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Posttranslational Processing and Identification of a Neutralization Domain of the GP_4 Protein Encoded by ORF4 of Lelystad Virus

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GP_4 is a minor structural glycoprotein encoded by ORF4 of Lelystad virus (LV). When it was immunoprecipitated from cell lysates and extracellular virus of CL2621 cells infected with LV, it was shown to have an apparent molecular mass of approximately 28 and 31 kDa, respectively. This difference in size occurred because its core *N*-glycans were modified to complex type *N*-glycans during the transport of the protein through the endoplasmic reticulum and Golgi compartment. A panel of 15 neutralizing monoclonal antibodies (MAbs) reacted with the native GP_4 protein expressed by LV and the recombinant GP_4 protein expressed in a Semliki Forest virus expression system. However, these MAbs did not react with the GP_4 protein of U.S. isolate VR2332. To map the binding site of the MAbs, chimeric constructs composed of ORF4 of LV and VR2332 were generated. The reactivity of these constructs indicated that all the MAbs were directed against a region spanning amino acids 40 to 79 of the GP_4 protein of LV. Six MAbs reacted with solid-phase synthetic dodecapeptides. The core of this site consists of amino acids 59 to 67 (SAAQEKISF). Comparison of the amino acid sequences of GP_4 proteins from various European and North American isolates indicated that the neutralization domain spanning amino acids 40 to 79 is the most variable region of GP_4. The neutralization domain of GP_4, described here, is the first identified for LV.

Lelystad virus (LV) is the etiological agent of a disease, currently called porcine reproductive and respiratory syndrome, which causes abortions in sows and respiratory distress in piglets. It was first isolated in Europe in 1991 (33), whereas a virus that causes similar clinical symptoms was isolated in the United States in 1992 and was designated porcine reproductive and respiratory syndrome virus (4). LV is a small, enveloped virus containing a positive-strand RNA genome. It grows preferentially in macrophages but can also grow in vitro in cell line CL2621 and other cell lines cloned from the monkey kidney cell line MA-104 (3, 4, 15). The genome of LV, a polyadenylated RNA of approximately 15 kb, was first sequenced in 1993 (5, 24). The nucleotide sequence, genome organization, and replication strategy indicated that LV is related to a group of small enveloped positive-strand RNA viruses, designated arteriviruses (24). This new family of viruses includes lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV), and simian hemorrhagic fever virus. Arteriviruses contain a genome of 12.5 to 15 kb and synthesize a 3′ nested set of six subgenomic RNAs during replication (6, 16, 23, 29, 34). These subgenomic RNAs contain a leader sequence which is derived from the 5′ end of the viral genome. Open reading frame 1a (ORF 1a) and ORF1b make up approximately two-thirds of the viral genome and encode the RNA-dependent RNA polymerase. Six smaller ORFs, ORF2 to ORF7, are located at the 3′ end of the viral genome. ORF2 to ORF6 probably encode envelope proteins, whereas ORF7 encodes the nucleocapsid protein (8–10, 26).

LV is the first arterivirus for which it has been demonstrated that all six proteins encoded by ORF2 to ORF7 are associated with the virion (25, 31). The 15-kDa N protein (encoded by ORF7) and the 18-kDa integral membrane protein M (ORF6) are not N glycosylated, in contrast to the 29- to 30-kDa GP_2 protein (ORF2), the 45- to 50-kDa GP_3 protein (ORF3), the 31- to 35-kDa GP_4 protein (ORF4), and the 25-kDa GP_5 protein (ORF5), which are. The N, M, and GP_5 proteins have also been detected in extracellular virus and lysates of cells infected with North American isolates, which are antigenically different from LV (2, 22, 28).

In a previous report, we described the isolation and characterization of a panel of LV-specific monoclonal antibodies (MAbs) that recognized GP_3, GP_4, M, and N (31). Interestingly, the MAbs directed against GP_4 neutralized LV, suggesting that at least part of the protein is exposed at the virion surface. In the present study, we have further investigated the processing and antigenic sites of the GP_4 protein. Using chimeric genes and pepscan analysis, we identified an antigenic domain in GP_4 of LV that can elicit neutralizing antibodies. This domain was shown to be highly variable among other strains of LV.

MATERIALS AND METHODS

Cells and viruses. The European prototype *Ter Huurne* strain of LV was isolated in 1991 (33). The U.S. prototype VR2332 strain was isolated in 1992 by Benfield et al. (3). Strain NL1 (isolated in The Netherlands in 1991) was isolated in our institute. The other strains tested were kindly provided as follows: strain NY2 (isolated in England in 1991) by T. Drew, strain DEN (isolated in Denmark in 1992) by A. Botner, strain LUX (isolated in Luxemburg in 1992) by A. Losch, SPA1 (isolated in Spain in 1992) by V. Shokouhi, SPA2 (isolated in Spain in 1992) by L. Espuna, and strain FRA (isolated in France in 1992) by Y. Leforban.

LV and VR2332 were grown on CL2621 cells as described previously (31). The seven different European isolates were grown in porcine alveolar macrophages. Macrophages were maintained as described previously (33). BHK-21 cells were maintained in Dulbecco's minimal essential medium supplemented with 5% fetal bovine serum and antibiotics. For transfection experiments, the BHK-21 cells were grown in Glasgow minimal essential medium (GIBCO-BRL/Life Technologies Ltd.) by the method of Liljeström and Garoff (21).

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TABLE 1. MAbs that reacted with GP_4 in Western blot analysis and were used in this study

MAb	Isotype	Ig concn (μg/ml)	Antibody titer[a]	Neutralization[b]	PEPSCAN result[c]
121.4	NT[d]	NT[d]	1	−	−
122.1	IgG2a	87	256	+	−
122.12	IgG1	87	512	++	±
122.20	IgG1	63	256	++	−
122.29	IgG1	89	1,024	+++	+
122.30	IgG1	105	512	+++	+
122.59	IgG1	64	256	++	−
122.66	IgG1	74	512	++	+
122.68	IgG1	70	32	++	−
122.70	IgG1	85	128	++	−
122.71	IgG1	81	256	+++	+
126.1	IgG2b	50	256	+++	−
126.7	IgG1	53	128	++	−
130.7	IgG2a	23	256	+++	+
138.28	IgG2b	40	1,024	+++	+

[a] Reciprocal of the highest dilution of hybridoma cell culture medium positive in an indirect immunoperoxidase assay of lung alveolar macrophages infected with LV.

[b] −, no plaque reduction by undiluted hybridoma medium; +, >50% plaque reduction by undiluted hybridoma medium; ++, >50% plaque reduction by undiluted and 1:10-diluted hybridoma medium; +++, >50% plaque reduction by undiluted, 1:10-diluted, and 1:100-diluted hybridoma medium.

[c] Reactivity of MAbs with the core sequence (SAAQEKISF; amino acids 59 to 67) in the PEPSCAN analysis; +, positive; −, negative; ±, weak.

[d] NT, the Ig production of this MAb was too low to determine its concentration and its isotype.

Antisera. Porcine anti-LV serum 21 and rabbit antipeptide serum 698 (directed against amino acids 62 to 77 of GP_4) have been used in previous experiments (26). Serum 700 is directed against amino acids 106 to 122 (CLFYASEMSEKGFKVIF) of GP_4 of LV and was obtained from a rabbit immunized as described previously (26). The production and characterization of four MAbs directed against the GP_4 protein have been described by van Nieuwstadt et al. (31). In addition to these, 11 other GP_4-specific MAbs were generated by immunizing mice with purified LV virions (31) and are listed in Table 1. The hybridomas producing these MAbs were derived from five consecutive fusion experiments; (i) MAb 121.4; (ii) MAbs 122.1, 122.12, 122.20, 122.29, 122.30, 122.59, 122.66, 122.68, 122.70, and 122.71; (iii) MAbs 126.1 and 126.7; (iv) MAb 130.7; and (v) MAb 138.28. The neutralizing activity of these MAbs was tested in a plaque reduction assay as described previously (31).

Plasmid constructions. Two oligonucleotides located upstream (LV13) and downstream (LV14) of ORF4 have been used previously to amplify and clone ORF4 in pGEM-4Z with the *Bam*HI and *Hin*dIII sites introduced in the primers (26). Table 2 shows the sequences of these primers and the primers listed below. The resulting plasmid was named pABV209. Two oligonucleotides, located at a similar position in relation to the initiation codon (PRRSV4) and the termination codon (PRRSV5) of ORF4 of VR2332 were used in reverse transcription-PCR (23) to amplify ORF4 of VR2332. The PCR fragment was digested with *Bam*HI and partially digested with *Hin*dIII, because ORF4 of VR2332 contains an internal *Hin*dIII site. The fragment was then cloned in pGEM-4Z, resulting in plasmid pABV270. Recombinant DNA techniques were performed essentially as described by Sambrook et al. (30). The nucleotide sequence of VR2332 ORF4 in pABV270 was determined on an automated DNA sequencer (Applied Biosystems) and was found to be identical to the published sequence (27). The ORF4 genes of LV and VR2332 were then transferred to the Semliki Forest virus expression vector pSFV1. Plasmids pABV209 and pABV270 were digested with *Bam*HI and *Hin*dIII (only partially for pABV270), the ORF4 fragments were treated with Klenow polymerase (Pharmacia) to create blunt ends, and these were then ligated in the *Sma*I site of pSFV1, which was dephosphorylated with calf intestinal alkaline phosphatase (Pharmacia). Plasmids containing ORF4 of LV (pABV265) and VR2332 (pABV271) in the correct orientation were further tested for expression of the GP_4 protein (see Fig. 3).

In addition, four different chimeric ORF4 genes of LV and VR2332 were made by using PCR to amplify fragments of pABV209 and pABV270 with primers containing the appropriate restriction sites to ligate the fragments in pGEM-4Z (see Fig. 3). The nucleotide sequence of ORF4 encoding amino acids 1 to 39 of the GP_4 protein of VR2332 was amplified with oligonucleotides PRRSV4 and PRRSV6 and was exchanged for that encoding amino acids 1 to 39 of LV in pABV209. This resulted in pABV306. The nucleotide sequence of ORF4 encoding amino acids 1 to 75 of GP_4 of VR2332 was amplified with oligonucleotides PRRSV4 and PRRSV9 and was ligated to the nucleotide sequence of ORF4 encoding amino acids 80 to 183 of the LV GP_4 protein, amplified with LV46 and LV14. This resulted in plasmid pABV308. In the same way, a complementary construct was created in pGEM-4Z; it consisted of the nucleotide sequence encoding amino acids 1 to 79 of the LV GP_4 protein amplified with LV13 and LV57 ligated to a fragment encoding amino acids 76 to 178 of VR2332, which was amplified with PRRSV10 and PRRSV5. This resulted in plasmid pABV314. A fourth chimeric construct consisted of a fragment encoding amino acids 40 to 79 of the LV GP_4 protein fused to fragments encoding amino acids 1 to 39 and amino acids 76 to 178 of the VR2332 GP_4 protein. This was achieved by ligating the *Bam*HI-*Sac*II ORF4 fragment of pABV270 and the *Sac*II-*Hin*dIII ORF4 fragment of pABV314 in pGEM-4Z digested with *Bam*HI and *Hin*dIII. This resulted in plasmid pABV325. Oligonucleotide sequencing was used to check plasmids pABV306, pABV308, pABV314, and pABV325 for the correct sequence. The chimeric ORF4 genes were transferred from pABV306, pABV308, pABV314, and pABV325 to pSFVI, as described above for the ORF4 genes of pABV209 and pABV270, resulting in pABV296, pABV305, pABV321, and pABV326, respectively (see Fig. 3).

To clone the ORF4 genes of seven different European isolates, we infected macrophages with NL1, NY2, DEN, FRA, SPA1, SPA2, and LUX. RNA was isolated as described by Meulenberg et al. (24). The ORF4 genes were amplified by reverse transcription PCR with oligonucleotides LV13 and LV14 by the method we used in an earlier study (23) and were cloned with *Bam*HI and *Hin*dIII in pGEM-4Z. For each strain, the nucleotide sequence of ORF4 of two clones derived from two independent PCRs was determined. The amino acid sequences derived from the nucleotide sequence were aligned with the multiple-sequence alignment program CLUSTAL of PCGene (IntelliGenetics Tm).

In vitro transcription and transfection of Semliki Forest virus ORF4 RNA. The pSFV1 plasmids containing different ORF4 constructs were linearized by digestion with *Spe*I and transcribed in vitro by the method of Liljeström and Garoff (20, 21). The synthesized RNA was transfected to BHK-21 cells in 15-mm wells of 24-well plates with Lipofectin as described by Liljeström and Garoff (21). Cells were fixed with ice-cold 50% (vol/vol) methanol-acetone, and the GP_4 protein expressed by the various ORF4 constructs was stained with MAbs in the immunoperoxidase monolayer assay (IPMA), essentially as described by Wensvoort et al. (32). To analyze the ORF4 expression products by immunoprecipitation, we transfected 10^7 BHK-21 cells with 10 μg of Semliki Forest virus ORF4 RNA (in vitro transcribed) by electroporation by the method of Liljeström and Garoff (21). The electroporated cells were plated in three 35-mm wells of six-well plates and labeled 18 h after transfection.

Radioactive labeling of proteins. BHK-21 cells electroporated with RNA derived from the various ORF4 constructs in pSFV1 were starved for 30 min in methionine-free Eagle's basal medium and were then labeled with 120 μCi of L-[^{35}S]methionine per ml for 4 h. Lysates were prepared as described by Meulenberg et al. (26) and were used for immunoprecipitation.

Metabolic labeling of CL2621 cells infected with LV and pulse-chase experiments were performed as described previously (25).

Immunoprecipitation, endoglycosidase treatment, and gel electrophoresis. Proteins were immunoprecipitated essentially by the method of Hulst et al. (14). Immunoprecipitates of LV proteins were either directly resuspended in Laemmli sample buffer (17) or were pretreated with endoglycosidases before being resuspended. In the latter treatment, the samples were resuspended in the appropriate endoglycosidase buffer and treated with peptide *N*-glycosidase F (PNGase F; Boehringer Mannheim) or endo-β-*N*-acetyl-D-glucosaminidase H (Endo H; Boehringer Mannheim) as described previously (26). Controls were treated similarly, except that the PNGase F or Endo H was omitted. Samples were resuspended in Laemmli sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

TABLE 2. Sequence of primers used in PCR to clone the ORF4 genes of LV and VR2332 and chimeric ORF4 genes in plasmid vectors pGEM-4Z and pSFV1

Name	Sequence[a]	Incorporated restriction site
LV13	5′ GGCAATT*GGATCC*ATTTGGA 3′	*Bam*HI
LV14	5′ AGAAGC*AAGCTT*GCGGAGTC 3′	*Hin*dIII
LV46	5′ GCCGTC*GGTACC*CCTCAGTACAT 3′	*Kpn*I
LV57	5′ ATGTACTGAGG*GGTACC*GACGGC 3′	*Kpn*I
PRRSV4	5′ GGCAATT*GGATCC*ACCTAGAATGGC 3′	*Bam*HI
PRRSV5	5′ GCGAGC*AAGCTT*CCGCGGTCAAGCATTTCT 3′	*Hin*dIII
PRRSV6	5′ CTTGCCG*CCGCGG*TGGTGTTG 3′	*Sac*II
PRRSV9	5′ ACAGCT*GGTACC*TATCGCCGTACGGCACTGA 3′	*Kpn*I
PRRSV10	5′ GCGATA*GGTACC*CCTGTGTATGTTACCAT 3′	*Kpn*I

[a] The underlined nucleotides in these primers are mutated with respect to the original sequence to create restriction sites or overhanging sequences or to avoid long stretches of one particular nucleotide. The restriction sites in the primers are shown in italics.

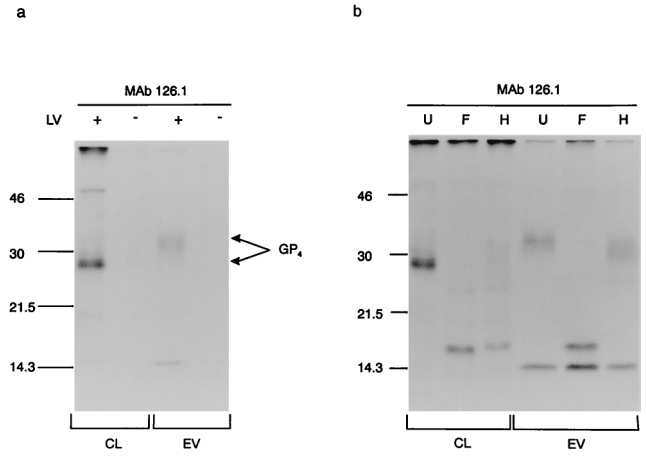

FIG. 1. Immunoprecipitation of GP_4 from lysates and extracellular virus of CL2621 cells infected with LV. For the preparation of radiolabeled cell lysates, CL2621 cells were infected with LV, and 18 h after infection they were labeled for 4 h with 90 μCi of L-[^{35}S]methionine per ml. For the preparation of radiolabeled virus, CL2621 cells were labeled between 8 and 32 h after LV infection with 60 μCi of L-[^{35}S]methionine per ml. (a) Cell lysates (CL) and extracellular virus (EV) of LV-infected (LV +), or mock-infected (LV −) CL2621 cells were immunoprecipitated with anti-GP_4 MAb 126.1. The position of the GP_4 protein is indicated by arrows. (b) Immunoprecipitation of GP_4 with MAb 126.1 from cell lysates (CL) and extracellular virus (EV) of CL2621 cells infected with LV. The immunoprecipitated GP_4 protein was either untreated (U), PNGase F treated (F), or Endo H treated (H). The samples were analyzed in an SDS–12.5% polyacrylamide gel under reducing conditions. The positions of the molecular weight markers are indicated on the left in thousands.

Epitope mapping by PEPSCAN procedures. A complete set of solid-phase overlapping dodecapeptides was synthesized from amino acids 25 to 94 of the ORF4 sequence of LV, which was determined previously (24). The synthesis of solid-phase peptides on polyethylene rods and immunoscreening by an enzyme-linked immunosorbent assay type of analysis were carried out by established PEPSCAN procedures (11). The hybridoma cell culture media were tested at a 1:5 dilution. Peptides were considered to represent antigenic sites if absorbance signals of two or more neighbouring peptides reproducibly amounted to more than twice the background.

RESULTS

Processing of GP_4. In an earlier study, we described a panel of neutralizing MAbs that reacted in Western immunoblot analysis with a diffuse 31- to 35-kDa protein of LV, which we designated GP_4 (31). GP_4 was shown to be a structural glycoprotein encoded by ORF4. In the present study, we used the immunoprecipitation technique to further investigate the processing of the GP_4 protein and to compare the GP_4 protein present in cell lysates and extracellular virus of CL2621 cells infected with LV. The GP_4 protein was immunoprecipitated by GP_4-specific MAb126.1 from lysates of LV-infected cells but not from lysates of mock-infected cells and migrated as a discrete band of 28 kDa together with a faint smear of somewhat higher apparent molecular mass (Fig. 1A). MAb 126.1 immunoprecipitated a diffuse GP_4 protein of about 31 kDa from the extracellular medium of LV-infected cells but not from the extracellular medium of mock-infected cells (Fig. 1A). The 15-kDa N protein was nonspecifically immunoprecipitated, as observed in previous immunoprecipitation experiments (25). The precipitation of N is not a result of a specific interaction between N and GP_4, because MAbs against N do not coprecipitate GP_4. When the immunoprecipitates were treated with PNGase F (an endoglycosidase that cleaves all N glycans irrespective of their maturation state) and with Endo H (an endoglycosidase that cleaves only noncomplex type N glycans), the GP_4 protein present in cell lysates was sensitive to both PNGase F and Endo H whereas the GP_4 protein present in extracellular virus was sensitive to PNGase F but resistant to Endo H (Fig. 1B). These findings indicate that the high-mannose N glycans of GP_4 had been modified to complex type N glycans during transport through the Golgi compartment, resulting in a more diffuse protein with a higher molecular mass (Fig. 1B). The difference in molecular mass between the PNGase F-treated and mock-treated GP_4 protein (approximately 10 kDa) suggests that all four putative N-glycosylation sites present in the amino acid sequence are used in vivo. A pulse-chase labeling experiment was performed to study the kinetics of the acquisition of Endo H-resistant N-glycans. The GP_4 protein migrated as a discrete band of 28 kDa after the pulse but faded during the chase, while a diffuse and slower-migrating species appeared (Fig. 2). The GP_4 protein was sensitive to PNGase F after the pulse but gradually became resistant to Endo H. After a chase of 180 min, most of the N glycans of the GP_4 protein had been converted to complex type N glycans. When the chase period was 400 min, the overall intensity of the GP_4 protein was significantly decreased, which may reflect the incorporation of this protein into virus particles followed by secretion of these particles into the medium (data not shown).

Identification of a neutralization domain in GP_4. In an earlier study, we demonstrated that four neutralizing MAbs specific for the GP_4 protein recognized LV but did not recognize

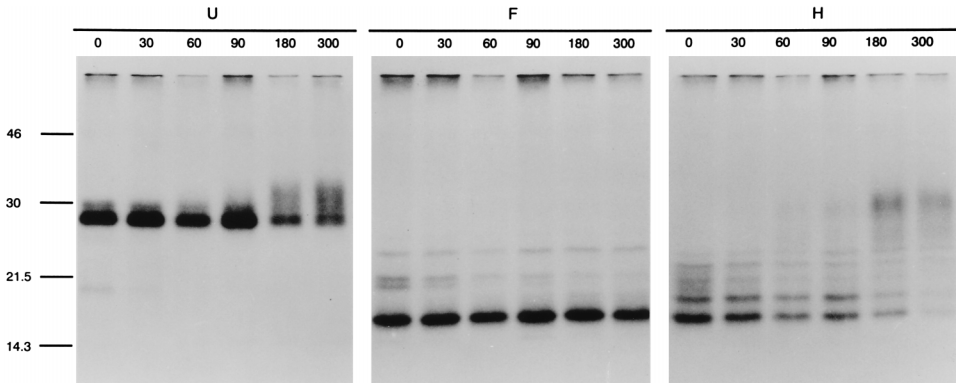

FIG. 2. Acquisition of Endo H resistance by the GP_4 protein. CL2621 cells were infected with LV, and 18 h after infection they were pulse-labeled for 30 min with 600 μCi of L-[^{35}S]methionine per ml and chased in medium containing 5 mM nonradioactive methionine. The length of the chase is indicated in minutes above the lanes. Lysates were prepared and immunoprecipitated with MAb 126.1. The immunoprecipitated proteins were treated with PNGase F (F) or Endo H (H) or were left untreated (U) before analysis by SDS-PAGE on a gel containing 12.5% acrylamide. The numbers on the left show the sizes of marker proteins in kilodaltons.

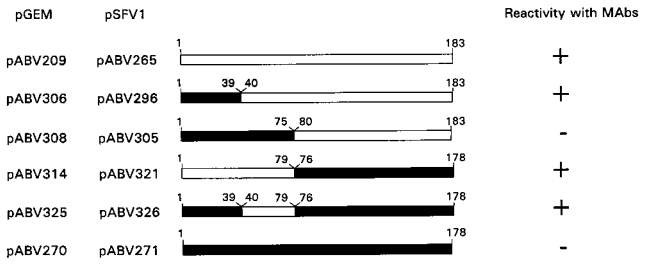

FIG. 3. Schematic diagram of GP_4 proteins expressed in pSFV1 and their reactivity with GP_4-specific MAbs. The names of the plasmids containing the different ORF4 genes are indicated. The genes were first inserted in PGEM-4Z and then transferred to pSFV1, as described in detail in Materials and Methods. Open bars represent the amino acid sequences derived from the GP_4 protein encoded by ORF4 of LV, and solid bars represent amino acid sequences derived from the GP_4 protein encoded by ORF4 of VR2332. The numbers of the amino acids are indicated above the bars and are derived from the amino acid sequences of GP_4 of LV and VR2332, as shown in Fig. 6B. The complete set of 15 GP_4-specific MAbs reacted identically with the different constructs in IPMA, and the reactivity is indicated as positive (+) or negative (−).

the U.S. isolate VR2332 (31). Besides these four MAbs, 11 other neutralizing GP_4-specific MAbs that had the same strain specificity were developed (Table 1). To identify the binding domain of these MAbs in the GP_4 protein, we made chimeric proteins of the GP_4 protein of LV and the U.S. isolate VR2332. These proteins were expressed in the Semliki Forest virus expression system developed by Liljeström and Garoff (20). First, RNA transcribed from pABV265, which consists of ORF4 of LV cloned in pSFV1, was transfected to BHK-21 cells, and 24 h later the cells were positively stained with each of a panel of 15 neutralizing MAbs in IPMA (Fig. 3), whereas BHK-21 cells transfected with pSFV1-RNA were not stained with the MAbs (data not shown). The GP_4 protein of U.S. isolate VR2332, cloned in pSFV1, was not recognized by the MAbs upon expression in BHK-21 cells (Fig. 3). Next, four chimeric genes of ORF4 of LV and VR2332 were constructed in pSFV1, which resulted in plasmids pABV296, pABV305, pABV321, and pABV326 (Fig. 3). When RNAs transcribed from these plasmids were transfected to BHK-21 cells and tested in IPMA with the GP_4-specific MAbs, these MAbs reacted identically (Fig. 3). The reaction pattern indicated that the binding sites of these 15 GP_4-specific MAbs were located in a region spanning amino acids 40 to 79 of the GP_4 protein, since the expression product of pABV326, consisting of amino acids 40 to 79 derived from the LV GP_4 protein and flanking sequences derived from the VR2332 GP_4 protein, was still recognized by the panel of MAbs.

To ensure that the different GP_4 proteins, especially those that were not recognized by the MAbs, were properly expressed in BHK-21 cells, they were immunoprecipitated from lysates of BHK-21 cells that were transfected with RNA transcribed in vitro from plasmids pABV265, pABV271, pABV296, pABV305, pABV321, and pABV326. Antipeptide serum 700 precipitated all six recombinant GP_4 proteins (Fig. 4). This was expected, since serum 700 is directed against amino acids 106 to 122 of the LV GP_4 protein, a sequence which, apart from amino acid 121, is identical in the VR2332 GP_4 protein. The recombinant GP_4 proteins had a similar size to the authentic GP_4 protein synthesized in CL2621 cells infected with LV (compare Fig. 2 and 4) and also contained PNGase F- and Endo H-sensitive N glycans (data not shown). The GP_4 proteins expressed by pABV305 and pABV271 migrated slightly faster than those expressed by pABV265, pABV296, pABV321, and pABV326, which is probably due to the deletion of 4 amino acids between amino acids 62 and 65 in the VR2332 sequence with respect to the LV sequence (as will be discussed in more detail below). MAb 126.1, specific to GP_4, recognized the GP_4 proteins expressed by pABV265, pABV296, pABV321, and pABV326 but did not recognize those expressed by pABV305 and pABV271, which confirmed the results obtained by IPMA (Fig. 3). Serum 698 had the same reaction profile as the MAbs. It contains antibodies that are directed against amino acids 62 to 77 of GP_4 of LV (26), which are located within the neutralizing domain of the GP_4 protein. Since the amino acid sequence in this region is completely different in VR2332 GP_4, the expression products containing the VR2332 sequence in this region were not recognized by this serum. Serum 21 recognized the LV GP_4 protein and the chimeric LV-VR2332 GP_4 proteins but did not recognize the VR2332 GP_4 protein. This indicated that this porcine anti-LV serum contains a variety of antibodies directed against different regions of the GP_4 protein.

Since the 15 MAbs all recognized the expression product of pABV326 and reacted with the reduced and denatured GP_4 protein in the Western blot analysis, they were expected to recognize a linear epitope in a region spanning amino acids 40 to 79 of GP_4. Therefore, their fine specificity was further investigated with synthetic peptides (12-mers) from this region by PEPSCAN analysis. MAbs 122.29, 122.30, 122.66, 122.71, 130.7, and 138.28 reacted positively with one specific antigenic site, consisting of amino acids 59 to 67 (SAAQEKISF) (Fig. 5). MAb 122.12 reacted only weakly with this antigenic site, whereas the remaining eight MAbs were negative in the PEPSCAN analysis.

Sequence analysis of the GP_4 protein of other European isolates. To analyze whether the antigenic domain, recognized by the GP_4-specific MAbs, was conserved among European isolates, the reactivity and neutralizing activity of the MAbs were further tested on seven European strains. The results indicated that these MAbs recognized and neutralized another Dutch isolate (NL1) and an English isolate (NY2) but not a Danish isolate (DEN), two Spanish isolates (SPA1 and SPA2),

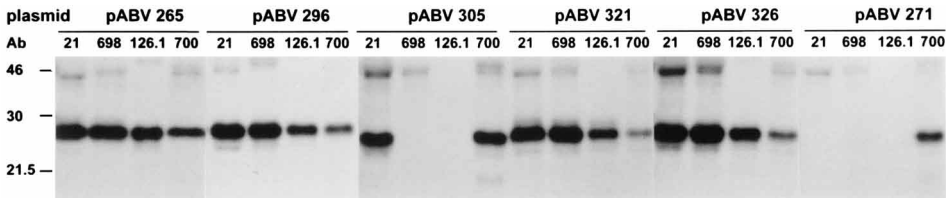

FIG. 4. Immunoprecipitation of GP_4 proteins of LV and VR2332 and chimeric GP_4 proteins derived from them. The plasmids pABV265, pABV296, pABV305, pABV321, pABV326, and pABV271 used to express the GP_4 proteins are indicated. The RNA transcribed from these plasmids was transfected to BHK-21 cells, and these cells were labeled with L-[^{35}S]methionine. Cell lysates were prepared and were immunoprecipitated with GP_4-specific antipeptide sera 689 and 700, MAbs 126.1, and porcine anti-LV serum 21. The immunoprecipitated proteins were analyzed by SDS-PAGE on a gel containing 12.5% acrylamide. The numbers on the left show the sizes of marker proteins in kilodaltons.

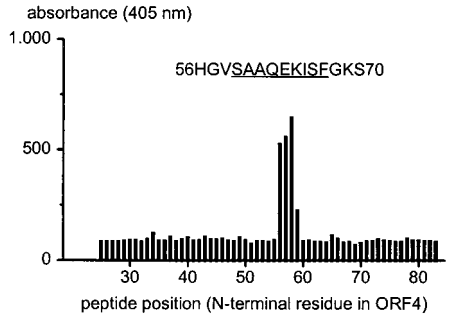

FIG. 5. PEPSCAN analysis of GP_4-specific MAbs with overlapping 12-mer peptides covering residues 25 to 94 of GP_4. The scan of neutralizing MAb 130.7, which recognizes four consecutive peptides, is shown. The amino acid sequence of the four antigenic peptides is shown, with the core of common residues underlined. Five other MAbs (122.29, 122.30, 122.66, 122.71, and 138.28) exhibited similar specificity.

a French isolate (FRA), or a Luxemburg isolate (LUX). Therefore, we were interested in the amino acid sequence in the region of the neutralization site of the GP_4 protein of these isolates. The ORF4 genes were cloned and sequenced. The amino acid sequences of the GP_4 proteins of the seven isolates were found to be 86 to 97% identical to those of LV. Aligning these amino acid sequences showed that the neutralization domain, in particular the region covered by amino acids 59 to 70, is much more diverse than the remaining part of the protein (Fig. 6A). The amino acid sequences of strains DEN, SPA1, SPA2, and FRA are especially different from LV in this region. This agrees with the finding that these strains are not recognized by the GP_4-specific MAbs and further confirms that this domain is not conserved among European isolates. Another region of heterogeneity was observed in the N-terminal part of the GP_4 protein. However, this part is not expected to contribute to the antigenicity of the protein, because it is part of the signal sequence which is supposed to be cleaved off (22a). Comparison of the amino acid sequence of the GP_4 protein of LV and that of VR2332 and other North American strains shows that the latter are also heterogeneous in the region spanning amino acids 40 to 79. Alignment of GP_4 of LV and VR2332 (Fig. 6B) or other North American isolates (data not shown) results in a gap and numerous amino acid differences at the neutralization site of GP_4 in the North American isolates, which confirms our observation that none of these isolates are recognized by the MABs.

DISCUSSION

We used a panel of 15 neutralizing MAbs to characterize the glycosylation and antigenic structure of GP_4, a minor structural protein encoded by ORF4 of LV. The results showed that all

A)

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LV-4        MAAATLFFLAGAQHIMVSEAFACKPCFSTHLSDIETNTTAAAGFMVLQDINCFRPHGVSAAQEKISFGKSSQCREAVGTPQYITITANVTDESYLYNADL   100
NL1-4                                                                                                              100
NY2-4             L                                                                                                100
PRRSV10-4         L                                                                                                100
LUX-4             L                          K                 L   R       E                                     100
SPA1-4         I      S   F                  K              N   L       T   N P   P         I                      100
SPA2-4       T I  L       L                  K              N D L       TT  N     P         I                      100
FRA-4          I  L       F                  K              N   L       T   N     P         I                      100
DEN-4          I  L       L                  K                T A    A EESQSVT N P         I                  H   100
             **.*.**.**.***.******************.*************.*.* *** .*.. ....*.*.********.****************.****

LV-4        LMLSACLFYASEMSEKGFKVIFGNVSGVVSACVNFTDYVAHVTQHTQQHHLVIDHIRLLHFLTPSAMRWATTIACLFAILLAI   183
NL1-4                                                                                                183
NY2-4                                                                                                183
PRRSV10-4                                                                                            183
LUX-4                                                                                                183
SPA1-4                                                                  T                            183
SPA2-4                                                               S                               183
FRA-4                                                                   T                            183
DEN-4                                                      A                                         183
             ************************************************.**********.**.*****************
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B)

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LV-4        MAAATLFFLAGAQHIMVSEAFACKPCFSTHLSDIETNTTAAAGFMVLQDINCFRPHGVSAAQEKISFGKSSQCREAVGTPQYITITANVTDESYLYNADL   100
VR2332-4      SSL  LVV FKCLL  Q         SS A  K       S A     S L HRDSASE----AIR IP   T I   V V         N  HSS     96
             **.. **...* . ..**.*********. *.**.*******.* *****.* *..  . .    .. * .***.*.*** *.*********.**...**

LV-4        LMLSACLFYASEMSEKGFKVIFGNVSGVVSACVNFTDYVAHVTQHTQQHHLVIDHIRLLHFLTPSAMRWATTIACLFAILLAI   183
VR2332-4       S               V      I AV     S  Q  KEF  -RS  V  V     M  ET     VL            178
             ****.***************.******.*..*****.**.** . ** . **.**.*****.**..****** .**********
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FIG. 6. Alignment of amino acid sequences of GP_4 proteins of eight different European isolates (A) and isolate VR2332, a representative of the North American antigenic group (B), with the amino acid sequence of GP_4 of LV. Only the amino acids which differ from the LV sequence are shown. Identical amino acids are indicated by *, and conserved amino acids are indicated by ·. The core peptide sequence, recognized by MAbs in the PEPSCAN analysis, is underlined. The LV sequence was derived from the work of Meulenberg et al. (24); the PRRSV10, a Dutch isolate, was sequenced by Conzelmann et al. (5); the VR2332 sequence was derived from the work of Murtaugh et al. (27); and the remaining sequences were determined in this work.

four N-glycosylation sites found in the amino acid sequence are probably used in vivo and that the core N glycans are converted very slowly to complex-type N glycans during transport from the endoplasmic reticulum through the Golgi compartment. All the MAbs recognized an antigenic site located between amino acids 40 and 79 of GP_4 of LV. In the PEPSCAN analysis, only six MAbs recognized a more defined area between amino acids 59 and 67 (SAAQEKISF), whereas the other nine MAbs did not react. MAbs are expected to differ in specificity since they are produced from independent hybridomas and thus should have a variable genetic constitution (18). Perhaps the affinity of the MAbs that did not react with linear peptides in the PEPSCAN analysis is too low, or their binding is more conformation dependent. Alternatively, they may recognize discontinuous epitopes adjacent to, partially overlapping with, or interacting with amino acids 59 to 67. Although all the MAbs recognized the reduced GP_4 protein in Western blot analysis, suggesting that they recognize linear epitopes, the GP_4 protein might be partially refolded during the blotting procedure. Preliminary results in competitive binding assays showed that the binding to GP_4 of a MAb that reacted with peptides containing the core sequence SAAQEKISF in the PEPSCAN analysis was blocked by MAbs that did not react with these peptides in the PEPSCAN analysis. This further supports our view that the antibody-binding sites of the 15 GP_4-specific MAbs are partially overlapping or in physical proximity. Interestingly, the 15 MAbs did not recognize and neutralize strain LUX, which had only three amino acid differences in the neutralization domain at positions 53, 57, and 64 of GP_4 compared to LV. This implies that amino acid 64 is most probably important for binding of the six MAbs that reacted with the core sequence spanning amino acids 59 to 67 in the PEPSCAN analysis, whereas at least one of these three amino acids is also important for binding of the nine MAbs that were negative in the PEPSCAN analysis. Further evidence for the importance of these amino acids for binding of the MAbs should be obtained by introducing single amino acid mutations at these positions in GP_4 of LV. It is not very likely that the glycosylation of the GP_4 protein plays a role in the binding of the MAbs since the N-glycosylation sites are located outside amino acids 40 to 79 and we have shown previously for a few GP_4-specific MAbs that they recognize the deglycosylated GP_4 protein in Western blot analysis (31).

Since the MAbs had neutralizing activity, their binding site is expected to be exposed at the viral surface. This is in line with the features characteristic for typical class I integral membrane proteins, which were identified in the amino acid sequence of GP_4. The N-terminal hydrophobic amino acids (amino acids 1 to 17) are expected to function as a signal sequence and might be cleaved off, whereas the hydrophobic amino acids (165 to 183) at the C terminus might anchor the protein in the membrane (26). The region in between, encompassing the antigenic domain identified here, is predicted to represent the ectodomain of the protein. The fact that 15 MAbs derived from five independent fusions of splenocytes from mice immunized with purified LV virions all recognized a region between amino acids 40 and 79 of GP_4 suggests that this is an immunodominant domain. However, it should be noted that the probability of isolating hybridoma clones which produce antibodies to a given epitope may be influenced by factors other than the immunogenicity of the epitope, such as fusion efficiency and clone stabilization. In addition, the recognition of an antigenic site might be species specific, as was shown for the capsid proteins of canine parvovirus (19). Therefore, the immunogenic significance of this epitope for pigs should be investigated further. Preliminary results indicated that certain porcine sera that are positive for LV also reacted in the PEPSCAN analysis with peptides at the neutralization site.

Comparison of the amino acid sequence of the GP_4 proteins of various European strains indicated that the neutralization domain was much more variable than other parts of the protein, suggesting that this domain is susceptible to immunoselection. Comparison of the GP_4 sequences of European and North American strains revealed a gap of 4 amino acids at the neutralization site in the North American sequence in comparison with the European sequences. Therefore, it remains to be investigated whether the conformation in this region of GP_4 is similar in North American strains and European strains and whether this region of North American strains is also able to induce neutralizing antibodies.

The neutralization domain of the GP_4 protein described here is the first site identified for LV. For two other arteriviruses, EAV and LDV, the neutralizing MAbs that were isolated were all directed against the major envelope protein G_L/VP3 encoded by ORF5 (1, 7, 12, 13). Therefore the G_L/VP3 protein is thought to interact with the receptor for EAV and LDV, respectively. By using neutralization-escape mutants, the neutralization site of EAV was mapped to specific amino acids in the ectodomain of G_L. Since GP_5 is also the major structural envelope protein of LV (26) and has a similar hydrophobicity profile to G_L/VP3, it might also be the protein that interacts with the receptor. Although porcine anti-LV sera recognize the GP_5 protein, we have not been able to isolate (neutralizing) MAbs against GP_5 of LV. If GP_5 interacts with the receptor of LV, the GP_4 protein might be in close proximity to it, since anti-GP_4 MAbs neutralize the virus. Future LV research should assess the role of GP_4, in particular its neutralization domain, and the role of GP_5 in immunity, cell attachment, and infection.

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