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The Two Major Envelope Proteins of Equine Arteritis Virus Associate into Disulfide-Linked Heterodimers

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In a coimmunoprecipitation assay with monospecific antisera, the two major envelope proteins G_L and M of equine arteritis virus were found to occur in heteromeric complexes in virions and infected cells. While the G_L protein associated with M rapidly and efficiently, newly synthesized M protein was incorporated into complexes at a slower rate, which implies that it interacts with G_L molecules synthesized earlier. Analysis under nonreducing conditions revealed that the G_L/M complexes consist of disulfide-linked heterodimeric structures. Pulse-chase experiments showed that virtually all G_L monomers ended up in heterodimers, whereas a fraction of the M protein persisted as monomers. The M protein also formed covalently linked homodimers, but only the heterodimers were incorporated into virus particles.

Equine arteritis virus (EAV) is the best-characterized member of the arteriviruses (38), a group of small enveloped viruses with a positive-stranded RNA genome about to be assigned family status; the cluster further includes lactate dehydrogenase-elevating virus, porcine reproductive and respiratory syndrome virus, and simian hemorrhagic fever virus (6, 18, 34). Arteriviruses resemble coronaviruses and toroviruses in their genome organization, transcription mechanism, expression strategy, and intracellular site of budding (38, 45). However, the virion architecture of arteriviruses is entirely different from that of coronaviruses and toroviruses and appears to be unique among animal viruses (8).

The spherical EAV particle has a diameter of 60 ± 13 nm and contains an infectious polyadenylated RNA genome of about 12,700 nucleotides that is packaged into an isometric nucleocapsid (6, 26). The core contains a phosphorylated nucleocapsid protein (N) of 14 kDa which is surrounded by a lipid bilayer (30, 50). Three transmembrane proteins are inserted into the viral envelope: a 16-kDa nonglycosylated membrane protein (M), a small envelope glycoprotein (G_S) of 25 kDa, and a large envelope glycoprotein (G_L) of 30 to 42 kDa (8, 30). N, M, and G_L are major structural proteins, whereas the G_S protein is only a minor virion constituent.

Like analogous proteins of coronaviruses and toroviruses (41), the M protein of EAV presumably contains three internal membrane-spanning segments and has its amino terminus exposed at the viral surface and its carboxy terminus buried within the virus interior. M probably plays an important role in the intracellular budding process (45). G_S is a class I integral membrane protein present as a disulfide-linked homodimer in virus particles (9). It has a single N-glycosylation site which becomes sialylated during export of the virions from the cell. Dimerization is inefficient and presumably occurs only during virion assembly. The G_L protein also has one N-linked oligosaccharide side chain which, in contrast to that of G_S, is heterogeneously modified with *N*-acetyllactosamine during its transport along the secretory pathway. Since only G_L is recognized by neutralizing antibodies (2, 7), it is most likely involved in cell attachment and/or membrane fusion.

Budding of enveloped viruses occurs either at the cell surface or at intracellular membranes (see the reviews by Dubois-Dalcq et al. [12], Griffiths and Rottier [21], Pettersson [37], and Stephens and Compans [46]). For an envelope protein to become incorporated into virus particles, it must be transported, either individually or in complexes, from the place of synthesis to that of virion assembly. Only properly folded and assembled transmembrane proteins are usually exported from the endoplasmic reticulum (ER) (29, 40); the rates and efficiencies of these events vary widely, depending on the polypeptide's primary structure (11). Accordingly, different virion proteins may enter virus particles with different kinetics (25, 35, 36). The functions of viral envelope proteins are diverse. With the exception of retroviruses, which do not require a viral transmembrane protein for capsid envelopment (28), virus budding relies on specific interactions between the envelope protein(s) and viral components gathered at the cytoplasmic side of the assembly sites. For most viruses, the membrane proteins seem to determine the place of virus maturation. Viruses which assemble at the plasma membrane have envelope proteins that are rapidly transported to the cell surface, whereas the membrane proteins of intracellularly budding viruses are generally retained at the budding site. In the presence of monensin, which blocks protein exit from the medial Golgi compartment, alphavirus budding is reallocated from the plasma membrane to the Golgi apparatus (20). Moreover, the C protein of rubella virus accumulates in the Golgi region only in the presence of the envelope proteins (23, 24, 39). Interactions between the viral envelope proteins can also affect their intracellular transport, e.g., Golgi localization of the bunyavirus G2 protein depends on its association with G1 (5) and the E1 protein of rubella virus is only routed to the Golgi complex in the presence of E2 (25). Other functions of the viral envelope proteins include binding of virus particles to host cell receptors, mediating of membrane fusion and penetration into cells, and destruction of receptor molecules to facilitate virus release and to prevent superinfection.

The morphogenesis of arteriviruses has not been looked at in detail, but assembly appears to take place in pre-Golgi

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membranes (33). Studies of the G_S protein have further suggested that the biosynthesis of the envelope proteins and the formation of virus particles are intimately related (9). In this paper, we show that the two major membrane proteins of EAV associate with different kinetics to form disulfide-linked heterodimeric structures which also occur in virions.

MATERIALS AND METHODS

Cells and viruses. Baby hamster kidney (BHK-21) and Crandell feline kidney (CRFK) cells (original and persistently infected with feline immunodeficiency virus [FIV]) were grown and 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). A concentrated stock of the Bucyrus strain of EAV (10) was prepared in BHK-21 cells (8).

Preparation of antisera. The production and characterization of rabbit antipeptide sera specific for the extreme carboxy termini of the N, M, and G_S proteins and of a rabbit antiserum directed against disrupted virions have been described previously (8). Antibodies against the G_L protein were obtained by subcutaneous injection of a New Zealand White rabbit with the synthetic peptide SP25 (NH_2-Thr-Phe-Gly-Thr-Asp-Cys-Asp-Asp-Thr-Tyr-Ala-Val-Pro-Val-Ala-Glu-Val-Leu-Glu-Gln-Ala-His-Gly-COOH). This peptide corresponds to amino acids 75 through 97 of the open reading frame 5 coding sequence and was predicted to represent the most immunogenic part of the G_L ectodomain (31). The oligopeptide was synthesized in a MilliGen/Biosearch 9400 Excell peptide synthesizer by the solid-phase Fmoc procedure (47). To increase its immunogenicity, SP25 was conjugated to keyhole limpet hemocyanin by using limiting amounts of glutaraldehyde (22). The rabbit was primed with 200 μg of the coupled peptide emulsified in Freund's complete adjuvant and was given boosters at monthly intervals with 500 μg of conjugate in Freund's incomplete adjuvant. The animal was bled 2 weeks after the fourth booster.

Metabolic labeling of infected cells with [^{35}S]methionine. Subconfluent cell monolayers in 10-cm^2 dishes were infected with EAV at 37°C as described by de Vries et al. (8). Unless stated otherwise, the cells were metabolically labeled at 8.5 h postinfection (p.i.). To deplete the intracellular pool of methionine, the cells were first incubated for 30 min in prewarmed methionine-free medium buffered with 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4). After the starvation period, the cells were (pulse-)labeled for the appropriate times in methionine-deficient medium containing 100 to 500 μCi of L-[^{35}S]-in vitro cell labeling mixture (>1,000 Ci/mmol; Amersham) per ml. The cells were then rapidly washed twice with chase medium (DMEM–10% FCS, 5 mM L-methionine, and 10 mM HEPES [pH 7.4]) at 37°C. Next, 800 μl of fresh chase medium and 200 μl of ice-cold lysis buffer (100 mM Tris-HCl [pH 7.6], 150 mM NaCl, 5% Nonidet P-40 [NP-40], 2.5% sodium deoxycholate, 0.5% sodium dodecyl sulfate [SDS] containing 5 μg [each] of aprotinin, leupeptin, and pepstatin A per ml) were added to the culture dishes. Alternatively, the cells were further incubated at 37°C for various times. Chase periods were terminated by replacing the supernatants with a mixture of 800 μl of fresh chase medium and 200 μl of lysis buffer; the same buffer was used to disintegrate virus particles in culture medium. The lysates were centrifuged for 20 min at $10^4 \times g$ and 4°C, the pellets were discarded, and the supernatants were adjusted to a concentration of 5 mM EDTA.

To analyze intra- and extracellular proteins simultaneously, a one-fourth volume of concentrated lysis buffer was added directly to the labeling or chase medium. When disulfide bonds were studied, iodoacetamide (IAA) was included in the lysis procedure to block free sulfhydryl groups and to prevent the formation of disulfide bridges during or after cell lysis (9).

[^{35}S]methionine-labeled virus was prepared essentially as described by de Vries et al. (8, 9).

Metabolic labeling of infected cells with [^{3}H]myristic acid. Infected and mock-infected BHK-21 cells were incubated at 37°C with 250 μCi of [9,10(*n*)-^{3}H]myristic acid (30 Ci/mmol; NEN) per ml from 8 to 12 h p.i. in complete medium containing 1% dimethyl sulfoxide. Uninfected CRFK cells and CRFK cells persistently infected with FIV (strain Ut113 [13]) were labeled in similar medium for 7 h.

Immunoprecipitation, gel electrophoresis, 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 total volume of 1 ml. In one experiment, the immunoprecipitation buffer was supplemented with dithiothreitol (DTT) to final concentrations of between 0 and 5 mM. The samples were incubated overnight at 4°C with 3 μl of rabbit antiserum and then for ≥2 h with 25 μl of a 10% suspension of formaldehyde-fixed and heat-inactivated group G *Streptococcus* sp. cells (Omnisorb; Calbiochem) or *Staphylococcus aureus* cells (Pansorbin; Calbiochem). The immune complexes were 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 antibody-antigen conjugates were eluted from the immunoadsorbent by incubation for 15 min at room temperature in 25 μl of Laemmli sample buffer without DTT for analysis under nonreducing conditions or with 50 mM DTT for analysis in a reducing environment. After centrifugation for 5 min at $10^4 \times g$, the supernatants were analyzed in SDS–15% polyacrylamide (PAA) gels (32). The gels were fixed in 10% acetic acid–10% methanol at room temperature for 15 min, impregnated with 2,5-diphenyloxazole (3), dried on Whatman 3MM filter paper, and exposed at −70°C to hypersensitized Fuji RX film.

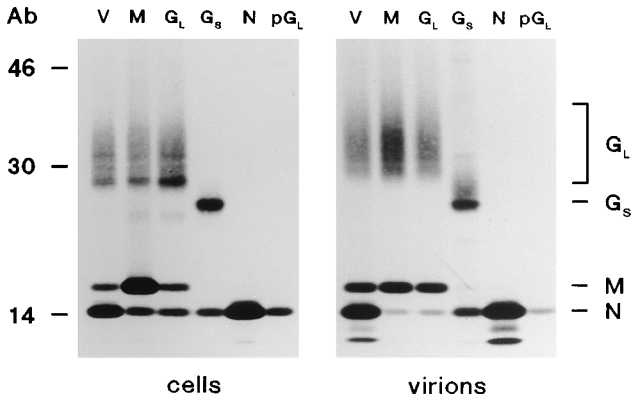

FIG. 1. Interactions between the structural proteins of EAV. (Left panel) EAV-infected BHK-21 cells were labeled for 1 h with L-[^{35}S]-in vitro cell labeling mixture at 8.5 h after infection. A true cell lysate was prepared and subjected to immunoprecipitation with an antiserum directed against disrupted virions (V), antipeptide sera recognizing each of the structural proteins (N, M, G_L, and G_S), and the preserum corresponding to the G_L-specific antipeptide serum (pG_L). (Right panel) EAV-infected BHK-21 cells were labeled for 4 h with L-[^{35}S]-in vitro cell labeling mixture, starting at 8.5 h p.i. and were then chased for 1 h. After removal of cell debris by low-speed centrifugation, the medium was mixed with a one-fourth volume of concentrated lysis buffer and subjected to immunoprecipitation with the aforementioned antisera. Since the G_S protein is only a minor virion component, five times more medium was incubated with the G_S-specific antipeptide serum than with the other sera. The samples were analyzed in an SDS–15% PAA gel under reducing conditions. The positions of the N, M, and G_S proteins are indicated by dashes; the bracket depicts the size limits of the heterogeneously glycosylated G_L protein. The positions and sizes (in kilodaltons) of marker proteins analyzed in the same gel are shown at the left. Ab, antibody.

Endoglycosidase treatment. Immunoprecipitates were digested with endoglycosidase F/N-glycosidase F (glyco F) (Boehringer Mannheim) in 200 μl of 50 mM sodium phosphate (pH 7.0)–10 mM EDTA, containing 100 μg of bovine serum albumin (BSA) per ml, 100 μg of phenylmethylsulfonyl fluoride per ml, and 1 μg (each) of aprotinin, leupeptin, and pepstatin A per ml. For treatment with endoglycosidase H (endo H) (Boehringer Mannheim) the samples were resuspended in 50 mM sodium acetate (pH 5.5)–10 mM EDTA containing 100 μg of BSA per ml, 100 μg of phenymethylsulfonyl fluoride per ml, and 1 μg of protease inhibitors per ml. For glyco F and endo H, 100 and 2 mU, respectively, of enzyme were employed and a mock control experiment was always carried out in parallel. After rotation of the samples for ≥16 h at 37°C, the immune complexes were collected by centrifugation and dissolved in Laemmli sample buffer.

Computer analysis of polypeptide sequences. Deduced polypeptide sequences were analyzed with the aid of the Sequence Analysis Software Package for VAX/VMS computers (version 7.1; University of Wisconsin Genetics Computer Group). To locate potential target sequences for posttranslational modifications, we used release 11.0 of the PROSITE protein pattern database (1).

RESULTS

Interactions between EAV structural proteins. Antipeptide sera specific for each virion protein were used in coimmunoprecipitation experiments with lysates of radiolabeled EAV-infected cells and detergent-treated culture supernatants from such cells. Apart from bringing the homologous protein down, the antibody directed against M also precipitated the G_L protein (Fig. 1). The G_L protein appears as a smear as a result of its heterogeneous glycosylation (as described below). The immunoprecipitate contains also some N protein as a result of its nonspecific adherence to the Omnisorb used to collect the immune complexes (see the pre-immune serum control). We have reported this phenomenon for *S. aureus* cells before (48). With the antibody to the G_L protein, the same result was obtained. In addition to G_L (and N), the M protein was now

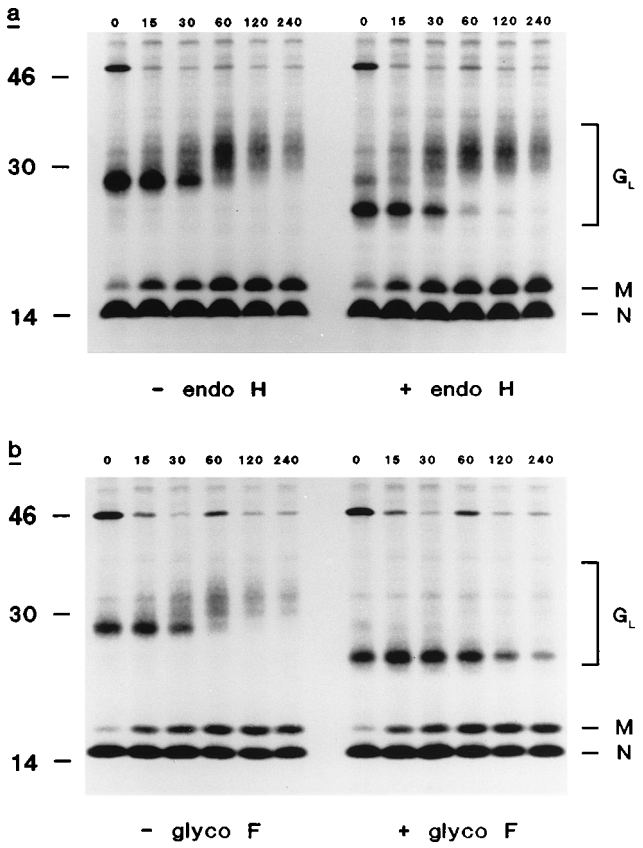

FIG. 2. Kinetics of endo H resistance acquisition and degradation of the G_L protein from EAV-infected BHK-21 cells. EAV-infected BHK-21 cells were pulse-labeled for 15 min with L-[^{35}S]-in vitro cell labeling mixture at 8.5 h after infection and chased with medium containing 5 mM nonradioactive methionine for the times indicated (in minutes) above the lanes. The G_L protein was immunoprecipitated from combined lysates of cells and medium with a specific antipeptide serum, the immunoprecipitates were split into four equal portions and treated (right-hand panels) or mock treated (left-hand panels) with endo H (a) or glyco F (b). The samples were analyzed in SDS–15% PAA gels under reducing conditions. The positions of the N and M proteins are indicated by dashes; the bracket depicts the location in the gel of the various forms of the G_L protein. The numbers on the left show size in kilodaltons.

coprecipitated. Antisera directed against G_S or N did not specifically bring down any additional polypeptides. These observations indicate that G_L and M interact in infected cells to form complexes which also appear in extracellular virions. Although additional interactions between the structural proteins may occur, they are not maintained under the current analytical conditions.

Kinetics of intracellular processing of the G_L protein. Next, we analyzed the maturation and stability of the G_L protein. Infected cells were pulse-labeled for 15 min and chased for various periods. Total lysates were prepared by adding concentrated lysis buffer to infectious culture medium, and immunoprecipitations were performed with the G_L-specific antipeptide serum. One set of experiments was designed to follow the acquisition of endo H resistance, a biochemical marker for transport of glycoproteins through the Golgi apparatus (49). The G_L protein appeared as a discrete species after the pulse, which faded during the next 120 min, while a diffusely migrating new species appeared (Fig. 2a, left-hand panel). After pulse-labeling, only a small fraction of the G_L molecules was found to be endo H resistant (Fig. 2a, right-hand panel); these had reached the medial Golgi compartment but had not yet passed through the *trans*-Golgi network, since they had not acquired heterogeneous poly-*N*-acetyllactosamine modifications. During the chases, the endo H-resistant fraction rapidly increased, with an estimated half-life of less than 30 min.

The overall intensity of labeled G_L protein decreased during the chases, indicating that the protein was subjected to degradation. To more precisely study the turnover of G_L, in a second set of experiments the samples were treated with glyco F. This enzyme removes *N*-glycans irrespective of their maturation state and hence facilitates quantification of the G_L protein. Figure 2b (right-hand panel) shows a marked decrease in the total amount of G_L, especially after extended chase periods; proteolysis thus occurred after the maturation of its N-linked oligosaccharide side chain. The deglycosylation of the G_L protein did not abolish or even reduce its association with M, which demonstrates that the *N*-glycan of G_L is not required to maintain the G_L/M complexes. In addition, the degradation of G_L was not attended by a clear reduction in the amount of coprecipitating M protein, which indicates that some of the radiolabeled M molecules were associated with unlabeled G_L synthesized during the chase (as discussed below).

The M protein is not myristoylated. Myristoylation of one or more of their structural proteins is obligatory in the assembly and/or entry of many animal viruses for the facilitation of protein-protein or protein-lipid interactions (for reviews, see the reports by Grand [19], Hruby and Franke [27], Schlesinger [43], and Schmidt [44]). Since the amino terminus of the EAV M protein contains a potential myristoylation site (NH_2-Met-Gly-Ala-Ile-Asp-Ser-Phe-Cys-Gly-Asp-. . .), we investigated whether myristic acid might contribute to the interaction between M and G_L. EAV-infected BHK-21 cells were labeled with [^{3}H]myristic acid, and the M protein was immunoprecipitated from both the cell lysate and the culture supernatant with a specific antipeptide serum and analyzed by SDS-PAA gel electrophoresis (PAGE). No [^{3}H]myristate-labeled proteins were observed, while [^{35}S]methionine clearly labeled the M protein (Fig. 3, left-hand panel). We analyzed FIV in parallel, with the FIV matrix (MA) protein (15) serving as a positive control. Antibodies from an experimentally infected cat specifically precipitated the labeled 15-kDa MA protein from the culture medium and a lysate of FIV-infected CRFK cells (Fig. 3, right-hand panel). The 50-kDa Gag precursor and some higher-molecular-weight species (probably Gag-Pol-related precursors [14]) were also precipitated from the lysate of FIV-infected but not uninfected CRFK cells.

G_L and M form disulfide-linked heterodimers. The failure to demonstrate myristoylation of the EAV M protein and the presence of a conserved cysteine near the amino terminus in all known arteriviral M proteins led us to investigate the role of disulfide bridges in M protein interaction with G_L. We first analyzed whether G_L/M complexes are sensitive to reducing agents. For this purpose, EAV-infected BHK-21 cells were metabolically labeled at 8.5 h p.i. for 30 min. A true cell lysate was then prepared and split into two equal portions to which either G_L- or M-specific antipeptide serum was added. Aliquots of these mixtures were incubated with increasing amounts of DTT, and the final immunoprecipitates were analyzed by SDS-PAGE under reducing conditions (Fig. 4). In the absence of the reducing agent, the M protein was again coprecipitated by the G_L-specific antipeptide serum. Similarly, with the antipeptide serum directed against M a clear amount of the G_L protein was brought down. The addition of DTT to the immunoprecipitation buffer had a distinct, concentration-dependent effect. With 0.1 mM DTT, the quantity of M and G_L coprecipitated by heterologous antiserum was decreased, and with DTT at concentrations above 0.5 mM coprecipitation was

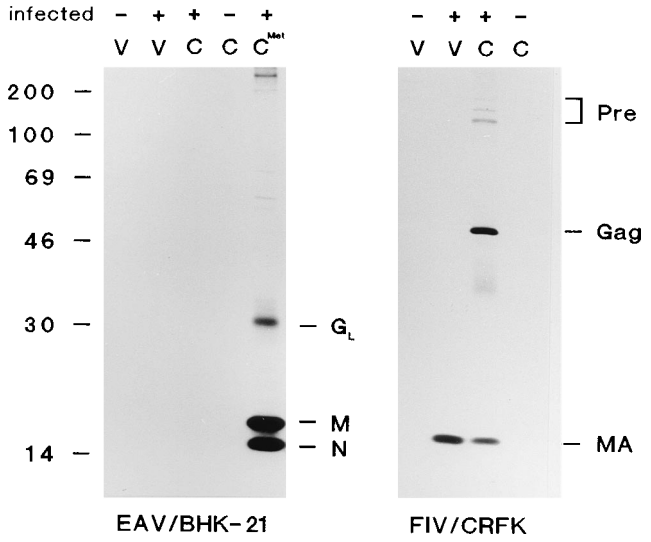

FIG. 3. [^{3}H]myristic acid labeling of mock- and EAV-infected BHK-21 cells and of uninfected and FIV-infected CRFK cells. Mock-infected (−) and EAV-infected (+) BHK-21 cells (left-hand panel) were labeled with [9,10(*n*)-^{3}H]myristic acid from 8 to 12 h after (mock-)infection. Uninfected (−) and FIV-infected (+) CRFK cells (right-hand panel) were incubated with [9,10(*n*)-^{3}H]myristic acid for 7 h. The cells (C) and media (V) were processed separately and subjected to immunoprecipitation with an M-specific antipeptide serum (left-hand panel) or serum from a FIV-infected cat (right-hand panel). As a control, EAV-infected BHK-21 cells from the same experiment were labeled for 30 min with L-[^{35}S]-in vitro cell labeling mixture and the cell lysate prepared from these cells (C^{Met}) was incubated with the M-specific antipeptide serum. The positions of the N, M, and G_L proteins of EAV and the MA and Gag proteins of FIV are indicated by dashes. The position of the presumptive Gag-Pol precursor (Pre) of FIV and large cleavage products thereof is depicted by a bracket. The numbers on the left show size in kilodaltons.

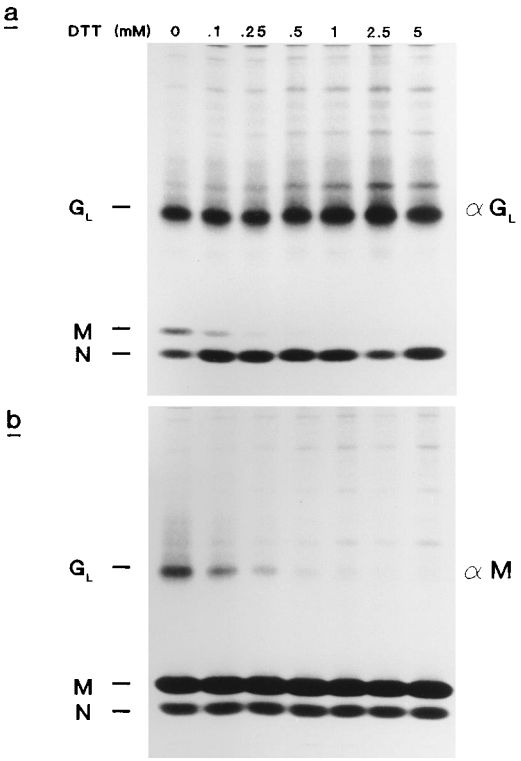

FIG. 4. Effect of DTT on the interaction between G_L and M. EAV-infected BHK-21 cells were labeled for 30 min with L-[^{35}S]-in vitro cell labeling mixture at 8.5 h after infection. A cell lysate was then prepared and incubated with a G_L-specific (a) or M-specific (b) antipeptide serum (αG_L and αM, respectively) in the presence of 0 to 5 mM DTT. The samples were analyzed by SDS-PAGE under reducing conditions. The positions of the N, M, and G_L proteins are indicated by dashes.

almost completely abrogated. DTT concentrations of ≤5 mM did not adversely affect antibody binding to homologous antigen. We hence conclude that disulfide bonds contribute to the association between M and G_L.

To further characterize the G_L/M complexes, we analyzed the proteins from pulse-chase experiments under nonreducing conditions. The formation of biologically irrelevant cysteine linkages during or after cell disruption was prevented by in situ alkylation of free sulfhydryl groups with IAA. Total culture lysates were incubated with G_L- or M-specific antipeptide sera, and immunoprecipitates were taken up in sample buffer with and without a reducing agent. Electrophoretic analysis of the reduced immunoprecipitates obtained with the G_L-specific antipeptide serum confirmed that increasing amounts of the M protein were coprecipitated as a function of time (Fig. 5a, left-hand panel). Alkylation resulted in a double band for the G_L protein, which suggests that it may adopt two (or more) conformations in EAV-infected cells. Electrophoresis under nonreducing conditions yielded a different picture (Fig. 5a, right-hand panel). The monomeric M protein was no longer observed, and only a small quantity of G_L monomers was detected. They migrated as a single band and to a lower-molecular-weight position than the reduced G_L protein, indicating a homogeneous population of disulfide-bonded molecules. This monomeric form had a relatively short half-life. After the pulse, most of the label accumulated higher in the gel, i.e., at a molecular weight position of about 40,000, again as a double band. It was converted into the characteristic smear during the chase, clearly representing disulfide-linked heterodimers of M and G_L. This interpretation was sustained by the results obtained with the antipeptide serum directed against M. Analysis under reducing conditions once more displayed the coprecipitation of G_L and M (Fig. 5b, left-hand panel). When DTT was omitted from the sample buffer, three M-related products were resolved (Fig. 5b, right-hand panel), the smallest of which corresponded to the monomeric M protein, since it comigrated with this protein analyzed under reducing conditions. The largest product was processed during the chase into diffusely migrating material and thus represents the G_L/M heterodimer. The band of intermediate mobility most likely consists of disulfide-linked homodimers of the M protein. The possibility that the putative G_L/M complex actually consists of one G_L molecule which is covalently coupled to a disulfide-linked homodimer of M can be ruled out since the M protein contains only one cysteine.

The amount of G_L protein coprecipitated by the M-specific antipeptide serum diminished in the course of the chase, while the M protein brought down by the G_L-specific antiserum increased concomitantly. These observations may relate to the stability of M (Fig. 5b, left-hand panel), which apparently remains association competent and allows interactions with G_L long after its synthesis. The gradual decrease in the amount of monomeric M protein during the chase supports this conception and suggests that this form is the precursor to the heterodimer. Accordingly, the intensity of the homodimeric species did not change much. Similarly, the amount of labeled G_L/M heterodimers did not significantly decrease between min 60 and 120 of the chase, while the amount of G_L was clearly reduced during this period. These observations provide further evidence for an association of preexisting M molecules with newly synthesized G_L molecules.

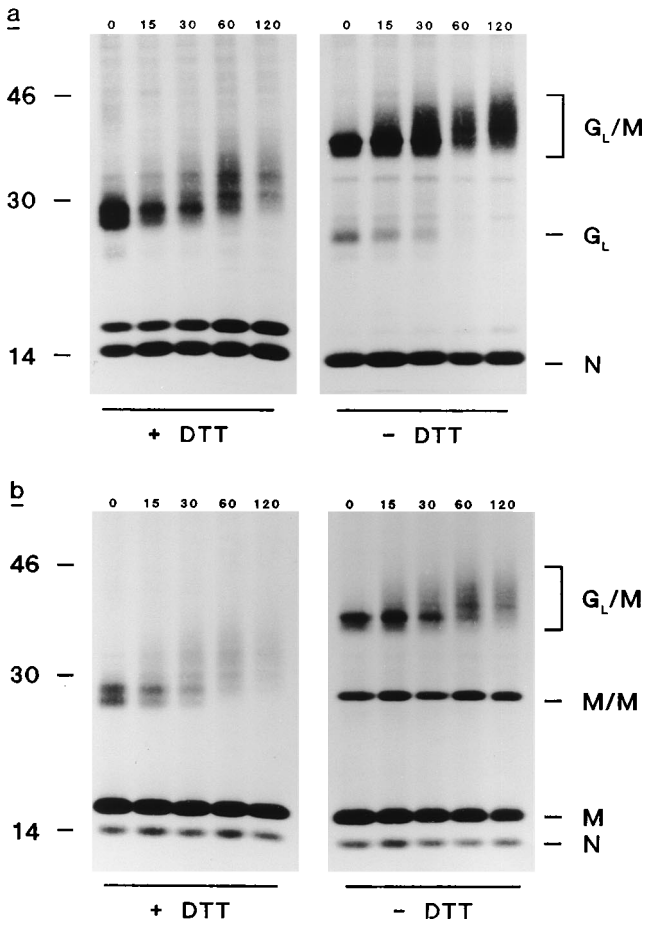

FIG. 5. Formation of G_L/M complexes in EAV-infected BHK-21 cells. EAV-infected BHK-21 cells were pulse-labeled for 15 min with L-[^{35}S]-in vitro cell labeling mixture at 8.5 h p.i. and chased with medium containing 5 mM nonradioactive methionine for the times indicated (in minutes) above the lanes. The G_L (a) and M (b) proteins were then precipitated from combined lysates of cells and medium with specific antisera, and the immunoprecipitates were divided into two equal parts. One half was analyzed by SDS-PAGE under reducing conditions (left-hand panels), and the other half was analyzed in the same gel under nonreducing conditions (right-hand panels). The positions of the N, M, and G_L monomers and the homodimeric M protein are indicated by dashes; the brackets depict the size limits of the G_L/M heterodimer. The numbers on the left show size in kilodaltons.

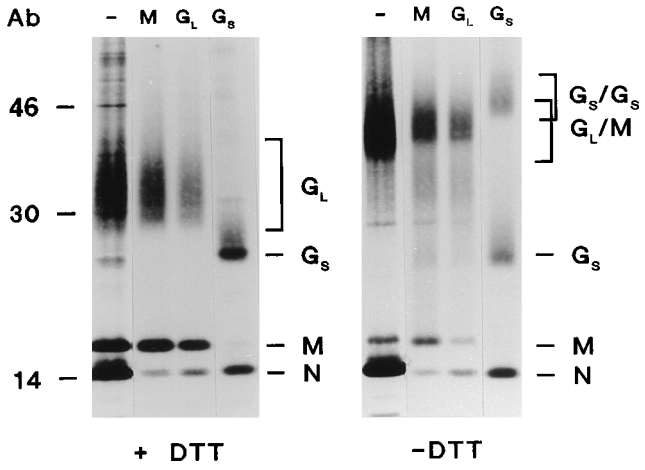

FIG. 6. Analysis of disulfide bonds between the envelope proteins in extracellular virions. EAV-infected BHK-21 cells were labeled for 4 h with L-[^{35}S]-in vitro cell labeling mixture, starting at 8.5 h p.i., and were then chased for 1 h. After removal of cell remnants by low-speed centrifugation, the medium was mixed with a one-fourth volume of concentrated lysis buffer and subjected to immunoprecipitation with M-, G_L-, and G_S-specific antipeptide sera. To compensate for the low abundance of the G_S protein in virus particles, five times more medium was incubated with the G_S-specific antipeptide serum than with the other sera. The immunoprecipitates were split into two halves and analyzed next to sucrose-gradient-purified virus (−) in an SDS–15% PAA gel under reducing (left-hand panel) and nonreducing (right-hand panel) conditions. The positions of the N, M, and G_S proteins are indicated by dashes; the brackets depict the size limits of the heterogeneously glycosylated G_L protein, the G_L/M heterodimer, and the homodimeric G_S protein. The numbers on the left show size in kilodaltons. Ab, antibody.

To exclude the possibility that M homodimers and G_L/M heterodimers arose during or after cell disruption as a result of the incomplete modification of reactive thiol groups by IAA, we performed a control experiment. EAV-infected cells were labeled with [^{35}S]methionine in the presence or absence of 5 mM DTT; addition of the reducing agent to the culture medium is known to prevent the formation of disulfide bonds in vivo (4). After labeling, the cells were washed twice with phosphate-buffered saline containing 50 mM IAA, lysed in the presence of the alkylating agent, and processed for immunoprecipitation with the antipeptide sera; thereafter, the immune complexes were analyzed under nonreducing conditions. From the DTT-treated cells, only G_L and M monomers were recovered whereas the lysate from untreated cells also yielded M homodimers and G_L/M heterodimers (data not shown). These results demonstrate that IAA was fully effective in blocking reactive sulfhydryl groups and confirm that the M homodimers and G_L/M heterodimers truly exist in EAV-infected cells.

G_L and M occur in heterodimeric complexes in virions. We finally wanted to establish the complexity of the G_L and M proteins in extracellular virions. A preparation of [^{35}S]methionine-labeled virus was used for analysis under reducing and nonreducing conditions without protective alkylation, either directly or after immunoprecipitation with G_L- or M-specific antibodies. Most G_L and M molecules were present in virions as heterodimers, while only small fractions of M and G_L were found in the monomeric form (Fig. 6). These monomers may have been generated artifactually during the experiment; G_S, which is present in virions exclusively as disulfide-linked homodimers (9), also appeared partly in its monomeric form under the experimental conditions used (Fig. 6).

DISCUSSION

We have previously demonstrated that the EAV membrane glycoprotein G_S occurs in virions in a homodimeric form (9). We now have shown that the other envelope proteins (G_L and M) exist as heterodimers. In infected cells, these proteins associate with different kinetics and efficiencies. Newly synthesized G_L molecules rapidly interact with M and are soon quantitatively incorporated into complexes. In contrast, de novo-synthesized M protein is converted into heterodimers slowly. The M molecules appear partly as homodimers, but most of them remain in their monomeric form. In the monomeric form, they remain association competent and continue to be recruited into complexes with newly synthesized G_L molecules.

The efficient recruitment of G_L molecules into G_L/M heterodimers requires the M protein to be present in the ER at high concentrations. Immunofluorescence studies indeed showed the M protein to accumulate in the ER (data not shown). The slow kinetics of incorporation of M into G_L/M complexes is consistent with newly synthesized M molecules entering a large pool of presynthesized M protein in the ER. The phenomenon of different viral structural proteins entering complexes at un-

equal rates is not without precedent. Opstelten et al. (35) found that the M protein of mouse hepatitis virus entered noncovalently linked M/S complexes almost immediately after its synthesis whereas S started to appear in such complexes 10 to 20 min later. For mouse hepatitis virus, folding of the large S protein appeared to be rate limiting. The fast assembly of the G_L protein into G_L/M heterodimers indicates that a similar situation does not exist for EAV, as might have been anticipated on the basis of the small size of the polypeptide.

Our data do not show whether formation of the G_L/M heterodimers is a prerequisite for rather than a consequence of the virus assembly process. Double-expression experiments using the vaccinia virus T7 transient expression system (17) have revealed that in the absence of other EAV components G_L and M can form disulfide-linked heterodimers, albeit with a low degree of efficiency (data not shown). It is also unknown whether G_L/M complexes are incorporated quantitatively into virions. If not, then the fraction excluded from viral particles is efficiently transported through the Golgi complex on its own, as judged from the rapid and complete processing of the N-linked oligosaccharide side chain of G_L. We speculate that heterodimerization is mandatory for the transport of the complexes along the secretory pathway. If free G_L/M heterodimers are indeed capable of leaving the ER, then other viral components must be important in determining the budding site of EAV.

From our experiments, it follows that the degradation of the G_L protein occurs primarily in a post-Golgi complex area and involves (only) G_L molecules that are covalently linked to the M protein. However, the exact status of the labile G_L molecules remains obscure. The decrease in the total amount of G_L may result from the endosomal and/or lysosomal degradation of free G_L/M heterodimers, cell-associated virions, and reinternalized virus particles but may also involve the degradation of secreted virions by extracellular proteases. In this context, it is interesting that the cytoplasmic tails of the M proteins of all arteriviruses sequenced to date accommodate putative leucine- and/or tyrosine-based endosomal-lysosomal sorting motifs (42). Evidence for the degradation of complete virus particles came from a pulse-chase experiment in which we studied the transport and secretion of the G_S protein. While the G_S protein is supposed to leave the ER only after its incorporation into virions (9), a marked reduction in the total amount of endo H-resistant G_S molecules was evident between min 120 and 240 of the chase (data not shown).

Virion assembly requires that the structural proteins are incorporated in constant relative amounts. The formation of G_L/M heterodimers may allow the virus to control the molar ratio of the two major envelope proteins in virions. We have previously shown that G_L and M are indeed present in EAV particles in roughly equimolar amounts (8). Electron microscopic images of EAV have shown small ringlike surface substructures (30) which may be formed by the G_L/M heterodimeric units. Recently, Faaberg et al. (16) showed that the two major envelope proteins of lactate dehydrogenase-elevating virus (VP-3 and VP-2 or M) also form disulfide-linked heterocomplexes and speculated that the cylindrical structures which are released from the viral envelope during the manipulation of virions are composed of these heterodimers.

In infected cells, but not in virions, a significant fraction of the M protein is present in the form of homodimers. It is unclear whether these structures function as intermediates in the formation of G_L/M heterodimers or merely constitute dead-end products. If they act as intermediates, their presence in a constant amount over extended periods would suggest a controlled balance between their formation and their conversion into heterodimers. If they are dead-end products, a certain small fraction of the M molecules would dimerize rapidly during or immediately after synthesis. These dimers might then be withdrawn from the productive folding process through the formation of noncovalent complexes. The participation of Cys-8 of the M protein in the formation of an intermolecular disulfide bond with G_L or with itself further suggests that the amino terminus of M is exposed to the oxidizing environment of the ER and is consistent with the $N_{exo}C_{cyt}$ transmembrane orientation proposed for this protein (6).

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