40 min, probably reflecting the trimming of the N-linked glycans (26, 38). From ~60 min on the G1 band became more diffuse due to sialylation (14, 26). As expected, G1 had the same mobility throughout the chase when analyzed under reducing conditions (Fig. 5 B). The difference in mobility of G1 analyzed under reduced and nonreduced conditions was small, suggesting only a minor effect of disulfide bond formation on the conformation of G1. In contrast, G2 displayed a quite different pattern. Heterogeneous products



**Figure 6.** Newly synthesized G1 dimerizes with old G2. Virus-infected cells were labeled for 2 min with [<sup>35</sup>S]methionine and chased for 10 min (lanes 1 and 3) or 30 min (lanes 2 and 4). Detergent-solubilized lysates were immunoprecipitated with either a monoclonal antibody against G2, known to precipitate the complex between G1 and G2 (lanes 1 and 2) or a mixture of G1- and G2-specific antisera (lanes 3 and 4). Electrophoresis was under reducing conditions.

migrating more slowly than mature G2 were seen during the first 60 min of the chase. The proportion of these products decreased during the chase. A band migrating at the position of mature G2 was observed from ~20 min on and appeared with a  $t_{1/2}$  of ~30–45 min. Under reducing conditions G2 had the same mobility during the whole chase. The difference between the mobility of G2 analyzed under reducing and nonreducing conditions was much greater than for G1, suggesting an important role for disulfide bonds in the creation of the mature conformation. As discussed below, BiP coprecipitated with G1, and in particular with G2, in all cases. We interpret these results to mean that the disulfide bonds are formed much faster (within 10 min) in G1 than in G2. For G2, it appears that a portion of the molecules have not matured even after 60–90 min.

#### Newly Synthesized G1 Dimerizes with “Old” G2

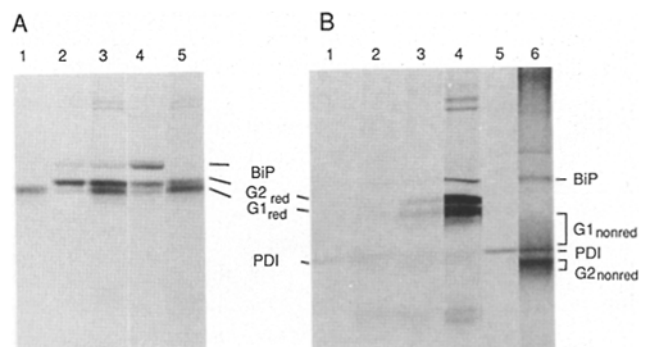
The results presented above suggested that G1 folds more rapidly than G2. If only properly folded G1 and G2 can form dimers, one consequence of this would be that newly made G1 (labeled) is able to dimerize only with a mature G2 (unlabeled) made before the pulse. To test this, cells were pulse labeled for 2 min and chased for either 10 or 30 min followed by immunoprecipitation with a G2-specific monoclonal antibody known to precipitate the heterodimeric complex. A portion of the same lysate was precipitated with polyclonal G1/G2 antiserum. The samples were reduced and alkylated before analysis by SDS-PAGE. This procedure results in sharp bands and good separation between G1 and G2. As shown in Fig. 7 A (lanes 2 and 3), the order of migration is, however, reversed compared to the situation in Figs. 1 and 2. After a 10-min chase, the G2-specific monoclonal antibody precipitated exclusively labeled G1, whereas G2 was unlabeled (Fig. 6, lane 1). Even after the 30-min chase, more label was recovered in G1 than in G2 (Fig. 6, lane 2). The polyclonal antibody precipitated equal amounts of G1 and G2 at both time points (Fig. 6, lanes 3 and 4). Thus, shortly after the pulse unlabeled G2 was complexed to labeled G1 in conformity with the model proposed above.

#### BiP and PDI Coprecipitate with the Glycoproteins

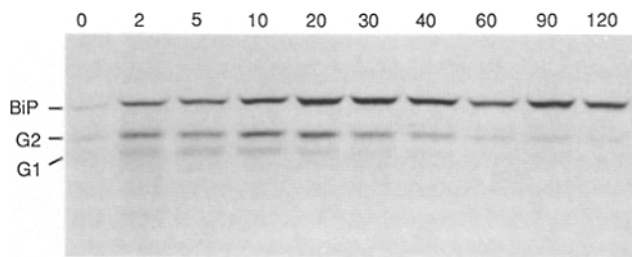
During the course of our studies, we consistently observed

two proteins with molecular mass of 70 and 54 kD that coprecipitated with our glycoprotein antibodies. The 70-kD protein can readily be seen in Fig. 5, A–D, while the 54-kD protein is difficult to detect after short pulse-labelings and because it comigrated with G2 under nonreducing conditions (Fig. 7 B, lane 6). The two proteins were identified as BiP and PDI, respectively, by immunoprecipitation with specific antibodies. To further analyze these associations, cells were labeled for 10 min with [<sup>35</sup>S]methionine and then immunoprecipitated with anti-G1/G2, anti-BiP, or anti-PDI antisera. As shown in Fig. 7 A, the anti-G2 and anti-G1/G2 antisera precipitated BiP (lanes 2 and 3). Anti-BiP precipitated both G1 and G2, with a preference for G2 (Fig. 7, lane 4). When the supernatant remaining after anti-BiP precipitation was reprecipitated with the anti-G1/G2 antiserum, no BiP was found to coprecipitate (Fig. 7, lane 5), indicating that glycoproteins associated with BiP had been quantitatively removed by the anti-BiP antiserum.

As shown in Fig. 7 B (lanes 1–3), increasing amounts of antibodies to PDI were able to precipitate G1 and G2 (lane 3). Efficient coprecipitation of pulse-labeled PDI with G1 and G2 was hampered by the large pool of unlabeled PDI present in the ER. Large amounts of antiserum had to be used, resulting in the partial displacement of the PDI by the reduced IgG (Fig. 7 B, lanes 1–3). Furthermore, PDI run under reduced conditions had a fuzzy appearance (Fig. 7 B, lanes 1 and 2), whereas nonreduced conditions gave a much sharper band (lanes 5 and 6). Newly synthesized G1 and G2 also migrated as fuzzy bands under nonreduced conditions (Fig. 7 B, lane 6). Coprecipitation of PDI (and BiP) with anti-G1/G2 antiserum could best be demonstrated if the samples were analyzed under nonreducing conditions. Thus, we conclude that the viral glycoproteins are complexed to both BiP and PDI. The association of BiP is much more pronounced to G2 (Fig. 5, C and D) than to G1 (Fig. 5, A and B) (Fig. 7 A, lane 4).



**Figure 7.** G1 and G2 associate to BiP and PDI. A lysate of virus-infected cells, labeled with [<sup>35</sup>S]methionine for 10 min was used in immunoprecipitation analyses with different antibodies (all polyclonal antibodies except the  $\alpha$ -BiP, which is a monoclonal one). (A) Lane 1,  $\alpha$ -G1; 2,  $\alpha$ -G2; 3,  $\alpha$ -G1 +  $\alpha$ -G2; 4 and 5, first precipitation with  $\alpha$ -BiP (4) and then with  $\alpha$ -G1 +  $\alpha$ -G2 (5). (B) Lanes 1–3, increasing amount of  $\alpha$ -PDI. Lanes 2, 6, and 10  $\mu$ l antiserum; 4,  $\alpha$ -G1 +  $\alpha$ -G2; 5,  $\alpha$ -PDI; 6,  $\alpha$ -G1 +  $\alpha$ -G2. Note that the samples in B, lanes 5 and 6 were analyzed under nonreducing conditions, whereas all the other samples were reduced.



**Figure 8.** Transient binding of G1 and G2 to BiP. Virus-infected cells were labeled with [<sup>35</sup>S]methionine for 2 min and chased for 0, 2, 5, 10, 20, 30, 40, 60, 90, and 120 min. Cells were detergent solubilized, the lysates immunoprecipitated with  $\alpha$ -BiP, and then analyzed by SDS-PAGE under reducing condition. The mobility of G1 and G2 is indicated.

### ***BiP Is Transiently Associated with G2 for a Longer Time Than with G1***

To study the possible transient nature of the BiP association to G1 and G2, infected cells were pulse labeled for 2 min, followed by chases up to 120 min. The lysates were then subjected to immunoprecipitation with anti-BiP antiserum. As shown in Fig. 8, the BiP antiserum coprecipitated both G1 and G2. Coprecipitation of G1 was observed during the first 20 min of the chase with a peak at 5–10 min. In contrast, G2 could be coprecipitated during the whole 120 min chase. However, the amount of coprecipitating G2 decreased rapidly after 40 min, reaching a low stable level between 60 and 120 min after the pulse. Lack of BiP antiserum has precluded further analysis of whether BiP is associated with the immature forms of G2 (see Fig. 5 C). However, the results further strengthen the conclusion that G2 folds more slowly than G1.

### ***Discussion***

The early steps after translocation of nascent polypeptide chains into the lumen of the ER have recently been the subject of intense studies. These steps include core glycosylation, folding, disulfide bond formation, assembly of oligomers, as well as other posttranslational modifications characteristic for individual proteins. From these studies it has become clear that proteins undergo different maturation steps that result in structures competent to exit from the ER. A “quality control” system (19), or “architectural editing” (24) seems to operate to ensure that only correctly folded and oligomerized membrane proteins may leave the ER (19). For soluble proteins, the requirement for oligomerization for the exit from the ER may be less stringent (49).

Here we have studied the synthesis, folding, oligomerization, and intracellular transport of two membrane glycoproteins, G1 and G2, of Uukuniemi virus, a member of the bunyavirus family. These proteins form the surface projections (spikes) on the virus particles (51). Our results can be summarized as follows. (a) Newly synthesized G1 is transported from the ER to the GC  $\sim$ 30–45 min faster than G2. This result was obtained either by analyzing the incorporation of newly synthesized (pulse-labeled) proteins into extracellular virions maturing in the GC, or by following the intracellular transport by subcellular fractionation. Pretreatment of cells with cycloheximide before the pulse chase, or chasing in the presence of the drug, clearly indicated that efficient transport to the GC of G2 required continuous syn-

thesis of G1. In other words, G1 and G2 are dependent on each other for transport to the GC. (b) Correct disulfide bond formation occurred much faster for G1 ( $t_{1/2}$  <10 min) than for G2 ( $t_{1/2}$   $\sim$ 30–45 min). (c) G1 and G2 were found only as heterodimers in virions. Newly synthesized G1 oligomerized with a G2 synthesized before the pulse. (d) Both BiP and PDI could be coprecipitated with G1/G2, suggesting a possible role for these proteins in the folding process.

Our initial results pointed to the possibility that G1 and G2 are transported independently of each other from the ER to the site of virus budding in the GC. This was an interesting hypothesis, since oligomerization of various membrane proteins, viral or cellular, have been found to occur without exceptions already in the ER (19). Our later results clearly indicated that this hypothesis was incorrect. Instead, we interpret our results in the following way. Heterodimerization of G1 and G2 occurs shortly after synthesis in the ER. Because G1 folds much faster than G2, newly synthesized G2 is unable to dimerize with newly synthesized G1, i.e., its own pair derived from the same p110 precursor. Instead, newly made G1 dimerizes with a mature, correctly folded, and unlabeled G2 made before the radioactive pulse. This was confirmed by immunoprecipitation with a G2-specific monoclonal antibody recognizing the heterodimers. Shortly after the pulse, heterodimeric complexes of labeled G1 and unlabeled G2 could be precipitated with the G2-specific antibody. After heterodimerization, the complexes are then transported to the GC, where they are incorporated into budding virions. In a pulse-chase experiment this means that newly synthesized G1 is scored as being transported faster than G2 to the GC.

Different rates of transport from the ER to the GC of both membrane proteins (10, 34) and secretory proteins (13, 30) have been observed. The molecular basis for these different rates has remained obscure. Several possibilities have been suggested: (a) positively acting transport signals with different affinities to some hypothetical ER receptors that would carry the proteins to the GC (13, 30); (b) a leaky retrieval of proteins from an intermediate compartment located between the ER and the GC; (c) retention of proteins in the ER by binding to resident ER proteins with different affinities. This could also include selective retention of unfolded, immature, or unassembled proteins. Lack of retention would result in rapid transport by a default mechanism via bulk flow (19, 23, 24, 42, 46). Evidence is rapidly accumulating in support of the retention model. Proteins in the process of folding are found transiently bound to BiP, a resident ER protein, while misfolded proteins may be stably associated with BiP and eventually degraded in the ER (see below). Our results support the notion that the rate of folding as reflected by disulfide bond formation and dimerization are important rate-limiting steps for exit from the ER.

Correct disulfide bond formation in the ER is thought to be an enzymatic process catalyzed by PDI. Disulfide bonds stabilize the folded conformation of proteins. During the folding process, PDI is assumed to break up incorrectly formed bonds and to catalyze the formation of the correct ones leading to the mature, correctly folded three-dimensional structure (11, 12). Results obtained with various model proteins indicate that the rate of folding varies between different proteins (19). The rate of  $\beta$ -chain folding and intrachain disulfide bond formation of glycoprotein hormones is, for example, the rate-limiting step in the  $\alpha$ - $\beta$  dimerization



(48). Likewise folding and trimerization of different procolagagens vary considerably (22). We estimated the rate of disulfide bond formation of G1 and G2 from the mobility shifts of pulse-chased labeled alkylated proteins on a non-reducing polyacrylamide gel. We found that G1 acquired a mobility similar to that of the mature protein already within 10 min, while G2 obtained its final mobility slowly over a period of up to 60 min with a half-time of  $\sim 30$ –45 min. This latter rate should be compared to that of VSV G protein, which acquires its fully disulfide-bonded form with a  $t_{1/2}$  of 2–3 min. Our finding of different rates of disulfide bond formation for G1 and G2 is interesting considering that their general structure is very similar. Both proteins have the same size ( $\sim 480$  amino acids), four N-linked glycans, and an identical high number of 26 cysteine residues in their ectodomains (43). Thus, one would expect a similar rate of folding. VSV G protein and influenza virus HA both have 12 cysteine residues in their ectodomains. Both proteins fold within minutes and trimerize with  $t_{1/2}$ s of 5–10 min (5–8, 16, 25). Since the same number of disulfide bonds have to be formed in G1 and G2, apparently other properties of their primary structures are responsible for the different rates of folding.

We also obtained evidence that BiP and PDI are associated with newly synthesized G1 and G2. BiP has been found to associate transiently to newly synthesized proteins during the folding process, or stably to misfolded proteins (1, 3, 9, 20, 31, 35, 37, 47). The role of BiP in the folding process is still somewhat unclear. BiP may assist in the folding process itself; prevent unfolded, immature proteins from aggregating; or prevent aggregated, misfolded proteins from exiting the ER (19, 37, 46). We found that BiP coprecipitated more strongly with an antiserum against G2 than with one against G1. Conversely, BiP antiserum coprecipitated G2 more efficiently than G1. Furthermore, BiP remained transiently associated with newly synthesized G2 for a longer time than with G1. These results are also in conformity with the conclusion that G2 folds slower than G1. The finding that PDI could also be coprecipitated with G1/G2 was unexpected, since such coprecipitations without cross-linking have not to our knowledge been reported before. Cross-linking of PDI to newly synthesized IgG has, however, been reported, suggesting a direct role of PDI in protein folding (45). PDI is a dominant protein in the ER. In addition, PDI has a very slow turnover. Thus, during the 2–10 min radioactive pulses used here, only a minor fraction of the PDI pool becomes labeled. Therefore detection of labeled PDI in the coprecipitates was difficult and studies on the possible transient nature of PDI binding has so far not been possible.

We believe that our model system offers some clear advantages in comparison to many other systems. It allows the simultaneous analysis of the maturation of two structurally similar membrane glycoproteins that have very different folding properties and are synthesized synchronously from a common precursor.

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