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Identification and Characterization of a Sixth Structural Protein of Lelystad Virus:

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Previously we have shown that Lelystad virus (LV) contains five structural proteins, a nucleocapsid protein N, an integral membrane protein M, and three glycoproteins GP_3, GP_4, and GP_5. In this study we identified a sixth structural protein of Lelystad virus. The protein has an apparent molecular weight of 29 to 30 kDa, was recognized by an ORF2-specific antipeptide serum in Western immunoblotting using sucrose gradient purified LV virions, and was shown to be *N*-glycosylated. It was therefore designated GP_2. The GP_2 protein was also immunoprecipitated by ORF2-specific antipeptide serum from lysates and extracellular virus of CL2621 cells infected with LV. A fraction of the GP_2 protein present in these lysates contained an intrachain disulfide bond. Endoglycosidase H treatment of immunoprecipitates indicated that the endoglycosidase H-sensitive *N*-glycans of the GP_2 protein become endoglycosidase H-resistant during passage through the Golgi compartment in cells infected with LV. In contrast, the *N*-glycans of the GP_2 protein expressed individually in a recombinant Semliki Forest virus remained endoglycosidase H-sensitive, indicating that the GP_2 protein was retained in the endoplasmic reticulum. LV is the first arterivirus which has been demonstrated to contain six virion-associated proteins, one nucleocapsid protein, one integral membrane protein, and four glycoproteins.

INTRODUCTION

Lelystad virus (LV) is the European prototype of porcine reproductive and respiratory syndrome virus and causes respiratory problems in pigs and abortions in sows (Wensvoort *et al.,* 1991). LV belongs to a family of enveloped positive-strand RNA viruses called arteriviruses. Other viruses in this family are equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (Meulenberg *et al.,* 1993; Plagemann and Moennig, 1993). Arteriviruses resemble togaviruses in their physicochemical properties and virion morphology, but resemble coronaviruses in their genome organization and replication strategy.

LV contains a positive-strand RNA genome of 15.1 kb, and during replication a nested set of six subgenomic RNAs is synthesized (Meulenberg *et al.,* 1993; Conzelmann *et al.,* 1993). The genes located at the 5′ end of the viral genome (ORFs 1a and 1b) occupy approximately 80% of the viral genome and encode the RNA-dependent RNA polymerase. Six smaller ORFs (ORFs 2 to 7) are located at the 3′ end of the viral genome. ORFs 3 to 7 encode structural proteins of LV. The 15-kDa nucleocapsid protein (N) encoded by ORF7, the 18-kDa integral membrane protein (M) encoded by ORF6, and the major 25-kDa envelope glycoprotein (GP_5) were identified in virions with gene-specific antipeptide sera (Meulenberg *et al.,* 1995). Similar proteins have also been detected in lysates of cells infected with a North American isolate, VR2332, which is antigenically different from LV (Nelson *et al.,* 1993). Recently we isolated monoclonal antibodies (MAbs) directed against LV and used them to demonstrate that GP_3, which is a 45- to 50-kDa protein that is *N*-glycosylated and encoded by ORF3, and GP_4, which is a 31- to 35-kDa protein that is *N*-glycosylated and encoded by ORF4, are also structural glycoproteins (van Nieuwstadt *et al.,* 1996). Because the MAbs directed against GP_4 were neutralizing, at least part of this protein is probably exposed at the surface of the virion.

In this report we describe the identification and characterization of a sixth structural protein, designated GP_2. By Western immunoblotting and immunoprecipitation we demonstrate that GP_2 is a 29- to 30-kDa *N*-glycosylated protein encoded by ORF2 and is incorporated in virus particles. The folding and intracellular transport of the GP_2 protein are analyzed.

MATERIALS AND METHODS

Cells and viruses

The European prototype *Ter Huurne* strain of LV was isolated in 1991 (Wensvoort *et al.,* 1991). To prepare LV virions, we grew LV in CL2621 cells. The virus was concentrated and purified on sucrose gradients as described earlier (van Nieuwstadt *et al.,* 1996).

BHK-21 cells were maintained in Dulbecco's minimal essential medium supplemented with 5% fetal bovine se-

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rum and antibiotics. For transfection experiments, BHK-21 cells were grown in Glasgow minimal essential medium (GIBCO-BRL/Life Technologies Ltd.), according to the method of Liljeström and Garoff (1993).

Expression of GP_2 in recombinant Semliki Forest virus

ORF2 was excised from plasmid pABV210, by digesting with *Sma*I and *Sph*I (partially). This plasmid was shown earlier to express the GP_2 protein properly in *in vitro* transcription and translation experiments (Meulenberg *et al.,* 1995). The ORF2 fragment was treated with Klenow polymerase (Pharmacia) to create blunt ends and was ligated in the *Sma*I site of expression vector pSFV1 Liljeström and Garoff (1993), which was dephosphorylated with calf intestinal alkaline phosphatase (Pharmacia). A plasmid containing ORF2 of LV (pABV262) in the correct orientation was used to obtain recombinant Semliki Forest viruses expressing the GP_2 protein. The plasmids pABV262 and pSFV-Helper2 were linearized with *Spe*I, and RNA was transcribed *in vitro* as described by Liljeström and Garoff (1993). These RNAs were cotransfected into BHK-21 cells by electroporation (Liljeström and Garoff, 1993) and the resulting recombinant virus (SFV-ORF2) was tested for GP_2 expression by immunoprecipitation.

Western blot analysis

Western blot analysis of purified virions of LV was performed as described previously (van Nieuwstadt *et al.,* 1996). The nitrocellulose strips were incubated with MAbs 126.2 (GP_3) and 130.7 (GP_4), GP_5-specific antipeptide serum 704, GP_2-specific antipeptide serum 689, the corresponding preserum 689-p, and porcine anti-LV serum 21 (Meulenberg *et al.,* 1995; van Nieuwstadt *et al.,* 1996). The hybridoma cell culture medium was used undiluted, but the antipeptide sera 704 and 689, the corresponding preserum 689-p, and serum 21 were diluted 1:25, 1:5, 1:5, and 1:50, respectively.

Metabolic labeling of cells and virus

Confluent monolayers of CL2621 cells were infected with LV at a multiplicity of infection of 1, and after 18 hr cells were labeled with 90 μCi L-[^{35}S]methionine/ml methionine-free Eagle's basal medium. The cells were labeled for 4 hr, after which lysates were prepared in PBS-TDS (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 1 m*M* phenylmethylsulfonyl fluoride in PBS), according to Meulenberg *et al.* (1995). For pulse–chase experiments, CL2621 cells were infected with LV at a multiplicity of infection of 1, and after 18 hr cells were starved for 45 min in methionine-free Eagle's basal medium. The cells were pulse-labeled with 600 μCi L-[^{35}S]methionine/ml methionine-free Eagle's basal medium for 30 min, washed in Eagles basal medium containing 5 m*M* nonradioactive L-methionine and 10% fetal bovine serum, and chased in the same medium for various intervals. Lysates were prepared from these cells and were used in immunoprecipitation.

In order to immunoprecipitate GP_2 expressed by SFV-ORF2, we first activated SFV-ORF2 particles by cleaving them with chymotrypsin according to the method of Berglund *et al.* (1993). CL2621 or BHK-21 cells were then inoculated with the activated SFV-ORF2 stock at a multiplicity of infection of 5. The cells were labeled and lysed as described for the CL2621 cells infected with LV.

For the preparation of radiolabeled virus, CL2621 cells were infected with LV at a multiplicity of infection of 1, and after 8 hr they were labeled for 24 hr with 60 μCi L-[^{35}S]methionine/ml Eagle's basal medium containing 0.5 m*M* nonradioactive L-methionine and 1% fetal bovine serum. The culture supernatant was harvested, layered on a sucrose cushion of 0.5 *M* sucrose in TNE, and centrifuged for 6 hr at 26,000 rpm in a SW40 rotor (Beckmann). The pellet of radiolabeled virus was resuspended in TNE and the LV virions were disrupted by adding an equal volume of PBS-TDS.

Immunoprecipitation, endoglycosidase treatment, and gel electrophoresis

Cell lysates and extracellular virus of CL2621 cells infected with LV or SFV-ORF2 were diluted in PBS-TDS and immunoprecipitated with antipeptide sera 687, 689, 689-p, or the N-specific MAb 122.17. Antipeptide serum 687 is directed against amino acids 35 to 50 (CLGSPSQDGYWSFFSE) in the GP_2 protein. This peptide serum was obtained from a rabbit immunized twice with 1 mg peptide conjugated to keyhole limpet hemocyanin, as described previously (Meulenberg *et al.,* 1995). LV virions prepared as described above were diluted in PBS-TDS and were immunoprecipitated in a similar manner as were the lysates, according to the method of Hulst *et al.* (1993). When disulfide bonds were studied, iodoacetamide (IAA, 50 m*M*) or *N*-ethylmaleimide (NEM, 20 m*M*) was added to the PBS-TDS to block free sulfhydryl groups to prevent the formation of disulfide bridges during or after lysis of cells or virus. Immunoprecipitates of LV proteins were either directly resuspended in Laemmli sample buffer (Laemmli, 1970) with (reducing conditions) or without (nonreducing conditions) 10% (v/v) 2-mercaptoethanol, or they were first treated with endoglycosidases before being resuspended. In the latter case, the samples were resuspended in the appropriate endoglycosidase buffer and treated with peptide *N*-glycosidase F (PNGaseF; Boehringer Mannheim) or endo-β-*N*-acetyl-D-glycosaminidase H (EndoH; Boehringer Mannheim) as described previously (Meulenberg *et al.,* 1995). Samples were resuspended in Laemmli sample buffer and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

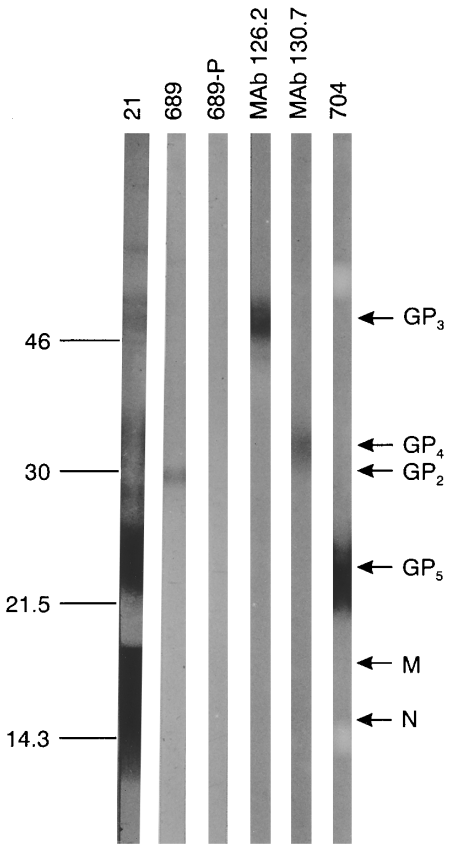

FIG. 1. Western immunoblot analysis of virion proteins of LV. The proteins were separated by SDS–PAGE on a gel containing 12.5% acrylamide under reducing conditions and transferred to nitrocellulose. Nitrocellulose strips, containing 10 μg of the purified virion proteins, were stained with MAb 126.2, MAb 130.7, antipeptide sera 704 and 689, the corresponding preserum 689-p, and porcine anti-LV serum 21. The position of the LV proteins GP_2, GP_3, GP_4, GP_5, M, and N are indicated with arrows. Numbers on the left show the sizes and position of the molecular weight markers.

RESULTS

Identification of the ORF2 gene product in virions of LV

To further examine structural proteins of LV, virions were purified and analyzed by Western blots. As shown earlier, MAb 126.2 recognized the 45- to 50-kDa GP_3 protein, MAb 130.7 recognized the 31- to 35-kDa GP_4 protein, antipeptide serum 704 recognized the 25-kDa GP_5 protein, whereas porcine anti-LV serum 21 recognized all three of these glycoproteins as well as the 18-kDa M protein and the 15-kDa N protein (Fig. 1; Meulenberg *et al.,* 1995; van Nieuwstadt *et al.,* 1996). The M and N proteins migrated as a smear because of the large amount of viral protein loaded in the gel. Although antipeptide sera 689 and 690, which were obtained from two rabbits immunized with the same peptide derived from the amino acid sequence of ORF2 (Meulenberg *et al.,* 1995), recognized *in vitro* translation products of ORF2 in the immunoprecipitation assay, we were unable to identify specific proteins with these sera in Western blot analysis in previous experiments. In these experiments 1 to 2 μg protein of the purified LV preparation was used per nitrocellulose strip, and the peptide serum was diluted 1:50. The improvement of virus growth and purification methods enabled us to repeat this experiment with 10 μg virion protein per nitrocellulose strip. This strip was stained with serum 689 diluted 1:5, and a protein with an apparent molecular weight of 29 to 30 kDa was observed. This protein was barely distinguishable in size from the GP_4 protein (Fig. 1). The 29- to 30-kDa protein was also stained by serum 690, although the staining was less intense (data not shown), but it was not stained by the corresponding preserum 689-p (Fig. 1). Hence, these results indicated that the 29- to 30-kDa protein encoded by ORF2 is a minor structural protein of LV.

N-glycosylation of the gene product of ORF2 of LV

The processing of the gene product of ORF2 was studied by immunoprecipitation and endoglycosidase treatment. In previous experiments we were unable to immunoprecipitate a specific protein with serum 689 from lysates of CL2621 cells that were infected with LV at a multiplicity of infection of 0.1 and that were labeled between 24 and 40 hr after infection. However, a specific 28-kDa protein and a faint, more diffuse species of 29 to 30 kDa could be immunoprecipitated from lysates of CL2621 cells infected at a multiplicity of infection of 1 and labeled between 18 and 22 hr after infection (Fig. 2a). These proteins were not immunoprecipitated by the corresponding preserum 689-p from lysates of LV-infected CL2621 cells. Some background bands were observed at about 46 kDa, which appeared to be caused by nonspecific reactions, since they were also recognized by preserum 689-p. The 28-kDa gene product of ORF2 recognized by serum 689 in the lysates was smaller than the gene product recognized in extracellular virus of CL2621 cells infected with LV (29 to 30 kDa; Fig. 2a). The 15-kDa N protein was nonspecifically immunoprecipitated from the extracellular virus by serum 689 and 689-p. Nonspecific immunoprecipitation of the N protein of LV was observed earlier, if N was present in large amounts in the sample, and it seems to be a common phenomenon, also reported for the EAV N protein (Deregt *et al.,* 1994). We do not understand why fewer N proteins were coprecipitated from the lysates. Perhaps the ratio of the N protein and the ORF2 gene product is higher in the extracellular virus than in the cell lysate of CL2621 cells infected with LV. This was also demonstrated for the N protein and the gene product of ORF2 (G_s) of EAV (de Vries *et al.,* 1992).

The gene product of ORF2, which was immunoprecipitated from the lysates and extracellular virus of CL2621 cells infected with LV, was treated with PNGaseF and EndoH and analyzed by SDS–PAGE. The size of this 28-kDa gene product was decreased to 24 kDa by PNGase

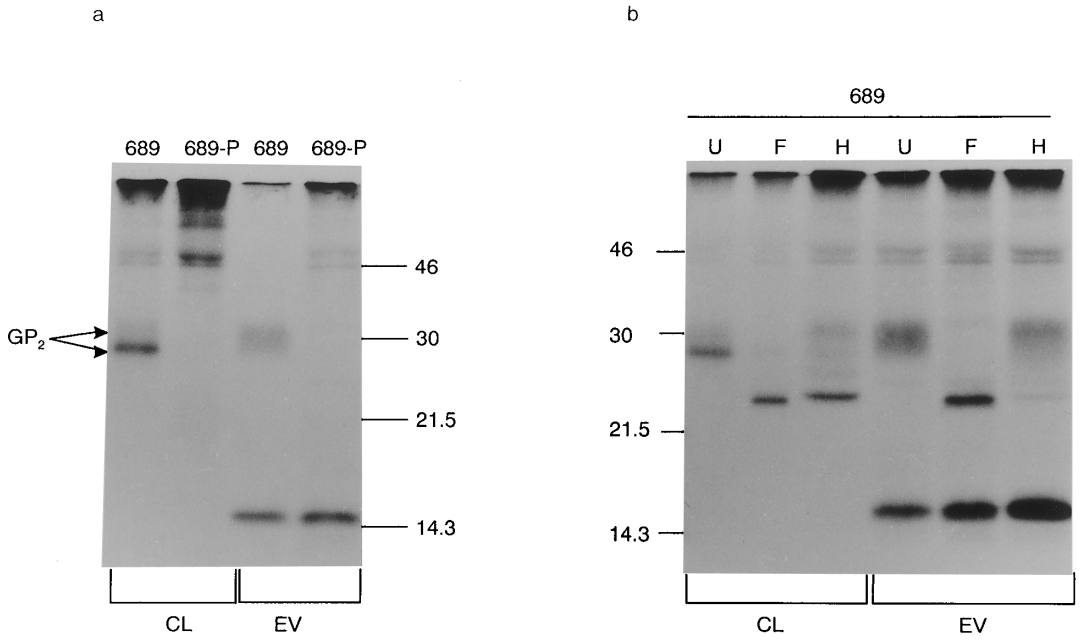

FIG. 2. Immunoprecipitation of GP_2 from lysates and extracellular virus of CL2621 cells infected with LV. For the preparation of radiolabeled cell lysates, CL2621 cells were infected with LV, and after 18 hr they were labeled for 4 hr with L-[^{35}S]methionine. For the preparation of radiolabeled virus, CL2621 cells were labeled with L-[^{35}S]methionine between 8 and 32 hr after infection with LV. (a) Cell lysates (CL) and extracellular virus (EV) of CL2621 cells infected with LV were immunoprecipitated with ORF2-specific antipeptide serum 689 and the corresponding preserum 689-p. (b) Immunoprecipitation of GP_2 with serum 689 from cell lysates (CL) and extracellular virus (EV) of CL2621 cells infected with LV. The immunoprecipitated GP_2 protein was either untreated (U), PNGaseF treated (F), or EndoH treated (H). The samples were analyzed in a SDS–12.5% polyacrylamide gel under reducing conditions. The position of the molecular weight markers is indicated on the left.

F (Fig. 2b). The size of the 28-kDa protein was also reduced to 24 kDa, but the diffuse smear of 29 to 30 kDa was EndoH resistant. The size of the 29- to 30-kDa protein immunoprecipitated from extracellular virus was also decreased to 24 kDa after treatment with PNGaseF, but did not change after treatment with EndoH (Fig. 2b). Therefore we concluded that the protein encoded by ORF2 is an *N*-glycosylated structural protein that acquires complex type *N*-glycans. This protein was designated GP_2. The difference in size (approximately 6 kDa) between the glycosylated and the unglycosylated forms of the protein suggests that both of the putative *N*-glycosylation sites identified in the amino acid sequence of ORF2 are used *in vivo.*

A pulse–chase experiment was performed to examine the acquisition of EndoH-resistant *N*-glycans by the GP_2 protein in time. The apparent molecular weight of the 28-kDa GP_2 protein that was immunoprecipitated from cells infected with LV after the pulse increased during the chase, resulting in a more diffuse protein of approximately 29 to 30 kDa (Fig. 3). Whereas the *N*-glycans of the GP_2 protein remained PNGase sensitive throughout the chase, the GP_2 protein faded and acquired EndoH-resistant *N*-glycans. Another partially EndoH-resistant 28-kDa form of the GP_2 protein became visible during the chase. This might be an intermediate containing one EndoH-resistant and one EndoH-sensitive *N*-glycan or might be due to incomplete digestion.

The GP_2 protein was also immunoprecipitated from CL2621 cells infected with SFV-ORF2. Although SFV-ORF2 infected the CL2621 cells less efficiently than the BHK-21 cells, sufficient amounts of GP_2 could be immunoprecipitated to compare the processing of this recombinant GP_2 protein with the processing of GP_2 in CL2621 cells infected with LV in a pulse–chase experiment. The apparent molecular weight of the GP_2 protein expressed by SFV-ORF2 was similar to that of GP_2 expressed in CL2621 cells infected with LV (Fig. 4 compared to Fig. 3). However, unlike the latter, it remained EndoH-sensitive throughout the chase (Fig. 4). In addition to the 24-kDa unglycosylated form of GP_2, observed earlier (Fig. 3), a fainter band of approximately 26 kDa was observed in all samples digested with EndoH. This 26-kDa band most likely results from incomplete EndoH digestion. The results suggested that the GP_2 protein expressed individually by SFV-ORF2 is retained in the endoplasmic reticulum (ER) and that it must interact with other viral proteins to be transported through the Golgi complex. The GP_2 protein expressed by SFV-ORF2 was also recognized by serum 21 in immunoprecipitation (data not shown). This further supported our finding that GP_2 is a minor structural protein.

Disulfide bonds in GP_2

The mobility of the GP_2 protein immunoprecipitated from cell lysates and extracellular medium was also stud-

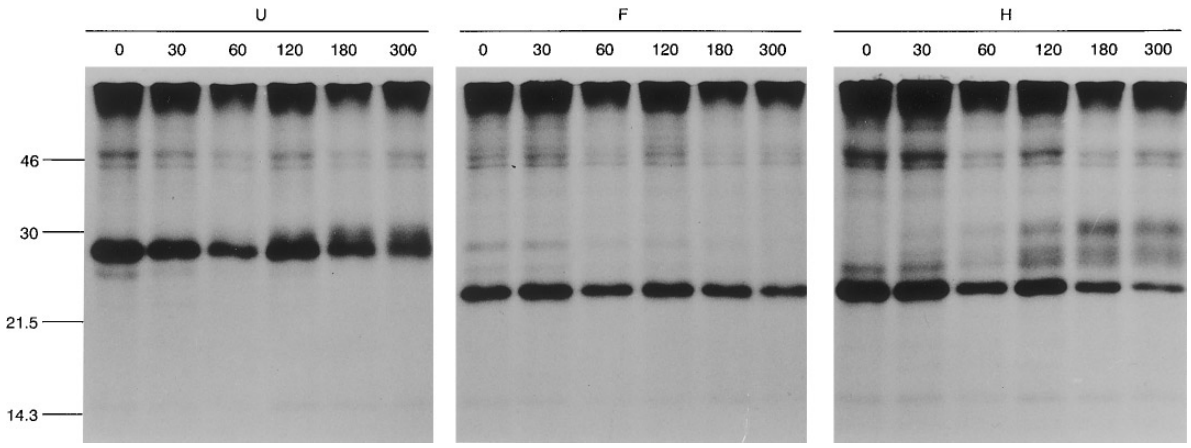

FIG. 3. Acquisition of EndoH resistance by the GP_2 protein. CL2621 cells were infected with LV, and 18 hr after infection they were pulse-labeled for 30 min with L-[^{35}S]methionine and chased in medium containing 5 m*M* nonradioactive methionine. The length of the chase is indicated in minutes above the lanes. Lysates were prepared and immunoprecipitated with antipeptide serum 689. The immunoprecipitated proteins were treated with PNGaseF (F) or EndoH (H) or were left untreated (U) before analysis by SDS–PAGE on a gel containing 12.5% acrylamide under reducing conditions. The numbers on the left show the sizes of marker proteins in kDa.

ied under nonreducing conditions by leaving out the 2-mercaptoethanol from the sample buffer. No difference in migration of GP_2 immunoprecipitated from extracellular virus was observed when GP_2 was analyzed by SDS–PAGE under nonreducing conditions compared to reducing conditions (Fig. 5). It may be that GP_2 dimers are present in virions of LV but are not recognized by the antipeptide serum 689, which could happen if the binding site in the GP_2 dimer is inaccessible to the antibodies of the antipeptide serum. However, this seems unlikely, because we also detected only monomeric GP_2 proteins after immunoprecipitation of extracellular virus with peptide serum 687, which is directed to a different site (Fig. 5). Hence, we conclude that the GP_2 protein does not form dimers by means of disulfide bridges. The N protein, which was nonspecifically coprecipitated from extracellular virus by sera 687 and 689 and which migrated at 15 kDa under reducing conditions, could not be detected under nonreducing conditions (Fig. 5), because disulfide-linked dimers of the N protein were present. These di-

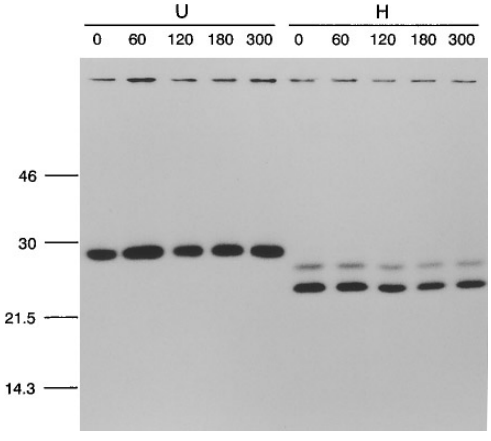

FIG. 4. Processing of GP_2 expressed by SFV-ORF2. The CL2621 cells were infected with SFV-ORF2, and 18 hr after infection they were pulse-labeled for 30 min with L-[^{35}S]methionine and chased in medium containing 5 m*M* nonradioactive methionine. The length of the chase is indicated in minutes above the lanes. Lysates were prepared and immunoprecipitated with antipeptide serum 689. The immunoprecipitated proteins were treated with EndoH (H) or were left untreated (U) before analysis by SDS–PAGE on a gel containing 12.5% acrylamide under reducing conditions. The numbers on the left show the sizes of marker proteins in kDa.

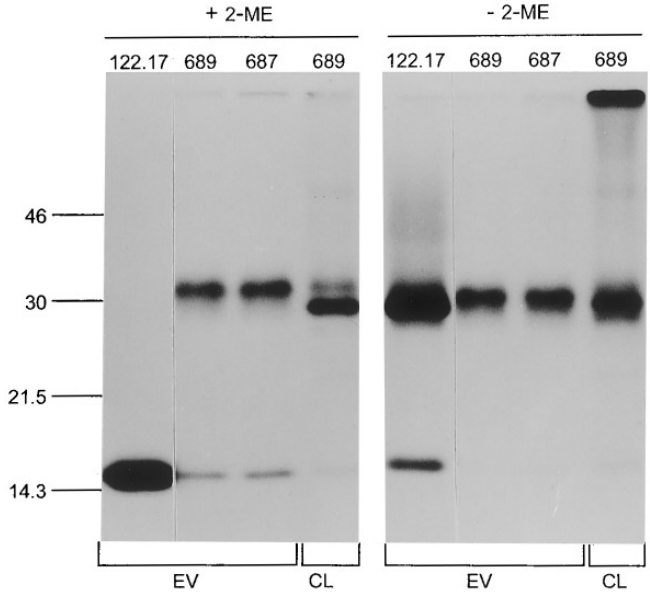

FIG. 5. SDS–PAGE of GP_2 under reducing and nonreducing conditions. Lysates (CL) and extracellular virus (EV) of CL2621 cells infected with LV and labeled with L-[^{35}S]methionine were immunoprecipitated with ORF2-specific antipeptide sera 687 and 689, and N-specific MAb 122.17. Immunoprecipitates were resuspended in Laemmli loading buffer with (reducing, +2-ME) or without (nonreducing, −2-ME) 10% (v/v) 2-mercaptoethanol. Samples were analyzed by SDS–PAGE on a gel containing 12.5% acrylamide. The numbers on the left show the sizes of marker proteins in kDa.

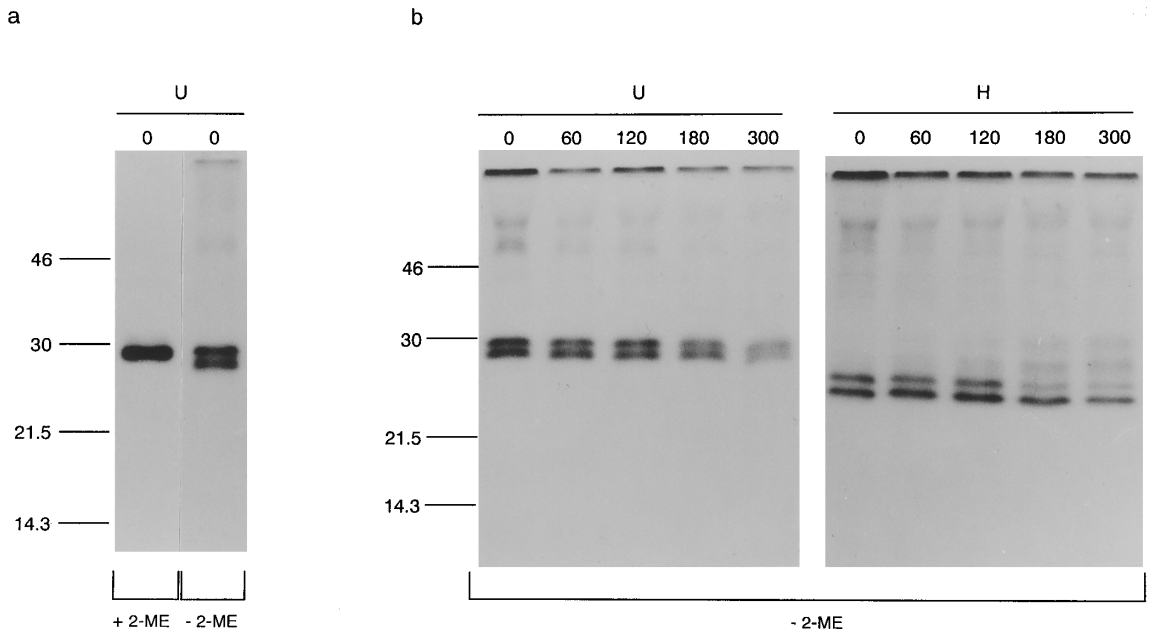

FIG. 6. Disulfide bonds detected in the GP_2 protein in CL2621 cells infected with LV. The CL2621 cells were infected with LV, and 18 hr after infection they were pulse-labeled for 30 min with L-[^{35}S]methionine and chased in medium containing 5 m*M* nonradioactive methionine. The length of the chase is indicated in minutes above the lanes. Cells were lysed in lysis buffer (PBS-TDS) containing 50 m*M* IAA and the lysates were immunoprecipitated with serum 689. (a) The immunoprecipitated GP_2 protein was analyzed by SDS–PAGE under reducing (+2-ME) and nonreducing (−2-ME) conditions. (b) The immunoprecipitated GP_2 protein was treated with EndoH (H) or was left untreated (U) before analysis by SDS–PAGE on a gel containing 12.5% acrylamide under nonreducing conditions. The numbers on the left show the sizes of marker proteins in kDa.

mers comigrated with the GP_2 protein at 29 kDa, as was shown by immunoprecipitation of extracellular virus with MAb 122.17, which is specific for N (Fig. 5). Although no slower migrating species were observed after immunoprecipitation of GP_2 from cell lysates of CL2621 cells infected with LV, a diffuse and slightly faster migrating protein species was observed under nonreducing conditions (Fig. 5). Since the mobility of proteins in SDS–PAGE increases as the protein acquires more compact conformations stabilized by disulfide bonds, we reasoned that intramolecular disulfide bonds are formed in GP_2. Therefore we investigated the GP_2 protein in another pulse–chase experiment, in which we lysed the LV-infected cells in the presence of 50 m*M* iodoacetamine (IAA) or 20 m*M* *N*-ethylmaleimide (NEM). These alkylating agents block free sulfhydryl groups and thus prevent nonnative disulfide bridges from forming during or after lysis of the cells. It has been shown that the resolution of multiple differentially disulfide-bonded species of viral glycoproteins is improved when artifactual disulfide bond formation is inhibited through IAA and NEM (de Vries *et al.,* 1995; Mulvey and Brown, 1994). The GP_2 protein was immunoprecipitated from CL2621 cells infected with LV, pulse-labeled for 30 min with L-[^{35}S]methionine, and lysed in the presence of IAA. Analysis of the sample under nonreducing conditions revealed two distinct forms of the protein (Fig. 6a). Identical results were obtained when NEM was added to the lysis buffer (data not shown). The two GP_2 forms differed only in their disulfide-bonded structure since they migrated as a single band of about 28 kDa under reducing conditions. The upper band observed under nonreducing conditions comigrated with the single GP_2 band detected under reducing conditions and therefore probably lacks intrachain disulfide bridges. The faster migrating form is expected to contain one intramolecular disulfide bridge, because the amino acid sequence of GP_2 contains two luminal cysteine residues, provided that the signal sequence is cleaved off between amino acids 29 and 30, according to von Heijne's rule (1986). Both species were sensitive to EndoH directly after the pulse and were converted during the chase to the same EndoH-resistant species, observed in the pulse–chase experiment that lacked IAA in the lysis buffer (Fig. 6b compared to Fig. 3). The intensity of the slower migrating, reduced form of GP_2 decreased somewhat faster during the chase than the faster migrating disulfide-bonded form of GP_2. This finding suggested that the reduced form of GP_2 is more transport-competent than the disulfide-bonded form. The reduced form of GP_2 is transported from the ER to the Golgi, where its *N*-glycans are modified to complex type *N*-glycans, whereas the disulfide-bonded form is mainly retained in the ER. The GP_2 protein immunoprecipitated from CL2621 cells infected with SFV-ORF2 also displayed two forms under nonreducing conditions (data not shown). These two forms remained equally EndoH-sensitive during the chase. These findings further supported the assumption that the reduced form of GP_2 is trans-

ported from the ER through the Golgi as long as other structural proteins of LV are present.

GP_2 dimers were not identified in CL2621 cells infected with LV and lysed in the presence of IAA or NEM. Furthermore, when GP_2 was immunoprecipitated from extracellular virus after disruption in lysis buffer with or without IAA or NEM, no dimeric or disulfide-bonded monomeric forms were detected under nonreducing conditions (data not shown). Hence, although an intramolecular disulfide bond may be formed during synthesis of GP_2, this disulfide bond was absent in the monomeric GP_2 proteins detected in virus particles.

DISCUSSION

In this study we identified and characterized a new virus-specific protein in CL2621 cells infected with LV and in LV virions. Our results demonstrate that the 29- to 30-kDa GP_2 protein encoded by ORF2 is a fourth virion-associated glycoprotein, in addition to GP_3, GP_4, and GP_5. The amino acid sequence of this protein shows features reminiscent of typical class I integral membrane glycoproteins. It contains an N-terminal signal sequence, a C-terminal hydrophobic sequence that may function as a membrane anchor, and two putative *N*-glycosylation sites. We have shown that most likely both *N*-glycosylation sites are used *in vivo* and that the high mannose oligosaccharides are converted very slowly to complex-type oligosaccharides before or during incorporation of GP_2 into virus particles. No disulfide-linked oligomers of GP_2 were detected in the lysates or extracellular virus of CL2621 cells infected with LV. We cannot exclude the possibility that multimeric forms of GP_2 are nonetheless present in virions, but are somehow disrupted when the viral envelopes are treated with detergent. The association of viral membrane proteins into homo- or hetero-oligomers by means of noncovalent interactions is commonly observed (Doms *et al.,* 1993). Interestingly, our findings are in contrast with those for the G_s protein encoded by ORF2 of EAV. The G_s protein was shown to be a minor structural glycoprotein incorporated in virus particles as a disulfide-linked dimer (de Vries *et al.,* 1992 and 1995). Furthermore, four monomeric species generated by the formation of alternative intrachain disulfide bridges were detected in lysates but not in the extracellular virus of BHK-21 cells infected with EAV. In about half of the GP_2 molecules present in lysates of CL2621 cells infected with LV or SFV-ORF2, an intramolecular disulfide bridge was formed between the two cysteines present. The monomeric GP_2 form lacking intrachain disulfide bonds appeared to be the most transport-competent species and appeared to be the precursor of GP_2 incorporated in virus particles. This is an interesting and surprising finding since proper disulfide bond formation is usually a prerequisite for transport competence. The formation of disulfide bridges within transmembrane and secretory proteins typically occurs within the ER and is an intrinsic part of the folding process, catalyzed by the enzyme protein disulfide isomerase (Bardwell and Beckwith, 1993; Freedman, 1993). The correct folding and transport of viral membrane proteins in the ER is often drastically impaired by *in vivo* reduction, as has been shown for the influenza HA protein (Braakman *et al.,* 1992), the vesicular stomatitis virus G protein (de Silva *et al.,* 1993), and the mouse hepatitis coronavirus S protein (Opstelten *et al.,* 1993).

In addition to the difference in dimerization, the rate at which the GP_2 proteins of LV and EAV are transported through the Golgi compartment, as measured by the acquirement of EndoH-resistant N-linked oligosaccharide chains, also appeared to differ. Pulse–chase experiments indicated that most of the N-linked glycans of GP_2 of LV were EndoH-resistant after a chase of 300 min, whereas de Vries *et al.* (1995) found that the *N*-glycans of G_s were already completely EndoH-resistant after a chase of 120 min. However, this difference might be caused by the fact that different cell types were used for these experiments, namely CL2621 cells for LV and BHK-21 cells for EAV. The GP_2 protein expressed individually by SFV-ORF2 was retained in the ER, suggesting that other structural proteins that are probably involved in virus assembly are needed for proper transportation of the GP_2 protein through the Golgi. This was also observed for the G_s protein of EAV by de Vries *et al.* (1995).

LV is the first arterivirus that has now been shown to contain six structural proteins, four glycoproteins (designated GP_2, GP_3, GP_4, and GP_5), an integral membrane protein (M), and a nucleocapsid protein (N). De Vries *et al.* (1992) identified four structural proteins of EAV. These were the glycoprotein G_l encoded by ORF5, the glycoprotein G_s encoded by ORF2, the M protein encoded by ORF6, and the N protein encoded by ORF7. VP1/N encoded by ORF7, VP2/M encoded by ORF6, and the major glycoprotein VP3 encoded by ORF5 were shown to be incorporated in virions of LDV (Harty and Plagemann, 1988, Faaberg *et al.,* 1995). The next steps in LV research must be to assess the function of the four structural glycoproteins of LV in virus assembly, cell attachment, and infection.

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