CHARACTERIZATION OF STRUCTURAL PROTEINS OF LELYSTAD VIRUS

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ABSTRACT

The genome of Lelystad virus (LV), a positive-strand RNA virus, is 15 kb in length and contains 8 open reading frames that encode putative viral proteins. Synthetic polypeptides of 15 to 17 amino acids were selected from the amino acid sequences of ORFs 2 to 7 and anti-peptide sera were raised in rabbits. Using these anti-peptide sera and porcine anti-LV serum, we identified three structural proteins and assigned their corresponding genes. Virions were found to contain a nucleocapsid protein of 15 kDa (N), an unglycosylated membrane protein of 18 kDa (M), and a glycosylated membrane protein of 25 kDa (E). The N protein is encoded by ORF7, the M protein is encoded by ORF6, and the E protein is encoded by ORF5.

INTRODUCTION

Lelystad virus (LV) is a small enveloped virus containing a positive-strand RNA genome. It was first identified in 1991 in the Netherlands by Wensvoort *et al.*[1] and in the United states by Collins *et al.*[2] as the causative agent of porcine reproductive respiratory syndrome (PRRS). PRRS is mainly characterized by reproductive failure in sows and respiratory problems in pigs of all ages[3].

The genome of LV is a polyadenylated RNA molecule of about 15 kb, which contains eight ORFs that probably encode the replicase genes (ORFs 1a and 1b), the envelope proteins

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(ORFS 2 to 6) and the nucleocapsid protein (ORF7)[4,5]. ORFs 2 to 7 are most likely expressed from six subgenomic RNAs, which are synthesized during replication[4,6].

LV (also named PRRS virus) resembles equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) and simian hemorrhagic fever virus (SHFV) in genome organization, replication strategy, amino acid sequence of the proteins and preference for infection of macrophages, both *in vivo* and *in vitro*[4,5]. Because of these similarities, proposals have been made to classify LV, EAV, SHFV, and LDV into a new virus family, tentatively named the *Arteriviridae*[4,5,7]. Arteriviruses have a genome organization and replication strategy similar to coronaviruses but the size of their genome is much smaller (12-15 kb) and they have different morphological and physicochemical properties.

Although the replication strategy of LV has been studied, and the complete nucleotide sequence of the viral genome has been determined, little is still known about the structural proteins of LV. In this paper these viral proteins were studied in more detail. The E, M, and, N proteins encoded by ORFs 5 to 7 respectively were shown to be structural proteins of LV.

MATERIALS AND METHODS

Cells and Viruses

LV was either grown on porcine alveolar macrophages or on CL2621 cells (courtesy of Boehringer-Ingelheim, St. Joseph, Mo.). Macrophages were maintained as described before[1]. CL2621 cells were maintained in Eagles basal medium supplemented with 5% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. To prepare concentrated and purified virions, confluent monolayers of CL2621 cells were infected at a multiplicity of infection (MOI) of 0.1. At the beginning of cytopathic changes (48-56 h after infection), the medium was harvested and centrifuged for 20 min at 1200 x *g*. The virus in the medium was concentrated by precipitating it with 6% polyethylene glycol 20,000 overnight at 4 °C, and was then centrifuged at 10,000 x *g* for 45 min. The pellet was resuspended in TNE buffer (0.01 M Tris-HCl pH 7.2, 0.1 M NaCl, 1 mM EDTA) and layered on a 30-0% glycerol 0-50%-di-K-tartrate gradient[8].

Endoglycosidase Treatment

Purified LV preparations were resuspended in 25 μl Endoglycosidase buffer (1% NP40, 1mM phenylmethylsulfonyl fluoride and 1 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin in phosphate-buffered saline (PBS)). Then 800 mU of peptide N-glycosidase F (PNGaseF; Boehringer Mannheim) was added and the reaction mixture was incubated overnight at 37 °C. Controls were treated similarly, except the PNGaseF was omitted.

Preparation of Antisera

Polyvalent antiserum 21 directed against LV was obtained from a specific-pathogen-free (SPF) pig infected intranasally with 10^5 $TCID_{50}$ of a fifth cell culture passage of LV (CDI-NL-91; Institute Pasteur I-1102). Blood samples were taken 42 days after infection. Gene-specific rabbit sera directed against ORFs 2 to 7 were obtained by use of synthetic peptides of 15 to 17 residues containing an amino acid sequence specific for each ORF. The peptides were conjugated to keyhole limpet hemocyanin. SPF rabbits were immunized intramuscularly and subcutaneously with 1 mg peptide conjugated to keyhole limpet hemocyanin in complete Freund's adjuvant. After one month, the rabbits were given a booster injection of the same amount of conjugated peptide in incomplete Freund's adjuvant. The rabbits were bled at 12 weeks after the first immunization. Sera were tested for their reactivity

with the various peptides in an enzyme-linked immuno sorbent assay (ELISA), using peptides coated to M96 plates[9]. Sera were also tested for their reactivity with viral antigen in an immunoperoxidase monolayer assay (IPMA) using LV infected alveolar lung macrophages, essentially as described by Wensvoort *et al.*[1].

Western Blot Analysis

Viral protein samples were suspended in Laemmli sample buffer[10], heated for 2 min at 100 °C, and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on gels containing 12.5% polyacrylamide. The separated proteins were transferred to nitrocellulose paper by electroblotting[11]. Polyclonal antiserum 21 and anti-peptide sera were diluted 1:50 in PBS containing 2% NaCl, 0.05% Tween-80, and 5% horse serum. Nitrocellulose strips were incubated with these diluted antisera for 1 h at 37 °C. The strips were washed three times with PBS containing 2% NaCl and 0.05% Tween-80. They were then incubated with rabbit anti-swine IgG horseradish peroxidase (1:500) or goat anti-rabbit IgG horseradish peroxidase (1:1000) diluted in PBS containing 2% NaCl, 0.05% Tween-80, and 5% horse serum for 1 h at 37 °C. Finally, the strips were washed three times in PBS and stained in a solution of 0.6 mg/ml 4-chloro-1-naphtol, 20% (v/v) methanol, and 0.3 µl/ml H_2O_2 (30%).

RESULTS AND DISCUSSION

Identification of Structural Proteins

Gene-specific antisera, containing antibodies directed against peptides of ORFs 2 to 7 were raised in rabbits. Six sera - - 690 (anti-ORF2), 694 (anti-ORF3), 698 (anti-ORF4), 704 (anti-ORF5), 710 (anti-ORF6), and 714 (anti-ORF7) - - were selected that reacted positively with the corresponding peptide in an ELISA (Table 1). Most of them also reacted positively in an IPMA with LV-infected alveolar macrophages and immunoprecipitated the *in vitro* translation products of their corresponding ORFs (Table 1). The generated gene-specific anti-peptide sera were used to identify the proteins incorporated in virus particles. Lelystad virus was purified on a glycerol-di-K-tartrate gradient, and infectious peak fractions, found at densities of 1.16-1.17 g/cm^3, were subjected to Western blot analyses using convalescent serum 21 and the gene-specific anti-peptide sera. Serum 21 recognized three structural proteins with an apparent molecular weight (Mw) of 25, 18, and 15 kDa (Fig.1a). Besides these three proteins, two faint bands of 28 and 42 kDa were observed. These were not detected on the control strip

Table 1. Reactivity of sera raised against LV-specific peptides of ORFs 2 to 7, using different test-systems

Serum	ORF	Amino acids[a]	Sequence	IPMA[b]	IVT[c]	WB[d]
690	2	64-78	CTLPNYRRSYEGLLPN	–	+	–
694	3	75-92	CKIGHDRCEERDHDELLM	+	+	–
698	4	62-77	CQEKISFGKSSQCREAV	+	+	–
704	5	145-161	CNFIVDDRGRVHRWKSPI	+	+	+
710	6	154-171	CVLGGKRAVKRGVVNLVKY	+	–	+
714	7	43-60	CGGQAKKKKPEKPHFP	+	+	+

[a]Location of the peptide sequence in each ORF.
[b]Immunoperoxidase monolayer assay on macrophages infected with Lelystad virus.
[c]Immunoprecipitation of radiolabeled *in vitro* translation products of ORFs 2 to 7.

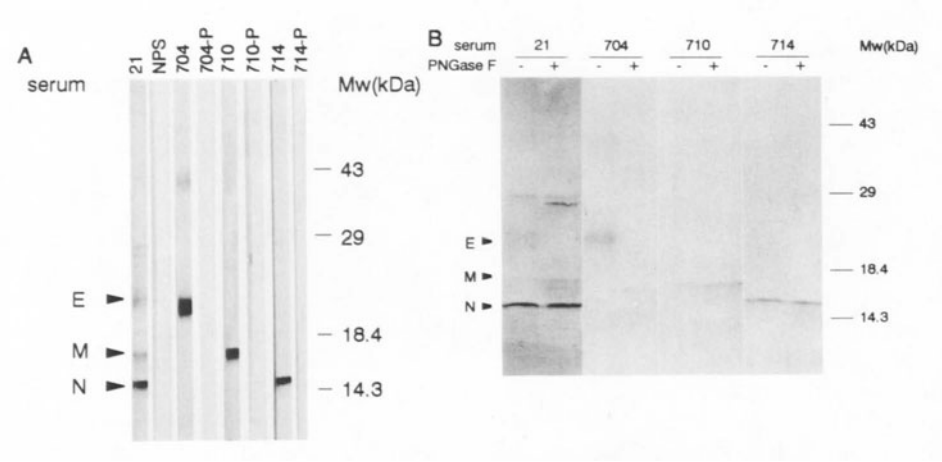

Figure 1. Western blot analysis of virions of LV. Virions were purified by isopycnic sedimentation on a glycerol-di-K-tartrate (A) Infectious fractions were resuspended in Laemmli sample buffer and were separated on a 12 5% polyacrylamide gel by SDS-PAGE. Proteins were transferred to nitrocellulose paper, and nitrocellulose strips were immunostained with porcine anti-LV serum 21, negative pig serum (NPS), gene-specific anti-peptide sera 704, 710, and 714, and their corresponding presera, 704-P, 710-P, and 714-P. The positions of the 15 kDa N protein, the 18 kDa M protein and the 25 kDa E protein are indicated with an arrow head. (B) Samples of LV virions were treated with PNGaseF (+) or left untreated (-) After SDS-PAGE and electrotransfer to nitrocellulose paper, proteins were immunostained with porcine anti-LV serum 21 and gene-specific anti-peptide sera 704, 710, and 714. The positions of the E, M, and N protein are indicated

incubated with a negative pig serum. Anti-peptide serum 704 (specific for ORF5), recognized the 25 kDa protein (E) and a faint band of 42 kDa. We concluded that the E protein is encoded by ORF5. The much fainter protein band observed at 42 kDa might be a dimer of the ORF5 encoded protein, still present to a limited extent under denaturing conditions. Anti-peptide serum 710 stained an 18 kDa protein (M), indicating that this structural protein is expressed from ORF6. Anti-peptide serum 714 reacted with a 15 kDa protein (major band) and a 28 kDa protein (minor band). This finding proves that the 15 kDa protein (N) is encoded by ORF7. The 28 kDa protein is probably a dimeric form of the N protein. No staining was observed when these Western blot strips were incubated with 704, 710, or 714 presera, or with anti-peptide sera specific for ORFs 2, 3, and 4.

Glycosidase Treatment of Purified LV

To establish which structural proteins of LV are glycosylated PNGaseF- treated and untreated virus preparations were analyzed on Western blots stained with convalescent serum 21 and the anti-peptide sera specific for ORFs 5, 6, and 7. As is shown in Figure 1b, after treatment with PNGaseF the apparent Mw of the E protein was reduced to approximately 17 to 18 kDa. The apparent Mw of the dimer of the E protein was reduced from 42 to 34 kDa. The size of the M and N proteins remained the same before and after treatment with PNGaseF. These results show that E is an N-glycosylated structural protein, whereas M and N are not. The size difference (± 7 kDa) between the unglycosylated and glycosylated E protein suggested that both putative N-glycosylation sites are functional *in vivo*.

Comparison of Structural Proteins of LV, EAV, LDV, and SHFV

Previous studies have shown that LV resembles EAV, LDV, and SHFV in genome size, virion architecture, genome organization, gene expression strategy, and amino acid sequences of

viral proteins. The identification of three major structural proteins, designated N, M, and E further confirms the relationship between LV and EAV, LDV, and SHFV. The amino acid sequence of the 15 kDa protein (N) encoded by ORF7 is extremely basic and is 41 and 20 % identical with the amino acid sequence nucleocapsid protein of LDV[12,13] and EAV[14] respectively. The identity of the nucleocapsid proteins of EAV and LDV was established by virus fractionation experiments. After the virus particles were treated with detergent only the N proteins of 15 to 16 kDa co-sedimented with the viral genome in the bottom fractions of a sucrose gradient[15]. In a sucrose gradient layered with NP40-treated LV-virions, the N protein was found in the bottom fraction of the gradient at a density of 1.18 g/cm^3, whereas the M and E protein cosedimented in the middle of the sucrose gradient at a density of 1.10 g/cm^3. This supplies further evidence for the assumption that the 15 kDa N protein of LV is the nucleocapsid protein.

The 18 kDa non-N-glycosylated envelope protein M encoded by ORF6 has the same hydrophobicity profile as the M protein of mouse hepatitis virus (MHV), infectious bronchitis virus (IBV), EAV, LDV, and the E protein of Berne torovirus[4]. These proteins are characterized by the presence of three hydrophobic segments at the N-terminus. Protease protection experiments have shown that the M proteins of MHV-A59 and IBV are type III integral membrane proteins[16,17]. They are anchored in the membrane by the three successive hydrophobic domains, whereas the C-terminal part is thought to be associated with the membrane surface.

The E protein of 25 kDa encoded by ORF5 was shown to be N-glycosylated, probably at two different sites. The E protein incorporated in virus particles was sensitive to PNGaseF (Fig. 1b), but partially resistant to EndoH (data not shown). These findings indicate that during virus maturation, E is transported through the Golgi apparatus and its N-linked oligosaccharides undergo Golgi-specific modifications. E is the counterpart of G_1, a structural envelope protein encoded by ORF5 of EAV[18]. G_1 migrated as a heterogeneous protein of 30 to 42 kDa on SDS-PAGE, because a variable number of lactosamine repeats were added to the N-linked core oligosaccharide. The E protein of LV, however, was not susceptible to Endo-ß-galactosidase (data not shown). Therefore the maturation of the N-linked oligosaccharide side chains of the E and G_1 protein is probably different.

Although the structural proteins of SHFV have not been studied in detail, a nucleocapsid protein of 12 kDa (N), an unglycosylated protein of 16-18 kDa (M), and a glycosylated glycoprotein of 50 kDa (counterpart of E and G_1) were identified in virus particles[7].

The N, M, and E proteins were also detected by Nelson *et al.*[19] in cell lysates of CL2621 cells infected with LV or with a United states isolate of LV. Although we have generated gene-specific anti-peptide sera, that recognized the N-glycosylated *in vitro* translation products of ORFs 2, 3, and 4 (Table 1) we were not able to detect the proteins encoded by these ORFs in cell lysates or purified LV. Perhaps these proteins were expressed only at very low levels in CL2621 cells and only small amounts of these proteins were incorporated in virus particles. Furthermore, the affinity of the polyvalent sera and monospecific peptide sera might not be high enough to detect such low amounts of protein. We do not yet know whether the gene products of ORFs 2 to 4 are structural proteins. The hydrophobicity profile of ORF2 of LV is similar to that of ORF2 of EAV and LDV. The gene product of ORF2, a 25 kDa glycoprotein designated G_s, was detected in a purified virus preparation of EAV[18]. It constituted only 1-2 % of the virion protein and was not recognized by a polyvalent anti-virion serum. Apparently this protein is only incidentally incorporated into virions.

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