The structural proteins of a porcine paramyxovirus (LPMV)

Anita Sundqvist,1 Mikael Berg,1 Pablo Hernandez-Jauregui,2 Tommy Linne1 and Jorge Moreno-López1*

1Department of Veterinary Microbiology, Section of Molecular Virology, BMC, Box 585, S-751 23 Uppsala, Sweden and 2Unidad de Investigacion Medica, Division de Patologia, Centro Medico Nacional, Mexico

The porcine paramyxovirus is a newly identified agent of a fatal disease in piglets, endemic in Mexico since 1980, where it was seen around the town of La Piedad, Michoacan, Mexico (hence LPM virus). At least six [35S]methionine-labelled proteins could be resolved by SDS–PAGE and five of them were clearly immuno-precipitated. Selective labelling of LPMV-infected cells with [3H]glucosamine revealed two bands with an Mr of about 66K and 59K, corresponding to the two viral glycoproteins, the haemagglutinin–neuraminidase protein and the fusion protein. Labelling of virus with [32p]orthophosphate disclosed one band with an Mr of 52K, corresponding to the phosphoprotein. Analysis of nucleocapsids obtained from purified virus or from a permanently infected cell line revealed one major band with an Mr of 68K, the nucleoprotein. Two other proteins were also identified, the large protein and the matrix protein, with apparent Mr of about 200K and 40K, respectively. The protein migration pattern of LPMV was compared, by SDS–PAGE, with that of Newcastle disease virus, bovine parainfluenza 3 virus and Sendai virus. Differences in the Mr of LPMV proteins and the proteins of these paramyxoviruses were observed. We propose that LPMV should be classified as a novel member of the genus Paramyxovirus.

Introduction

The family Paramyxoviridae is classified into three genera: Paramyxovirus, Morbillivirus and Pneumovirus (Matthews, 1982). These viruses have been identified as the causative agents of a variety of diseases both in humans and animals and some can affect the central nervous system, such as Newcastle disease virus in chickens (Brandly, 1964), canine distemper virus in dogs (Appel, 1987), and measles virus and mumps virus in humans (Shaffer et al., 1942; Kilham, 1949). The porcine paramyxovirus was isolated from pigs with nervous disorders, pneumonia, corneal opacity and infertility (Moreno-Lopez et al., 1986). The disease was observed in 1980 during an outbreak of encephalitis in piglets on farms around the town La Piedad, district of Michoacan, Mexico (hence it is called LPM virus) (Stephano et al., 1981). The disease has not been reported in other countries. The LPMV has properties in common with other paramyxoviruses and was therefore initially classified as a member of the genus Paramyxovirus (Moreno-Lopez et al., 1986; Stephano et al., 1988). However, no antigenic relationship to paramyxoviruses tested so far was found. Antisera to bovine parainfluenza virus 3 (PIV-3), Newcastle disease virus (NDV), human PIV-1, -2 and -3 and respiratory syncytial virus, as well as to mumps and measles viruses were cross-examined in appropriate assays (Moreno-Lopez et al., 1986). The aim of the present study was to identify, by SDS–PAGE, the structural proteins of LPMV and to compare them with the proteins of some other paramyxoviruses.

Methods

Viruses and cells. The viruses used were the porcine paramyxovirus LPMV, NDV (strains pigeon Sweden 83 and La Sota), bovine PIV-3 (strain U23) and Sendai virus. The LPMV was grown in pig kidney cell lines PK-15 and IBRS. A permanently infected PK-15 cell line, LPM-PI, was also established. NDV and Sendai virus were grown in 9-day-old embryonated hen eggs, whereas PIV-3 was grown in Madin-Darby bovine kidney cells.

Virus purification. Supernatants of virus-infected cell cultures or the allantoic fluids were clarified by low-speed centrifugation. The virus in the supernatants was concentrated by pelleting in a Sorvall centrifuge at 16000 g for 4 h. The pellets were dissolved in TEN buffer (10 mM-Tris–HCl pH 7.5, 1 mM-EDTA and 0.1 M-NaCl), layered on a linear 20 to 60% sucrose gradient and centrifuged at 100000 g for 3 h. The virus band was collected and pelleted through a 30% sucrose cushion. The virus was titrated in a haemagglutination test with chicken erythrocytes in a microsystem, and the protein concentration was determined according to Bradford (1976).

Preparation of antiserum. Antiserum to LPMV was prepared in rabbits by intramuscular inoculation of approximately 100 μg of purified virus, emulsified in Freund’s complete adjuvant (Difco). The same dose was injected 3 weeks later and the rabbits were bled 1 week after the last inoculation.
Purification of nucleocapsids (NCs). These were isolated from sucrose-purified virus as well as from LPM-P1 cells, essentially as described by Kolakofsky (1976). The purified virus was solubilized using 2% decanoyl-N-methylglucamide as a detergent and layered on a CsCl discontinuous gradient consisting of equal volumes of 40% CsCl (w/w), 30% CsCl, 25% CsCl and 5% sucrose in TEN buffer and centrifuged for 2 h at 220000 g. The visible band of NCs present in the middle of the original 30% CsCl step of the gradient was removed, dialysed against TEN buffer, concentrated and stored at 4°C until use. For the preparation of NCs from LPM-P1 cells, confluent monolayers were washed with PBS and treated with lysis buffer (0.15 M-NaCl, 0.005 M-Tris-HCl pH 8.0 and 0.6% NP40). The NCs in the cell lysates were purified as described above.

Electron microscopy. This was carried out as described previously (Moreno-López et al., 1986). Purified NCs were placed on a Formvar carbon-coated copper grid and then negatively stained with 2% ammonium molybdate. The grids were examined in a Philips 300 microscope at an accelerating voltage of 60 kV.

Radioactive labelling of virus and cells. Labelling of LPMV proteins with [35S]methionine was done essentially as described by Rydbeck et al. (1986). Confluent IBRS cells grown in Eagle's MEM were infected with LPMV at an m.o.i. of 5 to 10 p.f.u./cell. When the cells showed 25% c.p.e. the growth medium was changed to methionine-free Eagle's MEM. After incubation for 1 h, the LPMV-infected cells were radioactively labelled with 60 μCi/ml [35S]methionine and further incubated at 37 °C until an extensive c.p.e. was observed (at about 48 h). For preparation of cell-associated virus, virus-infected cells were harvested and pelleted at 1400 g for 10 min. Labelling of LPMV proteins with [3H]glucosamine and [32P]orthophosphate was performed similarly, but the cells were grown in glucose-free and phosphate-free Eagle's MEM, respectively.

Radioimmunoprecipitation. This was carried out essentially as described by Rydbeck et al. (1986). Mock-infected cells, LPMV-infected cells and purified virus, labelled as above, were disrupted on ice in L buffer (0.02 M-potassium phosphate, 0.1 M-NaCl, 0.5% deoxycholate 1% Triton X-100, 0.1% SDS and 1 mM-phenylmethylsulphonyl fluoride in 0.01 M-Tris–HCl pH 7.8) for 2 h, followed by centrifugation at 50000 g for 10 min. Using samples of 0.5 ml, 15 μl of preimmune or anti-LPMV rabbit serum was added to the supernatants and the tubes were shaken on ice overnight. Swine anti-rabbit immunoglobulins (Dako) were added to the mixtures, which were then incubated for 2 h on ice under continuous shaking. The complex was adsorbed by adding 150 μl of a 1:10 slurry of Staphylococcus aureus Protein A–Sepharose CL-4B (Pharmacia) in L buffer. The suspensions were incubated on ice for 1 h, shaken and washed four times in L buffer. After the last washing the pellets were resuspended in an SDS buffer (0.4 M-boric acid, 0.45 M-Tris–HCl, 3% SDS, 5% sucrose, 5 mM-EDTA, 1% 2-mercaptoethanol and 0.01% bromophenol blue) and boiled for 3 min; the supernatants were then analysed by SDS–PAGE.

SDS–PAGE. SDS–PAGE was carried out essentially as described by Laemmli (1970). The Pharmacia electrophoresis calibration kit was used as an M, marker and the gels were fixed and stained by Coomassie blue according to standard procedures. In experiments using radioactively labelled virus the gels (11% acrylamide) were vacuum-dried on filter paper before autoradiography.

Results

SDS–PAGE analysis of labelled LPMV proteins

Uninfected and LPMV-infected IBRS cells, as well as purified virus, were labelled with [35S]methionine or [3H]glucosamine. At least six [35S]methionine-labelled structural proteins could be resolved by SDS–PAGE from the purified virus preparation. The approximate M, of the six proteins were 200K, 68K, 66K, 59K, 52K and 40K (Fig. 1, lane 1). The same protein profile was revealed when immunoprecipitated samples were analysed (Fig. 2). However, in this figure the protein of 59K corresponding to the fusion (F) protein was seen as a...
faint, diffuse band. It is conceivable that this protein was insufficiently expressed to act as an immunogen for rabbits and, therefore, not immunoprecipitated to the same extent as the other proteins. The specific labelling of viral proteins with \[^{3}H\]glucosamine revealed glycosylation of the 66K and 59K bands (Fig. 1, lane 2).

For the identification of phosphorylated proteins, virus-infected IBRS cells were labelled with \[^{32}P\]orthophosphate. When the labelled purified virus was treated with RNase A before SDS-PAGE only one band (52K), that of the phosphoprotein (P) was observed (Fig. 1, lane 5). The massive band in Fig. 1 lane 4 was probably that of viral RNA because it disappeared after treatment with RNase.

In order to identify the nucleoprotein (NP), NCs were purified from \[^{35}S\]methionine-labelled LPMV as described in Methods. The analysis of purified NCs showed a single band of about 68K (Fig. 1, lane 3).

Protein migration pattern of LPMV compared to those of some other paramyxoviruses

The migration patterns of LPMV proteins were compared to those of NDV (strains pigeon Sweden 83 and La Sota), Sendai virus and bovine PIV-3 (strain U23).

The SDS–PAGE profiles are shown in Fig. 3. Both differences and similarities in the migration patterns between the four viruses were observed. The glycoprotein of 66K from LPMV migrated to a similar position as the haemagglutinin-neuraminidase (HN) proteins of PIV-3, NDV and Sendai virus (Fig. 3). The NP of LPMV (lane 5) migrated to a position similar to that of PIV-3 at around 68K (lane 4), whereas the mobility of the NP of the Sendai virus (lane 1) and NDV (lanes 2 and 3) NPs are somewhat different. The most striking difference was observed in the mobility of the P protein, which in LPMV was identified at about 52K; thus its mobility is similar to that of the P protein of NDV.
Table 1. Relative M_r of structural proteins of LPMV in comparison to those of other paramyxoviruses

<table>
<thead>
<tr>
<th>Protein</th>
<th>LPMV</th>
<th>PIV-3</th>
<th>Sendai</th>
<th>NDV</th>
<th>Mumps†</th>
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<td>39</td>
<td>38</td>
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</table>

* The M_r values for PIV-3, Sendai virus and NDV proteins are similar to those described by Panigrahi et al. (1987), Baker & Moyer (1988), Scheid & Choppin (1977), Nagai et al. (1976) and Smith & Hightower (1981).
† The M_r values according to Elango (1989).
‡ Values in bold indicate the inverse positions of the HN protein and the NP.

Electron microscopy

Electron microscopy of NCs isolated from purified virions was carried out as described in Methods. Two forms with the typical serrated appearance of paramyxovirus nucleocapsids were seen and a compact and a loose form, both with a diameter of 18 nm, are shown in Fig. 4. The fragments must have been derived from complete NC strands (such a strand is shown in the classical electron micrograph by Horne & Waterson, 1960).

Discussion

The structural proteins of LPMV corresponding to the L, NP, HN, F, P and M proteins were identified by SDS-PAGE using selective labelling and fractionation of virus particles. An almost identical protein profile was observed by immunoprecipitation using a rabbit anti-LPMV serum. Selective labelling of LPMV-infected cells with [3H]glucosamine showed two viral glycoproteins of 66K and 59K, corresponding to the HN and F proteins, respectively. These two glycoproteins, together with two other proteins, 40K (M) and 12K (F₂), were also seen when the membrane fraction was analysed after detergent treatment and interaction into a hydrophobic complex (not shown).

It was expected from previous studies on paramyxovirus NCs (Oglesbee et al., 1989) that SDS-PAGE analysis of NCs should reveal at least three different proteins, i.e. the major protein corresponding to the NP and two minor ones, the L and P proteins. However, our results show the presence of only one protein band, the NP (68K). This is in accordance with the studies of Hamaguchi et al. (1983), who reported that the bulk of the P and L proteins of NDV are not retained in the nucleocapsid preparation isolated by sucrose gradient centrifugation. To identify the viral P protein, LPMV-infected IBRS cells were labelled with [32P]orthophosphate. Two 32P-labelled bands were resolved by SDS-PAGE of purified virus, one of them of high M_r and the other one as a band of 52K (see Fig. 1, lane 4). Treatment of the labelled material with RNase A, before SDS-PAGE, resulted in the disappearance of the high M_r band, which presumably was the viral RNA. The band at 52K was considered to be the P protein (see Fig. 1, lane 5).
The L and M proteins were identified by their apparent Mr of about 200K and 40K, respectively. From the stained gel the most abundant proteins were the NP and M proteins. The HN protein migrated slightly faster than the NP (Fig. 1, lanes 1 to 3). Thus, when compared with the other paramyxoviruses tested, LPMV, HN and NP proteins migrated in reverse order (Table 1). In this table the Mr values of LPMV were compared with those of PIV-3, Sendai virus, NDV and mumps virus. The two viral glycoproteins (HN and F) of 66K and 59K, the P protein of 52K and the NP of 68K have been identified. We have no direct proof that the bands of 66K and 59K represent the HN and F proteins, respectively. The L protein was identified by its Mr of about 200K and the band of 40K probably corresponds to the M protein. The cellular actin protein (44K) was also present in purified virus. Since semi-purified virus had been used to immunize rabbits, the double L band in Fig. 2 lane 6 might represent a mixture of L protein and a protein of cellular origin. On the other hand, in Fig. 1 lane 1 a single band is present, and was considered to represent the L protein. The additional band, that of the highest Mr in lane 6, may also represent a cellular protein.

The LPMV structural proteins were compared to those of Sendai virus, NDV and bovine PIV-3 (Fig. 3). This shows that LPMV is strikingly similar to NDV as regards the mobility of the P protein (52K). A different mobility of the P proteins was observed in PIV-3 and Sendai virus (79K). LPMV is similar to PIV-3 as regards the mobility of their HN and NP proteins. These observations strongly suggest that LPMV is a genuine porcine virus. No serological relationship has been found with other paramyxoviruses tested so far (Moreno-López et al., 1986). The results of our study suggest that LPMV should be classified as a novel member of the genus Paramyxovirus.

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References


