JOURNAL OF VIROLOGY, July 1996, p. 4767–4772 Vol. 70, No. 7
0022-538X/96/$04.00+0

Proteins Encoded by Open Reading Frames 3 and 4 of the Genome of Lelystad Virus (*Arteriviridae*) Are Structural Proteins of the Virion

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Received 18 September 1995/Accepted 30 March 1996

Four structural proteins of Lelystad virus (*Arteriviridae*) were recognized by monoclonal antibodies in a Western immunoblotting experiment with purified virus. In addition to the 18-kDa integral membrane protein M and the 15-kDa nucleocapsid protein N, two new structural proteins with molecular masses of 45 to 50 kDa and 31 to 35 kDa, respectively, were detected. Monoclonal antibodies that recognized proteins of 45 to 50 kDa and 31 to 35 kDa immunoprecipitated similar proteins expressed from open reading frames (ORFs) 3 and 4 in baculovirus recombinants, respectively. Therefore, the 45- to 50-kDa protein is encoded by ORF3 and the 31- to 35-kDa protein is encoded by ORF4. Peptide-*N*-glycosidase F digestion of purified virus reduced the 45- to 50-kDa and 31- to 35-kDa proteins to core proteins of 29 and 16 kDa, respectively, which indicates N glycosylation of these proteins in the virion. Monoclonal antibodies specific for the 31- to 35-kDa protein neutralized Lelystad virus, which indicates that at least part of this protein is exposed at the virion surface. We propose that the 45- to 50-kDa and 31- to 35-kDa structural proteins of Lelystad virus be named GP3 and GP4, to reflect their glycosylation and the ORFs from which they are expressed. Antibodies specific for GP3 and GP4 were detected by a Western immunoblotting assay in swine serum after an infection with Lelystad virus.

An infection with Lelystad virus causes reproductive failure in sows. The causative agent was first isolated in The Netherlands (32). A related virus was isolated in North America (prototype strain ATCC VR2332) (4, 6) and is the causative agent of porcine reproductive and respiratory syndrome (PRRS); hence, the virus was named PRRS virus (PRRSV). Polyvalent antisera specific for European isolates of PRRSV cross-react with North American isolates in an immunoperoxidase assay on infected macrophages, and vice versa (30). However, European and North American isolates of PRRSV represent two distinct genotypes (18–20, 24, 31), and antigenic differences between European and North American isolates have been observed (25, 30).

PRRSV is related to lactate dehydrogenase-elevating virus of mice, equine arteritis virus, and simian hemorrhagic fever virus (7, 22, 26). These viruses have a single-stranded positive-sense RNA genome (15 kb in PRRSV, 14.2 kb in lactate dehydrogenase-elevating virus, and 12.7 kb in equine arteritis virus), a cubical nucleocapsid core, and a lipoprotein envelope. They belong to the newly proposed virus family *Arteriviridae* (5), which resembles the families *Coronaviridae* and *Toroviridae* in genome organization and replication strategy (8, 26).

The genome of members of the family *Arteriviridae* contains two large open reading frames (ORFs), ORFs 1a and 1b, and six smaller ORFs at the 3′ part of the genome, ORFs 2 to 7 (8, 13, 22). ORFs 1a and 1b code for proteins with polymerase and replicase activities. In a previous study, ORFs 5, 6, and 7 were proved to code for three structural proteins of Lelystad virus: a 25-kDa glycosylated protein (tentatively named E protein), an 18-kDa integral membrane protein M, and a 15-kDa nucleocapsid protein N (23). Although the predicted translation products of ORFs 2, 3, and 4 have the characteristics of membrane-associated proteins, these products have not been detected in virus-infected cell lysates or virions. Also, in equine arteritis virus, ORFs 2 and 5 were detected to code for a minor and a major envelope protein, respectively, but protein products of ORFs 3 and 4 were not identified (10).

In the present study, monoclonal antibodies (MAbs) specific for Lelystad virus were developed. Two more proteins of Lelystad virus could be identified by using these MAbs in a Western immunoblotting assay: a 45- to 50-kDa glycoprotein and a 31- to 35-kDa glycoprotein, which are encoded by ORFs 3 and 4, respectively, and appeared to be structural proteins of the virion.

Development of hybridomas producing MAbs specific for Lelystad virus. Mice were immunized with the prototype Ter Huurne isolate of Lelystad virus, which was isolated in 1991 on swine lung alveolar macrophages (32) and was adapted to multiply in the CL2621 cell line (3). Virus was concentrated from cell culture medium by ultracentrifugation in a Beckman type R19 rotor at 54,000 × *g* for 13 h. The virus pellet was resuspended in phosphate-buffered saline (PBS) (pH 7.2); this suspension was then layered on 20 to 50% (wt/vol) continuous sucrose gradients in PBS and centrifuged in a Beckman type SW40 Ti rotor at 200,000 × *g* for 16 h. Virus infectivity peaked in a fraction with a density of 1.17 g/cm^3. Fractions containing the virus were diluted with PBS and centrifuged at 200,000 × *g* for 12 h. The virus pellet was resuspended in 1 to 2 ml of PBS. This suspension contained pure virus, and a high number of intact virions was observed by electron microscopy. The protein concentration was determined by the Bradford protein assay method. From 1,300 ml of culture medium of infected cells, 100 to 300 μg of purified virus was obtained. Purified virus was used for immunization of mice and for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

BALB/c mice were immunized intraperitoneally first with 30 to 100 μg of purified Lelystad virus mixed with complete

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TABLE 1. MAbs used in this study

MAb	Protein specificity[a]	Isotype	Ig[b] concn (μg/ml)	Antibody titer[c]	
				Lelystad virus	VR2332
126.2	GP3 (45 to 50)	IgG1	56	256	<8
122.14	GP3 (45 to 50)	IgG2a	46	128	<8
122.1	GP4 (31 to 35)	IgG2a	87	256	<8
122.29	GP4 (31 to 35)	IgG1	89	1,024	<8
126.1	GP4 (31 to 35)	IgG2b	50	256	<8
130.7	GP4 (31 to 35)	IgG2a	23	256	<8
126.3	M (18)	IgG2a	11	256	<8
122.9	M (18)	IgG1	30	32	64
126.9	N (15)	IgG2b	12	256	256
122.17	N (15)	IgG1	26	1,024	1,024
SDOW17	N (15)	IgG1	117	256	1,024

[a] We propose that the ORF3-encoded 45- to 50-kDa protein and the ORF4-encoded 31- to 35-kDa protein of Lelystad virus be designated GP3 and GP4, respectively, to reflect the glycosylation of these proteins and the ORFs from which they are expressed. Numbers in parentheses indicate molecular mass in kilodaltons.

[b] Ig, immunoglobulin.

[c] Reciprocal of the highest dilution of hybridoma cell culture medium positive in an indirect immunoperoxidase assay of lung alveolar macrophages infected with Lelystad virus or CL2621 cells infected with the North American isolate VR2332.

Freund's adjuvant and 4 weeks later with the same dose in incomplete Freund's adjuvant. Four and 3 days before cell fusion was performed, mice were immunized with 100 to 200 μg of purified virus. The fusion protocol of Fazekas de StGroth and Scheidegger (12) was used to fuse splenocytes with nonproducing P3X63Ag8-653 murine myeloma cells (16), which were in the logarithmic phase of growth. An immunoperoxidase assay (15) performed with lung alveolar macrophages infected with Lelystad virus was used to screen hybridomas for production of specific antibody in cell culture medium. Hybridomas producing antibody specific for Lelystad virus were cloned two times by a limiting-dilution technique and were cultured to a high cell density (up to 10^6 cells per ml of cell culture medium) to produce 10 to 100 μg of immunoglobulin per ml of medium. A commercial immunoglobulin typing kit (SEROTEC, Oxford, England) was used to determine immunoglobulin isotypes of MAbs. A double-antibody sandwich enzyme-linked immunosorbent assay with antibody monospecific for mouse immunoglobulins for capture and detection was used to determine immunoglobulin concentration in hybridoma cell culture medium. In five fusion experiments, 31 stable hybridoma cell lines that produced MAb specific for Lelystad virus were established. The specificity of MAbs for Lelystad virus was confirmed when a positive reaction was obtained in an immunoperoxidase assay with lung alveolar macrophages infected with Lelystad virus and a negative result was obtained with uninfected lung alveolar macrophages. In a Western immunoblotting assay with purified virus, the MAbs recognized four structural proteins of Lelystad virus which had apparent molecular masses of 45 to 50, 31 to 35, 18, and 15 kDa, respectively. Table 1 shows the MAbs selected for this study, their isotypes, and the concentrations of immunoglobulin in cell culture medium. MAb SDOW17 was provided by D. Benfield (South Dakota State University, Brookings, S.Dak.). MAb SDOW17 was developed against the North American VR2332 isolate of PRRSV and recognized the 15-kDa N protein of North American and European isolates of PRRSV (25). In an immunoperoxidase assay with infected lung alveolar macrophages, the North American prototype isolate VR2332 was recognized by all three MAbs specific for the 15-kDa protein and by one of the two MAbs specific for the 18-kDa protein, but not by the other MAbs used in this study (Table 1). Similar results were obtained in a comparison between other European and North American isolates (results not shown), which confirms that European and North American isolates are antigenically diverse.

Virus protein specificity of the MAbs by Western immunoblotting. Proteins of purified virus were separated by SDS-PAGE, which was performed under reducing conditions by addition of 5% (vol/vol) β-mercaptoethanol to the Laemmli sample buffer (17). Virus proteins were transferred to nitrocellulose sheets by electroblotting (28), and nitrocellulose strips, each loaded with about 10 μg of virus protein, were incubated with hybridoma cell culture medium. Reaction patterns of MAbs were compared with those of porcine convalescent serum S21; negative pig serum; and antipeptide sera 690, 694, 698, 704, 710, and 714. These antipeptide sera are specific for protein products of ORFs 2, 3, 4, 5, 6, and 7, respectively (23). MAb 122.17, MAb 126.9, and MAb SDOW17 were specific for the 15-kDa nucleocapsid protein N, as the same 15-kDa protein was recognized by antipeptide serum 714 specific for the ORF 7 product, which is the N protein (Fig. 1A). Similarly, MAb 126.3 and MAb 122.9 were specific for the 18-kDa integral membrane protein M, as the same 18-kDa protein was recognized by antipeptide serum 710 specific for the ORF 6 product, which is the M protein (Fig. 1A). The 25-kDa structural glycoprotein, which was identified in a previous study as the product of ORF 5 by immunostaining with antipeptide serum 704 (23) (Fig. 1B), was not recognized by any MAb in our panel. MAb 122.29, MAb 130.7, MAb 122.1, and MAb 126.1 recognized a 31- to 35-kDa protein (Fig. 1B and C), and MAb 122.14 and MAb 126.2 recognized a 45- to 50-kDa protein (Fig. 1B and C). These must be two additional structural proteins, as purified virus was used for the immunoblotting. Swine convalescent serum recognized the 31- to 35-kDa and 45- to 50-kDa proteins in addition to the 25-kDa glycoprotein, the M protein, and the N protein (Fig. 1C). Antipeptide sera specific for the ORF 2, ORF 3, and ORF 4 products did not recognize the 31- to 35-kDa and 45- to 50-kDa proteins, however. A 28-kDa protein, which was recognized by swine convalescent serum, could not be assigned to any ORF product, as no reaction with the MAbs or antipeptide serum was observed. Because MAbs recognized the proteins on the blot even though reducing conditions were used for SDS-PAGE, epitopes recognized by these MAbs are probably continuous.

Immunoprecipitation of Lelystad virus proteins expressed by baculovirus recombinants. Since the three major structural proteins of Lelystad virus were found to be products of ORFs 5, 6, and 7, ORFs 2, 3, and 4 remained as candidates to encode the 31- to 35-kDa and 45- to 50-kDa proteins. Earlier we showed by in vitro transcription and translation experiments that these ORFs encode proteins of 30, 45, and 31 kDa (23). In the present study, we inserted ORFs 2 to 7 in baculovirus recombinants and tested the reactivity of the MAbs with the expressed proteins. Oligonucleotide primers complementary to sequences upstream and downstream of ORFs 2 to 7 of the Lelystad virus genome were synthesized, and *Bam*HI restriction sites were appended to forward and reverse primers. The following primers were used (*Bam*HI restriction sites are underlined): LV23 (5′ GATTGGATCCGAATTCGGGGTGAT GCAATG 3′) and LV25 (5′ AATCGGATCCTGGTCAGCT CGAATGATGTG 3′) for ORF2, LV26 (5′ GATTGGATCC GATTTCAGACAATGGCT 3′) and LV28 (5′ AATCGGAT CCGTTATCGTGATGTACTGGG 3′) for ORF3, LV29 (5′ GGCAATTGGATCCATTTGGA 3′) and LV30 (5′ AATTG

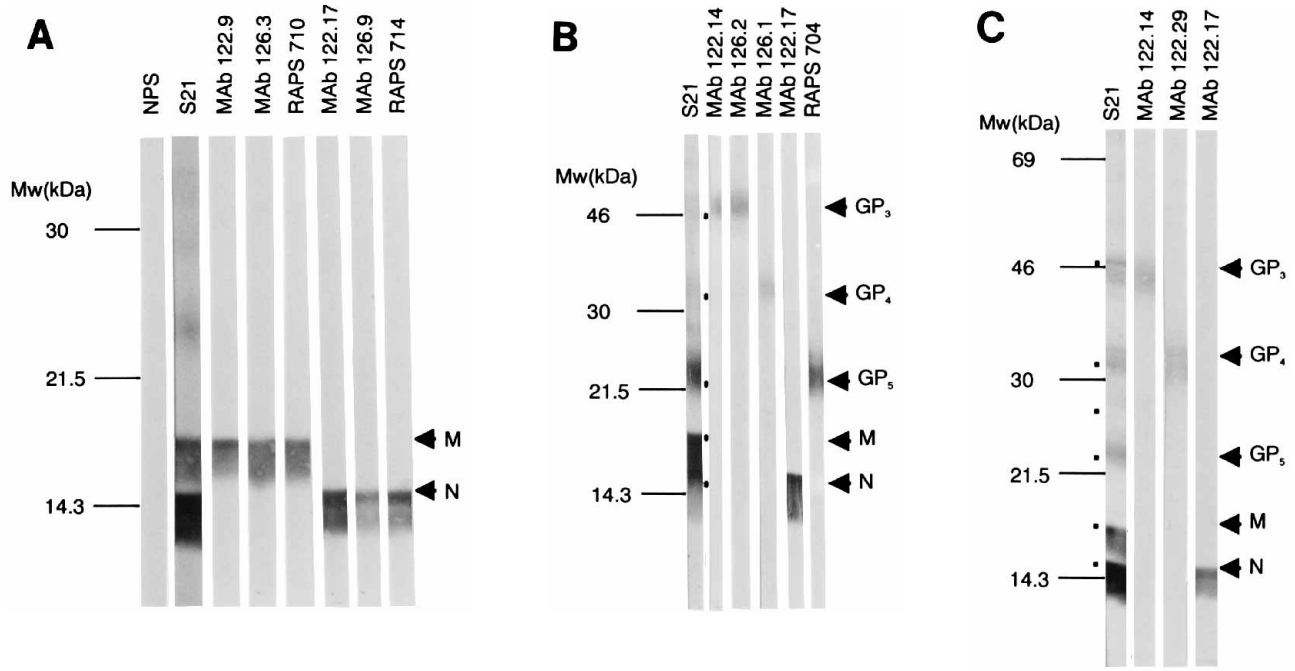

FIG. 1. Virions were purified by isopycnic sedimentation on a sucrose gradient, and infectious fractions were suspended in Laemmli sample buffer. Virion proteins were separated on a 12.5% polyacrylamide gel by SDS-PAGE and transferred to nitrocellulose strips. (A) Strips were immunostained with negative pig serum (NPS) or porcine anti-Lelystad virus serum (S21); antipeptide serum specific for the M protein (RAPS 710) or the N protein (RAPS 714); or MAb 122.9, MAb 126.3, MAb 122.17, or MAb 126.9. Binding of antibodies was detected with a peroxidase conjugate specific for mouse, rabbit, or swine immunoglobulins. The positions of the 15-kDa N protein and the 18-kDa M protein are marked with arrowheads. Positions of marker proteins are indicated. (B) Virion proteins on nitrocellulose strips were immunostained with porcine anti-Lelystad virus serum (S21); antipeptide serum 704 specific for the 25-kDa glycoprotein (GP5) (RAPS 704); or MAb 122.14, MAb 126.2, MAb 126.1, or MAb 122.17. Positions of the 45- to 50-kDa glycoprotein (GP3); the 31- to 35-kDa glycoprotein (GP4); and the GP5, M, and N proteins are marked. (C) Virion proteins on nitrocellulose strips were immunostained with porcine anti-Lelystad virus serum (S21) or with MAb 122.14, MAb 122.29, or MAb 122.17.

GATCCTCATATTGCCAAGAGAATGGC 3′) for ORF4, LV32 (5′ GATTGGATCCATTCTCTTGGCAATATG 3′) and LV33 (5′ GCGATAGGATCCTGCAAAAT 3′) for ORF5, LV36 (5′ GATTGGATCCTTCGGCTGAGCAATGGGA 3′) and LV38 (5′ AATCGGATCCTCTGGTTTTTACCGGCC 3′) for ORF6, and LV40 (5′ GATTGGATCCTCGTCAAGT ATGGCCGG 3′) and LV41 (5′ CTAAGGATCCTGTCAAA TTAACTTGCA 3′) for ORF7. Primers were used in a PCR to amplify ORFs 2 to 7 from cDNA clones as described before (21). After *Bam*HI digestion and gel purification, the PCR products were ligated downstream of the p10 promoter of baculovirus transfer vector pAcAS3 (29). Recombinant-DNA techniques were performed essentially as described by Sambrook et al. (27). Wild-type baculovirus DNA and DNA of pAcAS3 transfer vectors with inserts of ORFs 2 to 7 of the Lelystad virus genome were cotransfected in Sf21 insect cells as described by Hulst et al. (14). Recombinant virus expressing β-galactosidase was isolated and plaque purified three times. The expression of proteins of Lelystad virus was then tested by an immunoperoxidase assay of infected Sf21 cells with porcine anti-Lelystad virus serum, as described for a baculovirus recombinant expressing ORF7 (21). Recombinant viruses were designated bac-ORF2 to bac-ORF7.

In an immunoperoxidase assay, MAb 126.2 and MAb 122.14 stained Sf21 cells infected with bac-ORF3 but not Sf21 cells infected with the other baculovirus recombinants or with wild-type baculovirus (results not shown). This result suggests that MAb 126.2 and MAb 122.14 were directed at the protein expressed from ORF3. Similarly, MAb 122.1, MAb 122.29, MAb 126.1, and MAb 130.7 stained Sf21 cells infected with bac-ORF4 but not Sf21 cells infected with the other baculovirus recombinants or with wild-type baculovirus. This result suggests that these MAbs were directed at the protein expressed from ORF4. To confirm the protein specificities of the MAbs, radiolabeled proteins from lysates of Sf21 cells infected with recombinant baculovirus were immunoprecipitated with MAbs and the precipitated proteins were analyzed by SDS-PAGE, essentially as described for recombinant glycoprotein E1 of hog cholera virus (14). From lysates of Sf21 cells infected with bac-ORF3 recombinant virus, MAb 122.14 immunoprecipitated proteins with apparent molecular masses ranging from 28 to 44 kDa (Fig. 2A). The same proteins were immunoprecipitated by antipeptide serum 694, which is specific for the ORF3 gene product. After endoglycosidase digestion of the immunoprecipitate, a single protein of 28 kDa was detected (result not shown), indicating that the proteins synthesized in insect cells from the ORF3 gene were N glycosylated to various degrees. In contrast, MAb 122.14 did not precipitate such proteins from a lysate of cells infected with bac-ORF4 recombinant virus. Moreover, MAb 126.1, which recognized the protein encoded by ORF4 (see below), did not precipitate any protein from a lysate of cells infected with bac-ORF3 recombinant virus. Therefore, we concluded that MAb 122.14 is specific for the ORF3 gene product, which is the 45- to 50-kDa structural protein. MAb 126.2 had the same reaction profile and also recognized the ORF3 gene product. A similar MAb (WBE2) has been developed and characterized by Drew et al. (11). MAb WBE2 was directed at an epitope on a protein with an apparent molecular mass of 45 kDa, which was en-

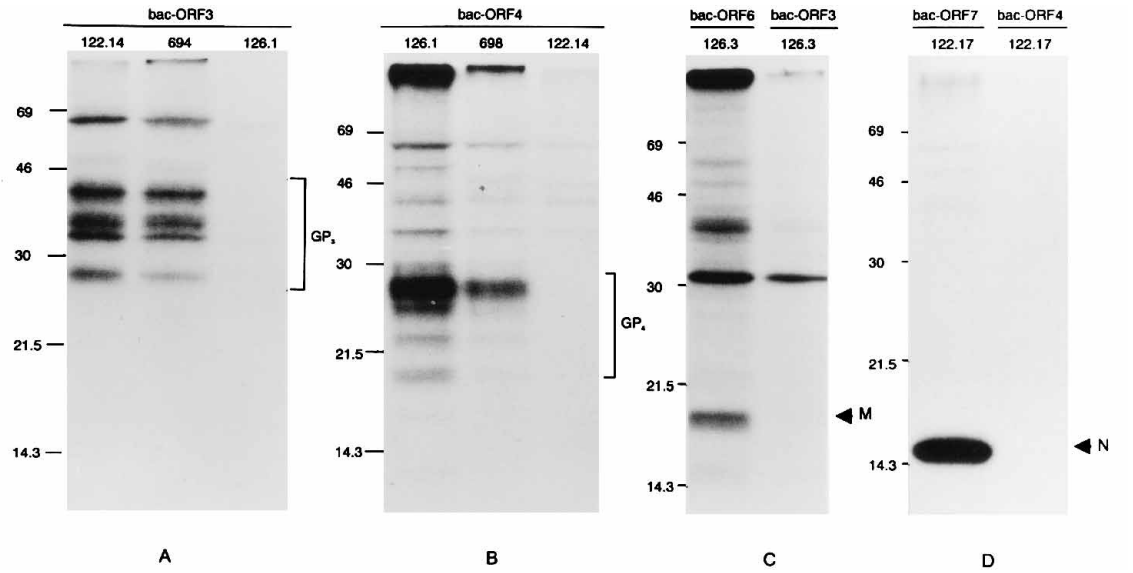

FIG. 2. Radioimmunoprecipitation of L-[^{35}S]methionine-labeled proteins from a lysate of Sf21 cells infected with baculovirus recombinants expressing ORF3 (bac-ORF3), ORF4 (bac-ORF4), ORF6 (bac-ORF6), or ORF7 (bac-ORF7) of the Lelystad virus genome, as indicated in panels A to D. Labeled proteins were precipitated with MAbs specific for Lelystad virus or gene-specific antipeptide sera and were analyzed by SDS-PAGE. The MAbs used were MAb 122.14, specific for GP3; MAb 126.1, specific for GP4; MAb 126.3, specific for M protein; and MAb 122.17, specific for N protein. The antipeptide sera used were 694, specific for ORF3 product, and 698, specific for ORF4 product.

coded by ORF3, as MAb WBE2 immunoprecipitated the in vitro-translated product of ORF3 (11).

From lysates of Sf21 cells infected with bac-ORF4 recombinant virus, MAb 126.1 immunoprecipitated proteins with apparent molecular masses ranging from 20 to 29 kDa (Fig. 2B). The same proteins were immunoprecipitated by antipeptide serum 698, which is specific for the ORF4 gene product. After peptide-*N*-glycosidase F (PNGase F) digestion of the immunoprecipitate, one major protein of 17 kDa was detected (result not shown), indicating that the proteins expressed in insect cells from the ORF4 gene were N glycosylated to various degrees. In contrast, MAb 126.1 did not precipitate such proteins from a lysate of cells infected with bac-ORF3 recombinant virus. A profile of protein bands as immunoprecipitated by MAb 126.1 was also observed with MAb 122.1, and we concluded that these two MAbs are specific for the ORF4 gene product, which is the 31- to 35-kDa structural protein.

MAb 126.3 immunoprecipitated the 18-kDa M protein from a lysate of Sf21 cells infected with bac-ORF6 recombinant virus, and MAb 122.17 immunoprecipitated the 15-kDa N protein from lysates of Sf21 cells infected with bac-ORF7 recombinant virus. The results confirm specificity of these MAbs. MAbs precipitated proteins with molecular masses higher than those of the proteins expressed from ORFs 3, 4, and 6. This indicates that proteins expressed from ORFs 3, 4, and 6 formed aggregates with cell membranes, which could not be separated by detergent in lysis buffer.

Endoglycosidase digestion of purified Lelystad virus. First, purified Lelystad virus was digested with endo-β-*N*-acetylglucosaminidase H (endo H) (Boehringer-Mannheim) or peptide-*N*-glycosidase F (PNGase F) (Boehringer-Mannheim). Next, digested virus and undigested control virus preparations were analyzed by SDS-PAGE and Western immunoblotting as described previously for the 25-kDa glycoprotein (23). Blots were incubated with MAb 122.14 (specific for the 45- to 50-kDa protein), MAb 122.29 (specific for the 31- to 35-kDa protein), or antipeptide serum 704 (specific for the 25-kDa glycoprotein). The test of the 25-kDa glycoprotein was included to check for enzyme activity, because we had previously shown that the 25-kDa protein is sensitive to PNGase F and resistant to endo H (23). Endo H digestion slightly reduced the size of the 31- to 35-kDa and 25-kDa proteins but not that of the 45- to 50-kDa protein (Fig. 3). A slight reduction in the size of the 25-kDa protein by endo H digestion was also detected in the previous study, but the effect could not be explained (23). In contrast, PNGase F digestion reduced the size of the 45- to 50-kDa protein to 29 kDa and that of the 31- to 35-kDa protein to 16 kDa, molecular masses which approximate those of unglycosylated products of ORF3 and ORF4. Because 45- to 50-kDa and 31- to 35-kDa proteins are largely endo H resis-

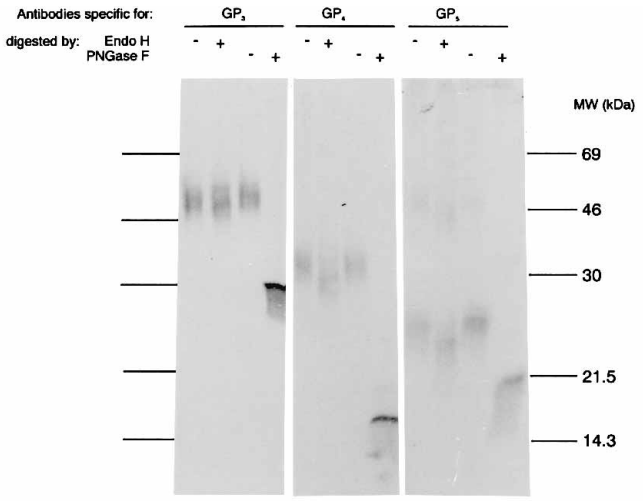

FIG. 3. Purified Lelystad virus was digested with endo H or PNGase F (+) or was left untreated (−). After SDS-PAGE and transfer to nitrocellulose strips, proteins were immunostained with MAb 122.14, specific for GP3; MAb 122.29, specific for GP4; or antipeptide serum 704, specific for GP5. Immunostaining with antipeptide serum 704 also revealed a dimer of GP5.

TABLE 2. Neutralization of PRRSV isolates by MAbs

MAb	Protein specificity	Neutralization of PRRSV isolates[a]:		
		Lelystad virus	Germ1/91	Germ2/92
126.2	GP3	−	−	−
122.1	GP4	++	++	−
122.29	GP4	+++	+++	−
126.1	GP4	+	++	−
130.7	GP4	++	++	−
126.3	M	−	−	−
126.9	N	−	−	−

[a] Germ1/91 was isolated from tissues of affected swine provided by H. Berner, Münich, Germany. Germ2/92 was isolated from tissues of affected swine provided by H. Neinhoff, Münster, Germany. These two isolates were adapted to multiply in CL2621 cells. +, >50% plaque reduction by undiluted hybridoma cell culture medium; ++, >50% plaque reduction by undiluted and 1:10 diluted medium; +++, >50% plaque reduction by undiluted, 1:10 diluted, and 1:100 diluted medium; −, no plaque reduction by undiluted medium.

tant, the asparagine-N-linked oligosaccharide chains are processed to complex type N glycans during transport through the Golgi system and during maturation of the virus. In this experiment, when the blot was stained with antipeptide serum 704, a dimer of the 25-kDa protein was also detected (Fig. 3).

Virus neutralization. Infectivity of the prototype Ter Huurne strain of Lelystad virus was quantified by a plaque assay on monolayers of CL2621 cells. After incubation for 4 days under an overlay medium solidified with 1% methylcellulose, plaques of infected cells developed. These could be visualized by immunoperoxidase staining and were counted by the naked eye. Plaques were reduced by 50% or more by incubating Lelystad virus with undiluted or diluted cell culture medium of hybridomas producing MAbs specific for the 31- to 35-kDa protein (Table 2). Virus was not neutralized by MAbs specific for the 45- to 50-kDa, M, or N proteins. To determine specificity of plaque reduction, the virus neutralization test was repeated by using two PRRSV isolates, Germ1/91 and Germ2/92, recovered from tissues of swine affected during outbreaks of porcine reproductive and respiratory disease in Germany. These isolates were adapted to multiply in CL2621 cells. In an immunoperoxidase assay, Germ1/91 was recognized by MAbs specific for the 31- to 35-kDa protein but Germ2/92 was not recognized. Germ1/91 was neutralized by MAbs specific for the 31- to 35-kDa protein, but Germ2/92 was not neutralized (Table 2).

This study showed that ORFs 3 and 4 code for structural glycoproteins of Lelystad virus: MAbs that recognized proteins of Lelystad virus with apparent molecular masses of 45 to 50 kDa and 31 to 35 kDa in a Western immunoblotting assay with purified virus also recognized similar proteins expressed from baculovirus recombinants with an insert of ORF 3 or 4. Moreover, PNGase F digestion of virions reduced the 45- to 50-kDa and 31- to 35-kDa glycoproteins to core proteins with apparent molecular masses of 29 and 16 kDa, which approximate molecular masses calculated from deduced amino acid sequences for the ORF3 product (with seven sites of potential glycosylation) and the ORF4 product (with four sites of potential glycosylation). An important finding was that MAbs specific for the 31- to 35-kDa glycoprotein neutralized Lelystad virus, confirming that this is a structural protein, part of which is exposed at the virion surface.

In the present study, the 45- to 50-kDa and 31- to 35-kDa glycoproteins were detected by polyclonal antibodies in swine convalescent serum, although this was not possible in a previous study (23). The sensitivity of Western immunoblotting was improved considerably in the present study by increasing the amount of purified virus on the nitrocellulose strips. A comparison of the staining intensities on the immunoblots showed that the M and N proteins predominate in the virion, whereas the 45- to 50-kDa and 31- to 35-kDa glycoproteins are only minor proteins. In order to visualize these proteins by immunostaining, therefore, a large amount of virus is required on the nitrocellulose strips. The 45- to 50-kDa and 31- to 35-kDa glycoproteins were not recognized by antipeptide sera, however, although these sera immunoprecipitated virus proteins expressed from ORFs 3 and 4 in baculovirus recombinants. This finding cannot be explained, since antibodies in antipeptide sera normally recognize linear epitopes exposed on the denatured protein on the blots. Apparently, epitopes of the 45- to 50-kDa and 31- to 35-kDa proteins recognized by MAbs are better exposed on the blots than are epitopes recognized by antipeptide sera.

Because of the homology among arteriviruses, we anticipate that ORFs 3 and 4 also code for structural proteins in equine arteritis virus and lactate dehydrogenase-elevating virus. De Vries et al. (10) studied structural proteins of equine arteritis virus and reported that ORFs 2 and 5 code for minor and major N-glycosylated membrane proteins, respectively, that have apparent molecular masses of 25 kDa (G_S) and 30 to 42 kDa (G_L). These proteins were detected in purified virus by immunoprecipitation with antibodies in sera of rabbits immunized with virions of equine arteritis virus. MAbs that recognized G_L were able to neutralize the virus, confirming that G_L is exposed at the virion surface (1, 2, 9). In Lelystad virus, a 25-kDa structural glycoprotein of the virus (provisionally named E protein) was translated from ORF5. However, because we had no MAbs specific for this 25-kDa glycoprotein, we could not confirm whether the ORF5-encoded protein of Lelystad virus also is a major target for virus-neutralizing antibodies. We have preliminary evidence that ORF2 of Lelystad virus also codes for a structural glycosylated protein. Since ORFs 2, 3, 4, and 5 of Lelystad virus appear to code for structural glycoproteins, we propose that these proteins be designated GP2, GP3 (45 to 50 kDa), GP4 (31 to 35 kDa), and GP5 (25 kDa), to reflect the glycosylation of these proteins and the ORFs from which they are expressed.

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