JOURNAL OF VIROLOGY, June 1995, p. 3441–3448 Vol. 69, No. 6
0022-538X/95/$04.00+0

The Small Envelope Glycoprotein (G_S) of Equine Arteritis Virus Folds into Three Distinct Monomers and a Disulfide-Linked Dimer

ANTOINE A. F. DE VRIES,* MARTIN J. B. RAAMSMAN, HENK A. VAN DIJK, MARIAN C. HORZINEK, AND PETER J. M. ROTTIER

Institute of Virology, Department of Infectious Diseases and Immunology, Veterinary Faculty, Utrecht University, 3584 CL Utrecht, The Netherlands

Received 15 August 1994/Accepted 28 February 1995

The small membrane glycoprotein (G_S) of equine arteritis virus (EAV) is a minor virion component but is abundantly expressed in EAV-infected cells. In this study, we have analyzed its membrane topology, folding, oligomerization, and intracellular transport. We show that G_S is a class I integral membrane protein with one functional N-glycosylation site. Gel electrophoresis under nonreducing conditions revealed that G_S occurs in EAV-infected cells in four monomeric conformations and as disulfide-linked homodimers. The slowest-migrating monomeric form corresponded to the fully reduced G_S protein; the three faster-migrating monomeric species are probably generated by the formation of alternative intrachain disulfide bonds between the three luminal cysteines in the molecule. The G_S monomers were selectively retained in the endoplasmic reticulum, as judged by their permanent susceptibility to endoglycosidase H, whereas the G_S dimers were specifically incorporated into virus particles and became endoglycosidase H resistant and sialylated during passage through the Golgi apparatus.

Equine arteritis virus (EAV) is the type species of a group of small, enveloped, positive-stranded RNA viruses, provisionally called arteriviruses, which also includes lactate dehydrogenase-elevating virus, porcine reproductive and respiratory syndrome virus, and simian hemorrhagic fever virus (11, 20, 34). These intracellularly budding viruses (32, 36, 42, 46) resemble togaviruses in their physicochemical properties and virion morphology but are similar to coronaviruses with respect to genome organization, replication mechanism, and gene expression strategy (20, 41).

The virions of EAV consist of an isometric core particle of approximately 35 nm which is surrounded by an envelope with tiny surface projections (21, 32). EAV contains a unique set of four structural proteins: a 14-kDa phosphorylated nucleocapsid protein (N), a 16-kDa unglycosylated membrane protein (M), a 25-kDa N-glycosylated membrane protein (G_S), and a 30- to 42-kDa N-glycosylated membrane protein (G_L) (14, 24, 47). The N protein is encoded by open reading frame (ORF) 7 and encapsidates the 12.7-kb polyadenylated RNA genome; the M, G_L, and G_S proteins are encoded by ORFs 6, 5, and 2, respectively (14). The envelope glycoproteins both possess one N-glycosylation site, but the processing of their oligosaccharide side chains during the vesicular transport of virions proceeds entirely different. The G_L protein becomes heterogeneously glycosylated with *N*-acetyllactosamine in a cell-type-specific manner (15), whereas the G_S protein always occurs as a single distinct species (13). The N, M, and G_L proteins are abundantly expressed in EAV-infected cells and incorporated into virus particles in large amounts. The G_S protein, in contrast, is only a minor virion constituent, although its expression level in virus-infected cells is comparable to that of the M protein (14). The remarkable underrepresentation of the G_S protein in virions led us to investigate the intracellular fate of this protein.

The incorporation of viral membrane proteins into virus particles typically depends on their conformational maturation within the endoplasmic reticulum (ER), which involves the folding of the individual molecules and their assembly into oligomeric complexes (23, 37). Only proteins that have acquired the proper tertiary and quaternary structure are transported along the secretory pathway. The specific folding requirements may thus be of critical importance for the rate and efficiency with which a protein emerges from the ER (7, 17, 31, 33). To facilitate the folding and oligomerization of polypeptides, the ER accommodates a number of folding enzymes and molecular chaperones (reviewed by Gething and Sambrook [19]). Incorrectly and incompletely folded or assembled polypeptides usually do not exit the ER but are either stably retained (3, 22, 28) or selectively degraded (5, 18, 30, 44). The mechanisms involved in ER retention of folding intermediates and misfolded proteins are not fully understood but may involve the formation of covalently and noncovalently linked aggregates and the association with ER resident chaperones including immunoglobulin heavy-chain-binding protein and calnexin (3, 10, 12, 17, 28, 35). The presence of free sulfhydryl groups within a protein may also contribute to its retention in the ER (18, 40).

We have determined the membrane topology of the EAV G_S protein and analyzed its folding, oligomerization, and intracellular transport. Our data reveal that G_S is a class I transmembrane glycoprotein which adopts multiple monomeric conformations. We further show that a fraction of the protein associates into disulfide-linked dimers. These dimers are specifically incorporated into virions and become sialylated during transport of virus particles out of the cell, while the G_S monomers are selectively retained in the ER and probably subjected to pre-Golgi degradation.

MATERIALS AND METHODS

Cells, viruses, and antibodies. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml (DMEM–10% FCS). The Bucyrus strain of EAV (16) was propagated in baby hamster kidney (BHK-

* Corresponding author. Mailing address: Institute of Virology, Department of Infectious Diseases and Immunology, Veterinary Faculty, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands. Phone: 31-30-532463/532485. Fax: 31-30-536723. Electronic mail address: T.Vries@vetmic.dgk.ruu.nl.

21) cells. The preparation of a concentrated virus stock has been described previously (14). The production and characterization of a rabbit antipeptide serum specific for the extreme carboxy terminus of the G_S protein has been reported before (14).

Radioactive labeling of intracellular proteins. Infection of BHK-21 cells with EAV was performed essentially as described by de Vries et al. (14) except that the cells were always incubated at 37°C. At 8 h postinfection (p.i.), the medium was removed and prewarmed methionine-free medium buffered with 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4) was added to the cells. After starvation for 30 min, the methionine-free medium was replaced by similar medium supplemented with L-^{35}S in vitro cell labeling mix (>1,000 Ci/mmol; Amersham), and the cells were labeled for the indicated time periods. After the labeling, the cells were placed on ice and washed two times for 1 min each with ice-cold phosphate-buffered saline (PBS) containing either 20 mM *N*-ethylmaleimide (NEM) or 50 mM iodoacetamide (IAA) as indicated in the figure legends. Alternatively, the pulse-labeling was followed by two rapid washes with prewarmed HEPES-buffered chase medium (DMEM–10% FCS containing 5 mM methionine), and the cells were then incubated in similar medium for different time periods. Wherever indicated, cycloheximide (0.5 mM) was added to the chase medium to block further protein synthesis. For the posttranslational reduction of intracellular proteins, the cells were incubated immediately after the labeling in chase medium containing 5 mM dithiothreitol (DTT) for 5 min. To allow reoxidation of the proteins, the cells were washed twice with chase medium and further incubated in such medium. The chases were terminated by two sequential washes with ice-cold PBS containing one of the aforementioned sulfhydryl alkylating agents. Next, the cells were solubilized in ice-cold lysis buffer (20 mM Tris-HCl [pH 7.6]–150 mM NaCl–1% Nonidet P-40 [NP-40]–0.5% sodium deoxycholate–0.1% sodium dodecyl sulfate [SDS] containing 1 μg of aprotinin, leupeptin, and pepstatin A per ml and 20 mM NEM or 50 mM IAA). The lysates were centrifuged for 20 min at $10^4 \times g$ and 4°C, the pellets were discarded, and the supernatants supplemented with EDTA to a final concentration of 5 mM. To simultaneously analyze both intra- and extracellular viral proteins, so-called total lysates were prepared. For this purpose, the medium was collected, mixed with 1/4 volume of 5× lysis buffer with 150 mM NaCl, and added back to the cells, which had been washed twice with PBS-NEM or PBS-IAA. The samples were further processed as described above.

Preparation of radiolabeled virions. Subconfluent monolayers of BHK-21 cells were infected with EAV at a multiplicity of infection of ≥20. After incubation for 8 h at 37°C, the medium was replaced by methionine-free medium, and the cells were starved for 30 min and subsequently labeled for 3 h with 500 μCi of L-^{35}S in vitro cell labeling mix per ml. At 11.5 h p.i., an excess of unlabeled methionine (5 mM) was added to the culture medium. The medium was harvested and clarified by low-speed centrifugation at 12.5 h p.i.

In vitro transcription. Ten micrograms of plasmid pAVI02 (14) was cut downstream of ORF 2 with *Eco*RI and treated with proteinase K (100 μg/ml; Boehringer Mannheim) for 30 min at 37°C in the presence of 0.5% SDS. The linearized plasmid DNA was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and once with water-saturated ether. It was subsequently ethanol precipitated and dissolved in water. In vitro transcription was performed by incubating 1 μg of the DNA template for 90 min at 37°C in a 20-μl reaction mixture containing 40 mM Tris-HCl (pH 7.5 at 37°C), 10 mM NaCl, 6 mM $MgCl_2$, 10 mM DTT, 2 mM spermidine, 100 μg of acetylated bovine serum albumin (BSA; New England Biolabs) per ml, 0.5 mM each ATP, CTP, GTP, and UTP, 1 U of RNasin (Promega) per μl, and 0.5 U of T7 RNA polymerase (Pharmacia) per μl. The DNA template was degraded by incubation of the transcription reaction with 1 U of RQ1 DNase (Promega) for 15 min at 37°C. The RNA transcripts were purified by phenol extraction and isopropanol precipitation and finally dissolved in water.

In vitro translation and proteinase K digestion. Four hundred nanograms of RNA transcript was translated for 1 h at 30°C in a 20-μl micrococcal nuclease-treated rabbit reticulocyte system (Promega) according to the vendor's instructions and using L-^{35}S in vitro cell labeling mix instead of [^{35}S]methionine. Where indicated, the in vitro translation system was supplemented with canine pancreatic microsomal membranes (3.2 equivalents per reaction; Promega). For protease protection assays, an 8-μl aliquot of the in vitro translation reaction was mixed on ice with 16 μl of 50 mM Tris (pH 7.6)–25 mM $CaCl_2$. Subsequently, the sample was split into four identical portions. The first aliquot was adjusted to a final volume of 10 μl with water, the second portion was supplemented with 2 μl water and 2 μl of proteinase K (2 mg/ml), and the third aliquot received 2 μl of the protease stock and 2 μl of 10% Triton X-100 (TX-100). The samples were then incubated for 60 min at 0°C. The protease was inactivated afterwards by addition of 2 μl of 12.5 mg of phenylmethylsulfonyl fluoride (PMSF) per ml–10 mg each of leupeptin and aprotinin per ml and incubation for 5 min at 0°C. To the fourth aliquot, 2 μl of proteinase K solution and 2 μl of protease inhibitors were added, and the sample was kept on ice for 5 min, supplemented with TX-100, and further incubated for 1 h at 0°C. Two microliters of each sample was taken for direct analysis; the rest was subjected to immunoprecipitation using the antipeptide serum.

Immunoprecipitation, PAGE, and fluorography. Crude protein samples were diluted in immunoprecipitation buffer (20 mM Tris-HCl [pH 7.6], 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 1 μg of protease inhibitors per ml) to a final volume of 1 ml. The samples were supplemented with 3 μl of rabbit serum and incubated overnight at 4°C. The next day, 25 μl of a 10% suspension of heat-inactivated group G *Streptococcus* sp. cells (Omnisorb; Calbiochem) was added, and the samples were incubated for ≥2 h at 4°C. The immune complexes were then collected by centrifugation and washed three times in 20 mM Tris-HCl (pH 7.6)–150 mM NaCl–5 mM EDTA–0.1% NP-40 and once in 20 mM Tris-HCl (pH 7.6)–0.1% NP-40. The resulting pellets were resuspended in 25 μl of Laemmli sample buffer with or without 20 mM DTT and incubated for 5 min at 96°C. After centrifugation for 3 min at $10^4 \times g$, the supernatants were analyzed in SDS-polyacrylamide (PAA) gels (29). Following PAA gel electrophoresis (PAGE), the gels were fixed in 10% acetic acid–10% methanol at room temperature for 15 min, impregnated with 2,5-diphenyloxazole (6), dried on Whatman 3MM paper, and exposed at −70°C to hypersensitized Fuji RX films.

Endoglycosidase treatment. Immunoprecipitates were resuspended in 400 μl of endoglycosidase buffer (50 mM sodium acetate [pH 5.5], 100 μg of BSA per ml, 100 μg of PMSF per ml, 1 μg each of aprotinin, leupeptin, and pepstatin A per ml) and split in two equal portions. One of the aliquots was digested overnight at 37°C with 2 mU of endoglycosidase H (endo H; Boehringer Mannheim) or with 20 mU of neuraminidase (from *Arthrobacter ureafaciens*; Boehringer Mannheim) as indicated in the text; the other portion was incubated under the same conditions but without enzyme to serve as a control. Subsequently, the samples were centrifuged for 3 min at $10^4 \times g$, and the pellets were resuspended in 25 μl of Laemmli sample buffer. Glycopeptidase F (glyco F) digestions were carried out similarly in 50 mM sodium phosphate (pH 7.0)–10 mM EDTA–100 μg of BSA per ml–100 μg of PMSF per ml–1 μg of protease inhibitors per ml. Per digestion 100 mU of endoglycosidase F/N-glycosidase F (Boehringer Mannheim) was used.

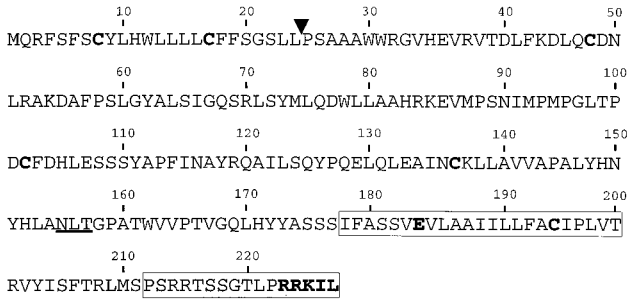

FIG. 1. Deduced amino acid sequence of the EAV G_S protein. The cysteine residues, the putative transmembrane glutamic acid residue, and the hypothetical carboxy-terminal ER retention signal are indicated in boldface; the single N-glycosylation site is underlined. The black triangle depicts the most likely signal sequence cleavage site as determined by the method of von Heijne (43). The white box indicates the putative membrane-spanning segment; the oligopeptide used to raise the G_S-specific antiserum is represented by a stippled box.

RESULTS

Membrane topology of the G_S protein. The position of the single N-glycosylation site (11) and the hydropathy profile (14), which reveals a potential signal sequence at the amino terminus and a possible membrane anchor near the carboxy terminus (Fig. 1), suggested that G_S is a class I ($N_{exo}C_{cyt}$) integral membrane protein. To establish the transmembrane orientation of G_S, in vitro transcripts encoding the protein were translated in a rabbit reticulocyte lysate in the absence and presence of microsomal membranes. Without microsomes, the translation yielded a polypeptide of 24 kDa that was recognized by the G_S-specific antipeptide serum (Fig. 2a). In the presence of microsomal membranes, the size of the product increased with about 2 kDa. To find out whether this increase in apparent molecular mass was exclusively caused by the addition of an N-linked oligosaccharide side chain or resulted from the combined effects of N glycosylation and the removal of a signal sequence, the core glycosylated ORF 2 product was treated with glyco F, which cleaves off all *N*-glycans. This reduced the size of the G_S protein to approximately 21 kDa, consistent with the presence of only one functional N-glycosylation site (14). Moreover, since the deglycosylated protein was smaller than

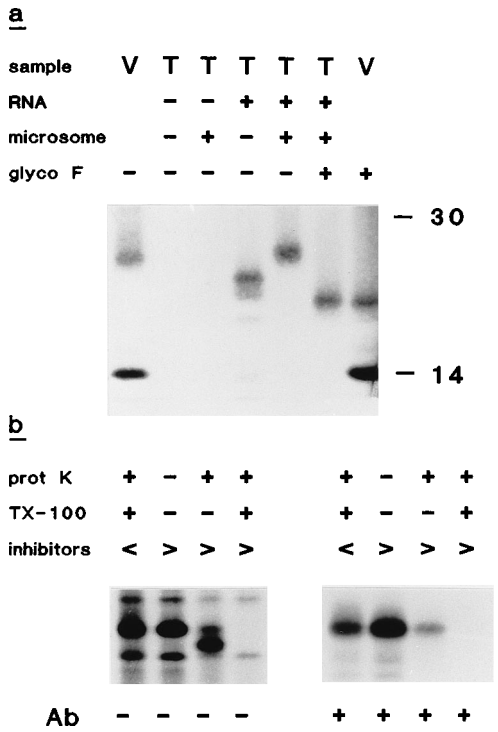

FIG. 2. Membrane topology of the EAV G_S protein. (a) ORF 2 transcripts were translated in vitro (T) in the absence or presence of canine pancreatic microsomes; the expression products were immunoprecipitated with a G_S-specific antipeptide serum and treated (+) or mock treated (−) with glyco F. For comparison, the antipeptide serum was applied to [^{35}S]methionine-labeled virus (V), and the resulting immunoprecipitates were treated or mock treated with glyco F. The antipeptide serum failed to bind any protein from lysates of in vitro translation reactions not supplemented with exogenous RNA. The samples were analyzed by SDS-PAGE under reducing conditions. The positions and sizes (in kilodaltons) of marker proteins analyzed in the same gel are indicated at the right. (b) ORF 2 transcripts were translated in a rabbit reticulocyte lysate in the presence of microsomes. Next, the in vitro translates were treated (+) or mock treated (−) with proteinase K for 1 h at 0°C in the absence of nonionic detergent or after dissolution of the microsomal membranes with TX-100. The proteinase K was inactivated by protease inhibitors which were added prior to (<) or after (>) the 1-h incubation period, and the samples were split into two portions. One aliquot was dissolved in Laemmli sample buffer containing 20 mM DTT and analyzed directly (left panel); the other aliquot was subjected to immunoprecipitation with the antipeptide serum (Ab) before application to an SDS–20% PAA gel (right panel).

the polypeptide synthesized in the absence of microsomes but was still recognized by the antipeptide serum which is directed against the extreme carboxy terminus of G_S, we conclude that the amino terminus had been cleaved off. This interpretation is supported by a computer analysis which predicts the amino terminus of the G_S protein to function as a signal sequence and identifies a possible cleavage site between Leu-24 and Pro-25 (43) (Fig. 1). The removal of 24 amino-terminal residues would release a peptide of 2.9 kDa, consistent with the size difference observed between the unprocessed G_S protein and the ORF 2 product made in the presence of microsomes and treated with glyco F. As the latter protein comigrates with the deglycosylated G_S protein from virions, the ORF 2 protein does not acquire apparent posttranslational modifications in addition to *N*-glycans.

To further investigate its membrane topology, the G_S protein synthesized in the presence of microsomal membranes was treated with proteinase K. This reduced its size by about 3 kDa (Fig. 2b). A small fraction of the protein was unaffected because of incomplete digestion. When the treatment was done in the presence of TX-100 to solubilize the membranes, the protein was completely degraded, indicating that it is not resistant to protease treatment per se. To identify the fragment removed by the protease, we used the antipeptide serum. We found that the mock-treated G_S protein was immunoprecipitable by the serum, in contrast to the product obtained after proteinase K digestion. Obviously, the carboxy terminus of the G_S protein is exposed from the membranes. This interpretation was supported by the observation that the antipeptide serum still recognized the small quantity of undigested G_S protein from the protease-treated sample (Fig. 2b). Clearly, the failure to immunoprecipitate the shortened ORF 2 protein was not due to antibody degradation during immunoprecipitation. The size of the protease-sensitive fragment is consistent with the removal of some 25 residues, indicating that the transmembrane domain of G_S probably extends to around Arg-201 (Fig. 1). Carbonate and Triton X-114 extractions further demonstrated that the G_S protein is not loosely associated with the lipid bilayer but represents a true integral membrane protein (data not shown). Altogether, our data indicate that G_S is a regular class I transmembrane integral protein.

Kinetics of intracellular processing of the G_S protein. In EAV-infected cells, the G_S protein is incorporated into virus particles which presumably assemble by budding into the lumen of the ER (32). The virions are thought to be transported in vesicular carriers through the Golgi complex to the plasma membrane, where they are liberated from the cell by exocytosis. To study the intracellular fate of G_S, EAV-infected BHK-21 cells were pulse-labeled for 15 min with [^{35}S]methionine and then chased for different time intervals. Next the cells were lysed, and the G_S protein was immunoprecipitated with the antipeptide serum and analyzed under reducing conditions in an SDS–15% PAA gel. As the left panel of Fig. 3a shows, a 27-kDa G_S band appeared after the pulse, the intensity of which drastically decreased during a 2-h chase period. The 1-kDa apparent molecular mass increase of the mature G_S protein compared with that in Fig. 2 is due to the alkylating agent NEM used during lysis for reasons described below. To investigate whether the decline in the intracellular amount of G_S could be accounted for by clearance of the protein with virions from the cells, its intracellular transport was analyzed biochemically by assaying the acquisition of resistance to endo H cleavage of its N-linked oligosaccharide side chain. N-glycosylated proteins are initially sensitive to this enzyme but usually become resistant upon passage through the medial-Golgi compartment (27). As illustrated by the right panel of Fig. 3a, the G_S protein synthesized during the pulse was fully endo H sensitive. In the course of the chase, the quantity of sensitive protein gradually decreased, while an increasing amount of endo H-resistant material appeared. At 60 min of chase, both forms were present at a nearly equimolar ratio. Thereafter, the amount of endo H-susceptible molecules further decreased. Simultaneously, a sharp reduction in the amount of endo H-resistant G_S protein, which most likely reflects the release of virus particles into the culture medium, was observed. Alternatively, after its processing in the Golgi apparatus, the G_S protein might be diverted to the lysosomal pathway to be rapidly destroyed. Likewise, the decrease in the endo H-susceptible form is perhaps not solely attributable to its conversion into the terminally glycosylated species but may also result from pre-Golgi degradation (5).

To determine to what extent secretion into the culture fluid and intracellular degradation contribute to the loss of G_S from the cells, we repeated the pulse-chase experiment but included the culture media into the lysates to obtain an overall picture. Once more, a clear albeit less dramatic reduction of the total

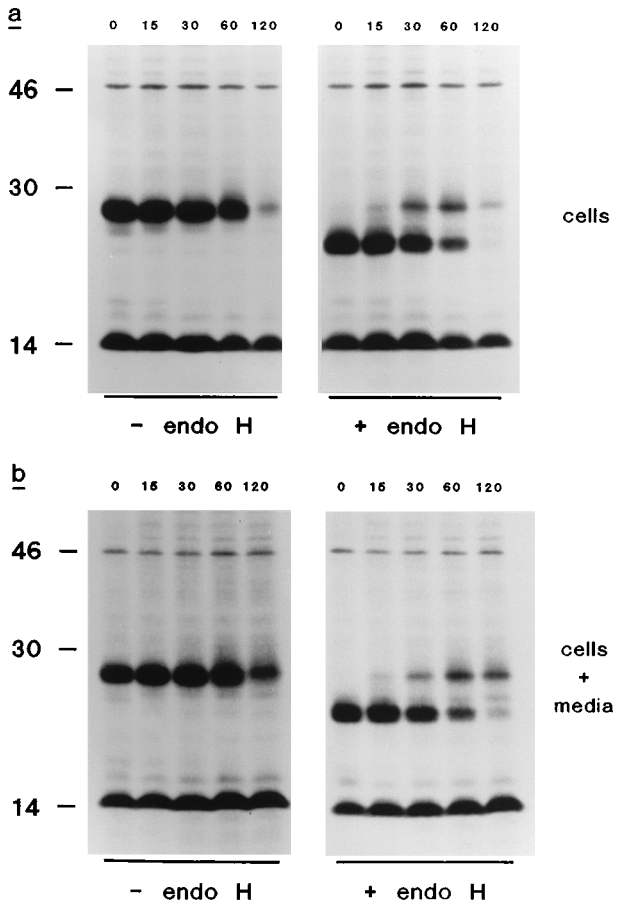

FIG. 3. Kinetics of endo H resistance acquisition of the G_S protein in EAV-infected BHK-21 cells. EAV-infected BHK-21 cells were pulse-labeled for 15 min with L-^{35}S in vitro cell labeling mix at 8.5 h after infection and chased with medium containing 5 mM nonradioactive methionine for the indicated time periods (in minutes). The G_S protein was then immunoprecipitated with the antipeptide serum from true cell lysates (a) or total lysates of cells and media (b). The immunoprecipitates were split in equal portions and treated (right panel) or mock treated (left panel) with endo H. The samples were finally dissolved in 25 μl of Laemmli sample buffer containing 20 mM DTT and analyzed by SDS-PAGE. Numbers on left refer to positions and sizes (in kilodaltons) of marker proteins.

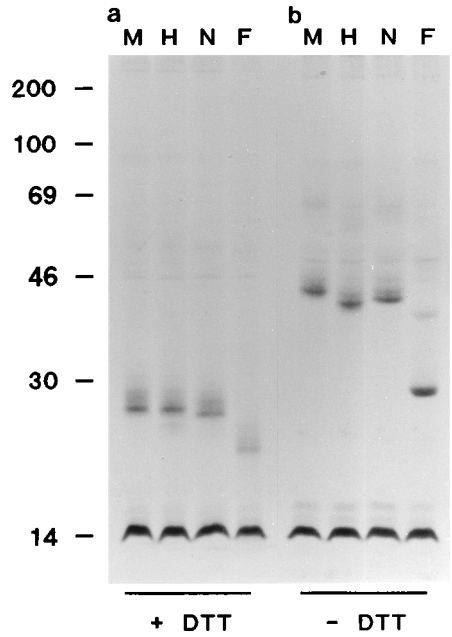

FIG. 4. Characterization of the extracellular G_S protein. EAV-infected BHK-21 cells were labeled for 3 h with L-^{35}S in vitro cell labeling mix starting at 8.5 h p.i. and then chased for 1 h. The G_S protein was immunoprecipitated from the culture medium by using the antipeptide serum, and the immunoprecipitates were divided in four equal portions. One of the aliquots was mock treated (M); the other aliquots were digested with endo H (H), neuraminidase (N), or glyco F (F). Next, each sample was split in two halves, which were analyzed by SDS-PAGE under reducing (a) and nonreducing (b) conditions, respectively. Numbers on left refer to positions and sizes (in kilodaltons) of marker proteins.

amount of G_S was noticed (Fig. 3b, left panel). The quantity of endo H-sensitive G_S protein also continued to decline throughout the experiment. In contrast, the amount of endo H-resistant G_S molecules again increased gradually during the first 60 min of chase but now remained constant afterwards. The endo H-resistant form of G_S is thus selectively incorporated into virions and in this way escapes from degradation. However, most of the G_S molecules apparently do not enter virus particles and are consequently not secreted but degraded intracellularly. Further evidence for the intracellular breakdown of G_S came from an experiment in which we independently expressed the protein from a vaccinia virus recombinant. Although the G_S protein stayed endo H sensitive even after a 2-h chase and was therefore not transported through the Golgi apparatus, it disappeared with a half-time of approximately 20 min (data not shown).

Analysis of infected cell culture medium confirmed that the G_S protein in extracellular virus particles carries only mature N-linked oligosaccharides (Fig. 4a). Its size was not affected by endo H, while the *N*-glycan was readily removed by incubation with glyco F, which cleaves off N-linked sugars irrespective of their maturation state. Moreover, treatment with neuraminidase subtly reduced its apparent molecular weight, which implies that the virion protein is sialylated. Since the addition of sialic acid occurs in *trans*-Golgi and/or the *trans*-Golgi network (38), these data confirm that the virus leaves the cell via the normal secretory pathway.

As is clearly shown in Fig. 4, the extracellular G_S protein is somewhat heterogeneous in size. Although less pronounced because of the relatively low intracellular amount of mature G_S protein, a similar heterogeneity is also evident in Fig. 3, but only after chase periods of ≥30 min. Since the fuzzy band appearance of G_S was not observed with virus grown in the presence of the Golgi α-mannosidase I inhibitor 1-deoxymannojirimycin (data not shown) and disappeared after glyco F treatment but not after neuraminidase digestion, the *N*-glycan of G_S apparently undergoes further late modifications in addition to sialylation.

Folding of the G_S protein in EAV-infected cells. Besides by specific retention signals, a protein can also be retained in the ER as a result of malfolding (23). Since the mature G_S protein most likely contains three cysteines in its ectodomain (Fig. 1) and might therefore become trapped into disulfide-linked aggregates, we investigated its folding in EAV-infected cells. For this purpose, samples of the total lysates from the pulse-chase experiment of Fig. 3b were analyzed under nonreducing conditions, thereby leaving all intra- and intermolecular disulfide bonds intact. To prevent the formation of nonnative disulfide bridges during or after cell lysis, free sulfhydryl groups had been alkylated with NEM prior to disruption of the cells. Surprisingly, the G_S protein appeared to be present in four monomeric forms (Fig. 5a); the two slower-migrating forms

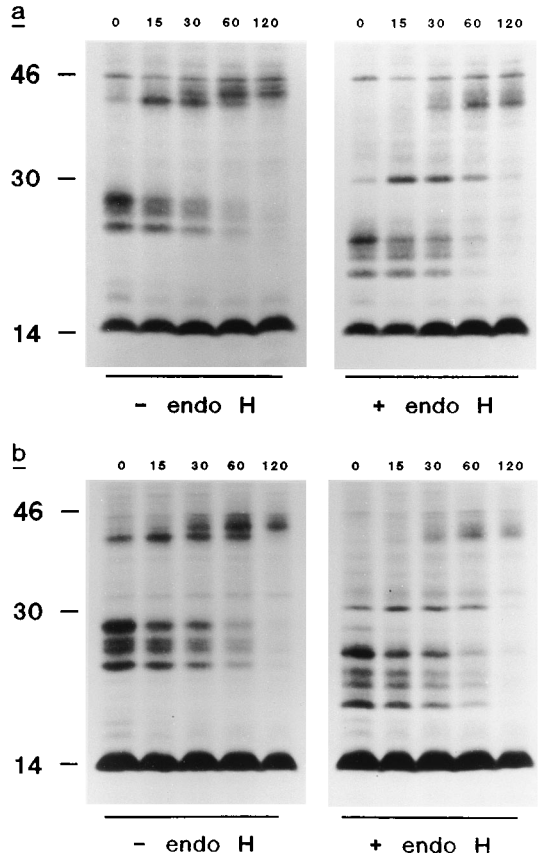

FIG. 5. Folding of the G_S protein in EAV-infected BHK-21 cells. EAV-infected BHK-21 cells were pulse-labeled for 15 min with L-^{35}S in vitro cell labeling mix at 8.5 h p.i. and chased for the indicated times (in minutes). Free sulfhydryl groups were blocked with NEM (a) or IAA (b) as described in Materials and Methods, and the G_S protein was immunoprecipitated with the antipeptide serum from total lysates of cells and media. The precipitates were split in equal portions and treated (right panel) or mock treated (left panel) with endo H. The small amount of 29-kDa G_S species observed in the right part of panel b immediately after the pulse resulted from incomplete Endo H digestion. The samples were finally dissolved in 25 μl of Laemmli sample buffer without DTT and applied to an SDS–15% PAA gel. Numbers on left refer to positions and sizes (in kilodaltons) of marker proteins.

were not always clearly resolved. In addition, a disulfide-linked species migrating at the position of a dimer was also observed. These conformational variants differed only in their disulfide-bonded structures, since all of them comigrated as a single band of 27 kDa under reducing conditions (Fig. 3b). The slowest-migrating monomer most likely lacks intrachain disulfide bridges because its mobility under nonreducing conditions corresponds to that of the fully reduced G_S protein.

The G_S dimers were evident after the 15-min pulse, but most of them arose during the first chase. The amount of monomeric G_S protein concomitantly declined, consistent with a precursor-product relationship. It is not clear which of the monomeric forms is the direct precursor of the dimers, although the amount of the slowest-migrating G_S species seemed to decrease somewhat faster than that of the other monomers. During the first 15 min of chase, the dimeric G_S protein started to be converted into a species with a slightly higher molecular weight. This process continued until finally all G_S dimers were of the slower-migrating type.

Endo H treatment of the nonreduced G_S protein revealed that the four monomeric forms of G_S remained fully endo H sensitive during the entire chase period. However, the dimeric form was gradually modified from a completely endo H-sensitive species into an endo H-resistant one. The endo H digestion reduced the apparent molecular mass of susceptible G_S dimers to about 30 kDa. The acquisition of endo H resistance by the G_S dimers coincided temporally with the shift to a higher molecular weight. Consequently, these data demonstrate that only the dimeric form of G_S is transported out of the ER and acquires complex N-linked oligosaccharides. The small increase in mobility of the endo H-resistant G_S dimers seen after the glycosidase treatment can be attributed to an unspecified activity within the endo H preparation which becomes manifest only under nonreducing conditions (see below).

Neuraminidase digestion of the G_S protein under nonreducing conditions yielded the opposite result. Whereas the G_S monomers and the faster-migrating form of the G_S dimer were not affected by the enzyme, the slower-migrating form of the G_S dimer was converted into a product that comigrated with the faster-migrating form of the G_S dimer and thus contained sialic acid (data not shown). The sialylation of only the dimeric form of G_S provides further evidence for the selective export of G_S dimers from the ER. In agreement with these data, incubation of EAV-infected cells with 1-deoxymannojirimycin did not impede formation of the G_S dimer but prevented its conversion to the slower-migrating form (data not shown). These G_S dimers were fully susceptible to endo H being converted into the 30-kDa product observed earlier.

The unexpected finding of multiple monomeric conformational forms of the G_S protein prompted us to reconfirm this result. We thus performed an additional pulse-chase experiment but used IAA instead of NEM as the thiol blocking agent. Although the migration pattern was somewhat different from that obtained with NEM, again four monomeric forms were clearly separated under nonreducing conditions even without endo H treatment (Fig. 5b). The slowest-migrating form again represents the fully reduced polypeptide, as its mobility was not affected by in vitro DTT treatment (data not shown). The covalent modifications introduced by the two alkylating agents evidently have different effects on the electrophoretic mobilities of the G_S monomers. This was corroborated by the finding that under reducing conditions, NEM-modified G_S and IAA-modified G_S migrated as polypeptides with different apparent molecular masses of 27 and 29 kDa, respectively (data not shown). The dissimilar effects of the two sulfhydryl modifying compounds probably relates to their specific chemical properties (9). The choice of the alkylating agent did not have a detectable effect on the mobility of the G_S dimers. After the pulse, the IAA-modified G_S dimers again migrated as a single band of 42 kDa whose apparent molecular mass gradually increased to 44 kDa during a 2-h chase. Endo H treatment of the IAA-modified G_S protein gave results similar to those obtained previously: the G_S monomers invariably remained endo H susceptible, whereas the dimeric G_S protein slowly became endo H resistant.

The conformation of the G_S protein in virions was as expected. When virus particles were analyzed under nonreducing conditions, the G_S protein appeared as a dimer of 44 kDa that was sialylated because it was susceptible to neuraminidase treatment. Removal of the oligosaccharides with glyco F reduced its apparent molecular mass to 28 kDa (Fig. 4b). Strangely, treatment with endo H also resulted in a slight increase in its electrophoretic mobility, but this was apparently not caused by a change in molecular weight since no effect of the treatment was observed under reducing conditions (see Fig. 4b and above). We hence speculate that a component in

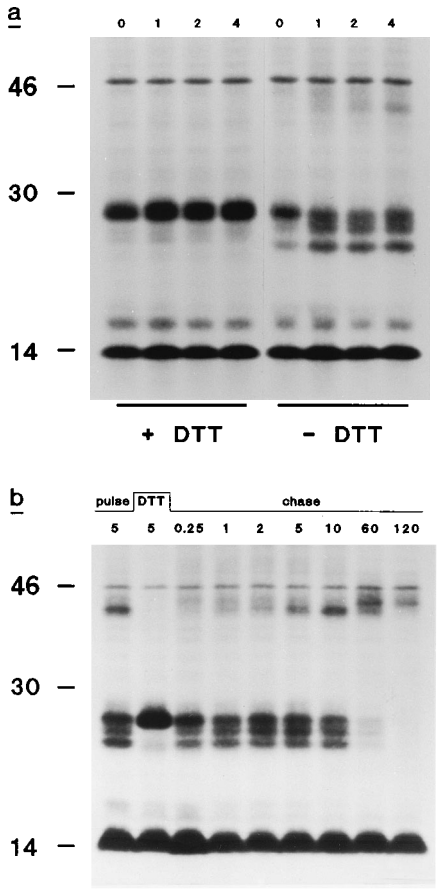

FIG. 6. Kinetics of disulfide bond formation in the G_S protein. (a) EAV-infected BHK-21 cells were pulse-labeled for 2 min with L-^{35}S in vitro cell labeling mix at 8.5 h p.i. and chased with medium containing 5 mM nonradioactive methionine and 0.5 mM cycloheximide for the indicated time periods (in minutes). The cells were lysed after two washes with PBS–20 mM NEM, the G_S protein was immunoprecipitated with the antipeptide serum, and the immune complexes were divided into two parts. One half was analyzed by SDS-PAGE under nonreducing conditions (right panel); the other half was analyzed in the same gel under reducing conditions (left panel). Numbers on the left show size in kilodaltons. (b) EAV-infected BHK-21 cells were pulse-labeled for 5 min with L-^{35}S in vitro cell labeling mix at 8.5 h p.i. and incubated with medium containing 5 mM nonradioactive methionine and 5 mM DTT for 5 min. The cells were then rapidly washed twice with chase medium to remove the DTT and incubated in similar medium for the indicated times (in minutes). The cells were lysed following two washes with PBS–20 mM NEM, the G_S protein was immunoprecipitated with the antipeptide serum, and the immune complexes were dissolved in 25 μl of Laemmli sample buffer without DTT and analyzed by SDS-PAGE. Numbers on left refer to positions and sizes (in kilodaltons) of marker proteins.

the endo H preparation somehow alters the conformation of the G_S dimer.

Kinetics of disulfide bond formation in the G_S protein. To further study the interrelationships between the different forms of the G_S protein, we determined the kinetics of their formation in EAV-infected cells after a very short (2-min) pulse, which was followed by chases of 1, 2, and 4 min in the presence of cycloheximide to block protein elongation. Immediately after the pulse, most G_S molecules were still without disulfide bonds since their electrophoretic mobility was insensitive to DTT (Fig. 6a). During the first chase, the majority of unfolded G_S molecules had already been converted into the three disulfide-containing monomeric forms. This situation did not change during the subsequent chase periods in which the ratios between the different monomers remained constant. The various intrachain cystine bridges are thus formed simultaneously and almost instantaneously after chain termination. A clear amount of the 42-kDa dimeric form of the G_S protein appeared only after the 4-min chase, although less condensed, apparently incompletely folded G_S dimers were already detectable after the first chase time. This result implies that the dimers contain both intra- and intermolecular disulfide bonds, which might explain why more time is required to produce a fully folded dimer than to generate the different G_S monomers. It also suggests that the formation of the intermolecular cystine bridges can precede intrachain disulfide bond formation. The nonoxidized G_S protein may thus be directly converted into a dimer. Whether the folded monomers can also give rise to dimers is hard to determine since the monomeric forms of the G_S protein may be readily interconvertible. Moreover, a rapid reshuffling of nonnative disulfide bonds immediately after multimerization may also lead to the formation of properly folded G_S dimers.

Further evidence for the existence of intrachain disulfide bonds within the G_S dimers came from an experiment in which we reduced newly synthesized G_S protein in vivo and then monitored its reoxidation. EAV-infected BHK-21 cells were pulse-labeled for 5 min; the label was then removed, and the cells were incubated for the same time in chase medium containing 5 mM DTT. Next, the cells were thoroughly washed with normal chase medium to remove the DTT and to restore the redox balance in the cell, and the incubation was continued in the same medium for various time periods. As demonstrated by analysis under nonreducing conditions (Fig. 6b), both the monomeric and dimeric forms of the G_S protein had been synthesized during the 5-min labeling period. The subsequent in vivo reduction with DTT collected of all the G_S protein into one reduced species of 27 kDa. After removal of the reducing agent, the monomeric forms of the G_S protein reappeared instantaneously. A fuzzy band of about 44 kDa was also observed following the ultrashort chase of 15 s. This band presumably represents newly formed dimers. Alternatively, it consists of reoxidizing dimers that did not fall apart as a consequence of the in vivo DTT treatment but only lost their disulfide-bonded structure. During the longer chase periods, a more distinct species emerged with an apparent molecular mass of 42 kDa, which was gradually converted into a 44-kDa product, the terminally glycosylated G_S dimer incorporated into virions.

DISCUSSION

In this report, we show that the EAV G_S protein is a typical class I integral membrane glycoprotein which assumes several distinct conformations. Four monomeric forms of G_S were resolved by gel electrophoresis under nonreducing conditions, three of which contain intrachain disulfide bonds, as was evident from comparative analyses under reducing conditions. The invariably immature state of their N-linked oligosaccharides indicated that the G_S monomers are arrested in a pre-Golgi compartment. A small fraction of the G_S protein assembles into disulfide-linked dimers. As judged by the resistance to endo H and the sialylation of their N-linked sugars, these dimeric structures are transported specifically through the Golgi complex. Analysis of virus particles revealed that they only contain the dimeric form of G_S. The transport and secretion of G_S dimers are therefore accounted for by virion release.

The identification of the different monomeric forms of the G_S protein was completely dependent on their protective alkylation with NEM or IAA prior to solubilization. Without chemical modification, no discrete species were resolved. It is

unlikely that the different species arose as an artifact of the alkylation procedure. After in vitro reduction, all forms of G_S migrated as one species, indicating that their multiple band appearance was not caused by heterogeneous modification with the thiol blocking agent. It is also improbably that the electrophoretic heterogeneity resulted from the incomplete alkylation of free sulfhydryl groups, since G_S molecules that were reduced with DTT and subsequently treated with NEM or IAA in vivo migrated as a single band under nonreducing conditions. The position of this band correlated with that of the alkylated G_S protein analyzed under reducing conditions (data not shown).

A number of mechanisms may be responsible for the selective retention of the G_S monomers within the ER. One is the signal-mediated mechanism operative in retaining ER resident class I transmembrane proteins. The extreme carboxy-terminal sequence RRKIL of the G_S protein (Fig. 1) indeed fits the consensus sequence of an established ER retention signal (25, 39). When the protein is incorporated into virions during virus assembly, which occurs in the ER (32), this signal becomes hidden within the interior of the virus. This would allow the protein to enter the exocytotic pathway and to be secreted as part of virions.

A second mechanism acting to restrict monomeric G_S protein to the ER may be its inability to pass the local quality control system (23). Proteins usually do not exit the ER unless properly folded and assembled. Since the G_S protein possesses three luminal cysteine residues (Fig. 1), it can and apparently does fold into three distinct structures with different intramolecular disulfide bonds. Preliminary data from mutation analyses support this view. Each of these monomeric structures still contains one reactive cysteine. Protein disulfide isomerase, one of the major folding factors in the ER, catalyzes the formation and interconversion of cystine bridges (2, 8). The exposure of a free sulfhydryl group might alert the enzyme as well as other components of the quality control system in their proofreading of the folding process (26). In this mode, protein disulfide isomerase may persist in the continual isomerization of the G_S molecule, since the only possible way to satisfy the quality control system is by oxidation of the free sulfhydryl group through intermolecular disulfide bond formation. Likewise, disulfide exchange reactions with proteins of the ER have been proposed in the retention of immunoglobulin M heavy chains inside this organelle (1, 40). The observation that the molar ratios of the monomeric species did not change during the different chase periods may support the idea that they are in a dynamic equilibrium. Since continuous disulfide exchange reactions would guarantee a permanent supply of dimerization-competent molecules, the apparent confinement of dimer formation to a short period after completion of protein synthesis might point to the requirement of additional de novo-synthesized proteins to assist in the proper folding of G_S or to stabilize correctly folded G_S homodimers.

As a third possible mechanism, the majority of the G_S molecules may be retained in the ER and excluded from the productive folding pathway by entering noncovalently linked aggregates (17). The formation of properly folded homodimers would then rely on the small fraction of newly synthesized G_S molecules whose misfolding immediately after polypeptide chain termination is prevented by the quick association with chaperones, folding enzymes, and/or other G_S molecules. In this scenario, the fully reduced G_S protein might serve as the direct precursor to the dimers and the partially oxidized monomers would represent dead-end products that continue to be present in the same relative molar amounts and are subjected to pre-Golgi degradation. The relatively fast decrease of particularly the nonoxidized form of G_S after a 15-min pulse and the observation that not all disulfide-linked dimers immediately possess the full complement of native cystine bridges are consistent with this model. Alternatively, the slower-migrating forms of the G_S dimer which are evident just after a very short pulse may represent incorrectly and/or incompletely folded molecules that quickly dissociate afterwards or undergo disulfide bond rearrangements.

If the nonoxidized form is the precursor to the dimers, it would explain two intriguing features of the G_S protein. One is its apparent overexpression in EAV-infected cells. Though a very minor virion constituent, the G_S protein is synthesized in these cells at a level comparable to that of the other structural proteins. As there are no indications that G_S serves additional functions to the virus, its overproduction might serve to provide a permanently high supply of assembly-competent molecules. The other feature is the instability of the protein. If the disulfide-containing monomers are indeed nonfunctional, then their accelerated removal from the ER would be desirable to help clear the compartment. Studies with the T-cell antigen receptor subunits have shown that the presence of a charged amino acid residue in a transmembrane segment of a protein can cause its retention and degradation within the ER (4, 45). Similarly, the presence of Glu-184 in its putative transmembrane segment may render the unassembled G_S monomers labile.

It is unclear whether the G_S protein is essential for EAV assembly. Although G_S is only a minor virion component, the selective and efficient incorporation of G_S dimers into virus particles implies that the protein is indispensable. In this context, it is interesting that EAV particles possess an icosahedral nucleocapsid. If the protein were to occupy the edges of the symmetrical virus particles, this would ascribe it a pivotal role in particle morphogenesis and at the same time explain its low abundance.

Although their presence in virions has not yet been demonstrated, G_S homologs are predicted in the sequences of all arteriviruses analyzed so far. Intriguingly, comparison of their amino acid sequences reveals that the ORF 2 proteins invariably carry three luminal cysteine residues, provided that the signal sequences are cleaved off at the positions predicted by von Heijne's rule (43). The evolutionary conservation of this feature may emphasize the importance of disulfide bridges for the function of the G_S protein in the biogenesis of arteriviruses. To learn more about the bizarre folding behavior of G_S, we are currently studying mutants of the protein in which the cysteine residues have been replaced by serine residues.

ACKNOWLEDGMENTS

We thank Dirk-Jan Opstelten for helpful discussions and suggestions and I. Braakman for critical reading of the manuscript.

Use of the services and facilities of the Dutch National Expertise Center CAOS/CAMM, under grant numbers SON 326-052 and STW NCH99.1751, is gratefully acknowledged.

REFERENCES

1. **Alberini, C. M., P. Bet, C. Milstein, and R. Sitia.** 1990. Secretion of immunoglobulin M assembly intermediates in the presence of reducing agents. Nature (London) **347:**485–487.
2. **Bardwell, J. C. A., and J. Beckwith.** 1993. The bonds that tie: catalyzed disulfide bond formation. Cell **74:**769–771.
3. **Bole, D. G., L. M. Hendershot, and J. F. Kearney.** 1986. Post-translational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. J. Cell Biol. **102:** 1558–1566.
4. **Bonifacino, J. S., P. Cosson, N. Shah, and R. D. Klausner.** 1991. Role of potentially charged transmembrane residues in targeting proteins for retention and degradation within the endoplasmic reticulum. EMBO J. **10:**2783–2793.

5. **Bonifacino, J. S., and J. Lippincott-Schwartz.** 1991. Degradation of proteins within the endoplasmic reticulum. Curr. Opin. Cell Biol. **3:**592–600.
6. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46:**83–88.
7. **Braakman, I., H. Hoover-Litty, K. R. Wagner, and A. Helenius.** 1991. Folding of influenza hemagglutinin in the endoplasmic reticulum. J. Cell Biol. **114:**401–411.
8. **Bulleid, N. J.** 1993. Protein disulfide-isomerase: role in biosynthesis of secretory proteins. Adv. Protein Chem. **44:**125–150.
9. **Chreighton, T. E. (ed.).** 1990. Protein structure: a practical approach. IRL Press, Oxford.
10. **Degen, E., and D. B. Williams.** 1991. Participation of a novel 88-kD protein in the biogenesis of murine class I histocompatibility molecules. J. Cell Biol. **112:**1099–1115.
11. **den Boon, J. A., E. J. Snijder, E. D. Chirnside, A. A. F. de Vries, M. C. Horzinek, and W. J. M. Spaan.** 1991. Equine arteritis virus is not a togavirus but belongs to the coronaviruslike superfamily. J. Virol. **65:**2910–2920.
12. **de Silva, A., I. Braakman, and A. Helenius.** 1993. Posttranslational folding of vesicular stomatitis virus G protein in the ER: involvement of noncovalent and covalent complexes. J. Cell Biol. **120:**647–655.
13. **de Vries, A. A. F.** Unpublished data.
14. **de Vries, A. A. F., E. D. Chirnside, M. C. Horzinek, and P. J. M. Rottier.** 1992. Structural proteins of equine arteritis virus. J. Virol. **66:**6294–6303.
15. **de Vries, A. A. F., E. D. Chirnside, M. C. Horzinek, and P. J. M. Rottier.** 1993. Equine arteritis virus contains a unique set of four structural proteins. Adv. Exp. Med. Biol. **342:**245–253.
16. **Doll, E. R., J. T. Bryans, W. H. McCollum, and M. E. W. Crowe.** 1957. Isolation of a filterable agent causing arteritis of horses and abortion by mares. Its differentiation from the equine abortion (influenza) virus. Cornell Vet. **47:**3–41.
17. **Doms, R. W., R. A. Lamb, J. K. Rose, and A. Helenius.** 1993. Folding and assembly of viral membrane proteins. Virology **193:**545–562.
18. **Fra, A. M., C. Fagioli, D. Finazzi, R. Sitia, and C. M. Alberini.** 1993. Quality control of ER synthesized proteins: an exposed thiol group as a three-way switch mediating assembly, retention and degradation. EMBO J. **12:**4755–4761.
19. **Gething, M.-J., and J. Sambrook.** 1992. Protein folding in the cell. Nature (London) **355:**33–45.
20. **Godeny, E. K., L. Chen, S. N. Kumar, S. L. Methven, E. V. Koonin, and M. A. Brinton.** 1993. Complete genomic sequence and phylogenetic analysis of the lactate dehydrogenase-elevating virus (LDV). Virology **194:**585–596.
21. **Horzinek, M., J. Maess, and R. Laufs.** 1971. Studies on the substructure of togaviruses. II. Analysis of equine arteritis, rubella, bovine viral diarrhea, and hog cholera viruses. Arch. Gesamte Virusforsch. **33:**306–318.
22. **Hurtley, S. M., D. G. Bole, H. Hoover-Litty, A. Helenius, and C. S. Copeland.** 1989. Interactions of misfolded influenza virus hemagglutinin with binding protein. J. Cell Biol. **108:**2117–2126.
23. **Hurtley, S. M., and A. Helenius.** 1989. Protein oligomerization in the endoplasmic reticulum. Annu. Rev. Cell Biol. **5:**277–307.
24. **Hyllseth, B.** 1973. Structural proteins of equine arteritis virus. Arch. Gesamte Virusforsch. **40:**177–180.
25. **Jackson, M. R., T. Nilsson, and P. A. Peterson.** 1990. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. EMBO J. **9:**3153–3162.
26. **Kaji, E. H., and H. F. Lodish.** 1993. *In vitro* unfolding of retinol-binding protein by dithiothreitol. J. Biol. Chem. **268:**22195–22202.
27. **Kornfeld, R., and S. Kornfeld.** 1985. Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem. **54:**631–664.
28. **Kozutsumi, Y., M. Segal, K. Normington, M.-J. Gething, and J. Sambrook.** 1988. The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. Nature (London) **332:**462–464.
29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
30. **Lippincott-Schwartz, J., J. S. Bonifacino, L. C. Yuan, and R. D. Klausner.** 1988. Degradation from the endoplasmic reticulum: disposing of newly synthesized proteins. Cell **54:**209–220.
31. **Lodish, H. F., N. Kong, M. Snider, and G. J. A. M. Strous.** 1983. Hepatoma secretory proteins migrate from rough endoplasmic reticulum to Golgi at characteristic rates. Nature (London) **304:**80–83.
32. **Magnusson, P., B. Hyllseth, and H. Marusyk.** 1970. Morphological studies on equine arteritis virus. Arch. Gesamte Virusforsch. **30:**105–112.
33. **Matter, H., and H.-P. Hauri.** 1991. Intracellular transport and conformational maturation of intestinal brush border hydrolases. Biochemistry **30:**1916–1923.
34. **Meulenberg, J. J. M., M. M. Hulst, E. J. de Meijer, P. L. J. M. Moonen, A. den Besten, E. P. de Kluyver, G. Wensvoort, and R. J. M. Moorman.** 1993. Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. Virology **192:**62–72.
35. **Ou, W.-J., P. H. Cameron, D. Y. Thomas, and J. J. M. Bergeron.** 1993. Association of folding intermediates of glycoproteins with calnexin during protein maturation. Nature (London) **364:**771–776.
36. **Pol, J., and F. Wagenaar.** 1992. Morphogenesis of Lelystad virus in porcine lung alveolar macrophages. Am. Assoc. Swine Pract. Newsl. **4:**29.
37. **Rose, J. K., and R. W. Doms.** 1988. Regulation of protein export from the endoplasmic reticulum. Annu. Rev. Cell Biol. **4:**257–288.
38. **Roth, J., D. J. Taatjes, J. M. Lucocq, J. Weinstein, and J. C. Paulson.** 1985. Demonstration of an extensive *trans*-tubular network continuous with the Golgi apparatus stack that may function in glycosylation. Cell **43:**287–295.
39. **Shin, J., R. L. Dunbrack, Jr., S. Lee, and J. L. Strominger.** 1991. Signals for retention of transmembrane proteins in the endoplasmic reticulum studied with CD4 truncation mutants. Proc. Natl. Acad. Sci. USA **88:**1918–1922.
40. **Sitia, R., M. Neuberger, C. Alberini, P. Bet, A. Fra, C. Valetti, G. Williams, and C. Milstein.** 1990. Developmental regulation of IgM secretion: the role of the carboxy-terminal cysteine. Cell **60:**781–790.
41. **Snijder, E. J., M. C. Horzinek, and W. J. M. Spaan.** 1993. The coronavirus-like superfamily. Adv. Exp. Med. Biol. **342:**235–244.
42. **Stueckemann, J. A., D. M. Ritzi, M. Holth, M. S. Smith, W. J. Swart, W. A. Cafruny, and P. G. W. Plagemann.** 1982. Replication of lactate dehydrogenase-elevating virus in macrophages. 1. Evidence for cytocidal replication. J. Gen. Virol. **59:**245–262.
43. **von Heijne, G.** 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14:**4683–4690.
44. **Wikström, L., and H. F. Lodish.** 1993. Unfolded H2b asialoglycoprotein receptor subunit polypeptides are selectively degraded within the endoplasmic reticulum. J. Biol. Chem. **268:**14412–14416.
45. **Wileman, T., G. R. Carson, F. F. Shih, M. F. Concino, and C. Terhorst.** 1990. The transmembrane domain of the T-cell antigen receptor β chain contains a structural determinant of pre-Golgi proteolysis. Cell Regul. **1:**907–919.
46. **Wood, O., N. Tauraso, and H. Liebhaber.** 1970. Electron microscopic study of tissue cultures infected with simian haemorrhagic fever virus. J. Gen. Virol. **67:**129–136.
47. **Zeegers, J. J. W., B. A. M. van der Zeijst, and M. C. Horzinek.** 1976. The structural proteins of equine arteritis virus. Virology **73:**200–205.