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Membrane association of the C-terminal half of the open reading frame 1a protein of lactate dehydrogenase-elevating virus

Brief Report

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Summary. ORF 1a of lactate dehydrogenase-elevating virus, strain P (LDV-P), encodes a protein of 2206 amino acids. Eisenberg hydrophobic moment analysis of the protein predicted the presence of eleven transmembrane segments in the C-terminal half of the molecule (amino acids 980–1852) that flank the serine protease domain. cDNAs encoding ORF 1a protein segments encompassing transmembrane segments 5 to 11 and its amphipathic C-terminal end as well as the N-terminal 80 amino acids of the downstream ORF 1b protein were transcribed and the transcripts in vitro translated in the absence and presence of microsomal membranes. The synthesis of the protein products with putative transmembrane segments was enhanced by the presence of the microsomal membranes and the proteins became membrane associated. When synthesized in the absence of membranes they were recovered in the supernatant upon ultracentrifugation of the translation reaction mixtures, whereas they were recovered in the membrane pellet when synthesized in the presence of membranes. Furthermore, the latter proteins were not released from the membranes by disruption of the membrane vesicles in carbonate buffer, pH 11.5, and large portions of the proteins were resistant to digestion by trypsin, chymotrypsin and proteinase K. No N-glycosylation was observed and only little, if any, processing of the protein by the putative serine protease. The results indicate that the C-terminal half of the ORF 1a protein represents a non-glycosylated integral membrane protein. Potential modes of synthesis and function of the protein are discussed. In addition, the results showed that the synthesis of the ORF 1a protein was generally terminated at its termination codon, but that read-through into the ORF 1b gene occurred with low frequency.

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Lactate dehydrogenase-elevating virus (LDV) belongs to a new group of positive-strand RNA viruses, presently classified as genus *Arterivirus* [3], which also includes equine arteritis virus (EAV), simian hemorrhagic fever virus, and porcine reproductive and respiratory syndrome virus (PRRSV) [22, 23]. Expression of the viral genomes of 12–15 kb in infected cells is via the formation of 3′coterminal nested sets of 6 or 7 subgenomic mRNAs. The major structural proteins of these viruses are translated from subgenomic mRNAs 5, 6, and 7. The 5′ three-quarters of the viral genome encodes two large proteins, 1a (1727–2396 amino acids) and 1b (1410–1448 amino acids) which are translated from genomic RNA. The ORF 1b protein is expressed via a frameshift mechanism involving a slippery sequence and a pseudoknot [8]. The ORF 1b proteins of these viruses possess several common functional motifs, i.e. replicase, helicase, and zinc finger motifs, and probably represent the RNA replicases of these viruses. The ORF 1a protein of EAV possesses an N-terminal papain-like cysteine proteinase (PCP) that autocatalytically cleaves off the N-terminal 29-kDa end of the protein; (nonstructural protein-1; nsp-1) downstream of the catalytic PCP residues [26]. The ORF 1a proteins of LDV and PRRSV possess two PCPs. Both are functionally active in cleaving off N-terminal products of about 21 (nsp-1α) and about 26 kDa (nsp-1β), respectively [7] (see Fig. 1). In the case of EAV, a 61 kDa product (nsp-2) is then removed by another cysteine proteinase CP [7]. In addition, the ORF 1a proteins of these viruses possess a serine proteinase (SP) motif in the C-terminal half of the molecule (see Fig. 1), and the SP has been suggested to be responsible for the further cleavage of the protein into functional units [7, 26].

The function of the ORF 1a proteins is unknown. However, hydrophobic moment analyses of the ORF 1a proteins of LDV, EAV, and PRRSV have identified similar 11 or 12 potential transmembrane segments in their C-terminal segments of about 1200 amino acids [20] which flank the SP motif, 4 to 7 segments on either side (see Fig. 1). The predicted structure is unique among virus-encoded proteins and implies some specific function of the ORF 1a proteins in arterivirus replication. The only other proteins with 11 or more transmembrane segments are membrane-associated transport proteins [2, 6, 15, 19]. In the present study we provide strong evidence that the segment of LDV ORF 1a protein with transmembrane segments 5–11 (see Fig. 1) becomes intimately associated with endoplasmic reticulum (ER) membranes during synthesis and that none of its potential N-glycosylation sites becomes glycosylated. Our approach has been to in vitro translate mRNAs representing portions of LDV ORF 1a in the absence and presence of microsomal membranes and then to examine the membrane association of the protein products and their susceptibility to degradation by proteinases. At the same time we have examined the efficiency of termination of the ORF 1a protein and of frameshift/readthrough at the slippery sequence at the end of LDV-P ORF 1a (^{6763}GCU UUA AAC UGU UGA...; slippery sequence and ORF 1a termination codon are underlined; [20]).

Plasmids pBSK$^-$A66, pBKS$^+$181-3 (G3), and pBKS$^+$4–6 carry overlapping segments of the 3′ end of LDV-P ORF 1a (see Fig. 1; nt 4202–4934, 4805–5945

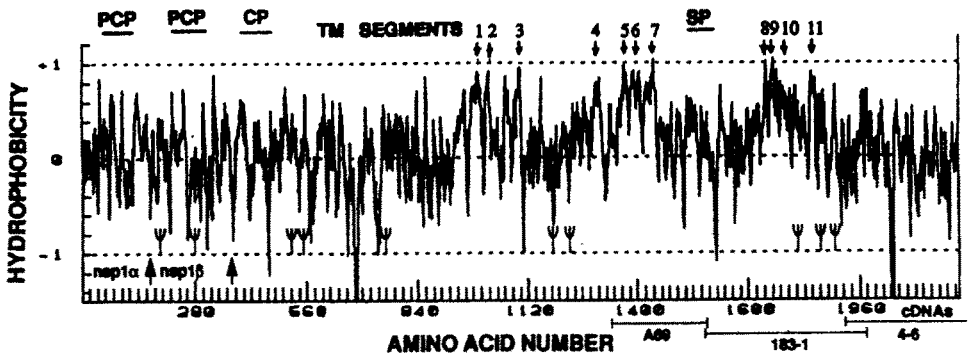

Fig. 1. Hydrophobic moment analysis of the ORF 1a protein of LDV-P by the method of Eisenberg et al. [9] and location of the predicted transmembrane segments (*TM*), of proteinase domains (*PCP* papain-like cysteine proteinase; *CP* cysteine proteinase; *SP* serine proteinase) of N-glycosylation sites (ψ), of the nsp 1α and nsp 1β cleavage products of the protein [7] and of the portions encoded by cDNAs A69, 183-1, and 4–6

and 5833–7043, respectively; GenBank accession number U15146) and have been generated in previous studies [20]. cDNA 4–6 also encompasses the 5′-terminal end of ORF 1b including the putative pseudoknot that is postulated to play a role in frame-shifting between ORF 1a and 1b. cDNA 4–6 was excised from its Bluescript vector with restriction endonucleases BamHI and AatII, incubated with Klenow DNA polymerase in the presence of deoxynucleotide triphosphates to obtain blunt ends and ligated into the MscI site of the pCITE-1 vector (Novagen, Madison, WI) which supplied an AUG initiation codon 12 nucleotides upstream of the ORF-1a segment in pC4.6. The DNAs of $pBSK^-$ A69, $pBSK^+$ 181-3 and pC4.6 were linearized and transcribed with T7 RNA polymerases or with T3 RNA polymerase in the case of $pBSK^-$ A69 as described previously [11]. The integrity of the RNA was verified by agarose gel electrophoresis (see later, Fig. 3A). The transcribed RNAs were in vitro translated in a rabbit reticulocyte lysate system in the absence and presence of canine pancreatic microsomal membranes as also described previously [10, 11]. For investigating their association with microsomal membranes, the products of translation were incubated and centrifuged under different buffer conditions [5, 13, 21]. The translation reaction mixtures were diluted about 100-fold with Tris-buffered sucrose (250 mM sucrose, 25 mM Tris-HCl, pH 7.5) or 100 mM sodium carbonate, pH 11.5. The mixtures were incubated on ice for 1 h and then centrifuged for 1 h at about 100 000 × **g** at 4 °C. When incubated in isotonic sucrose buffer, the microsomal membranes remain closed vesicles and all proteins associated with the membrane and those located within the vesicles will be located in the membrane pellet (P), whereas proteins synthesized on free ribosomes will be recovered in the supernatant (S). In contrast, when translation products are incubated in 100 mM Na^+-carbonate at pH 11.5, the membrane vesicles are disrupted and only integral membrane proteins remain associated

with the pelleted membranes, whereas secreted glycoproteins and peripheral proteins are recovered in the supernatant [13].

After centrifugation, the proteins in the supernatant (S) were precipitated with trichloroacetic acid, washed with acetone, and air dried. The pelleted material (P) was washed once in sterile water. The S and P proteins were suspended in reducing sample buffer and analyzed by tricine sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) [25] using 16.5%T: 3%C or 12.5%T: 3%C gels (T refers to total percentage of acrylamide and bisacrylamide monomers and C refers to the percentage of crosslinker relative to T [17]) as described previously [11].

Only the ORF 1a segment present in each transcript could yield a protein of significant size since the other two reading frames in each transcript did not encode any protein longer than 34 amino acids. The main product of the A69 transcript whether translated in the absence or presence of microsomal membranes was a protein of about 20–22 kDa (Fig. 2A, lanes 1 and 4). However, the presence of membranes enhanced translation of the A69 transcript and the product was almost exclusively recovered in the membrane pellet and thus associated with membranes, whereas the protein synthesized in the absence of membranes was mainly recovered in the S fraction. Incubation in carbonate buffer, pH 11.5, did not cause a significant release of the protein from the membranes (Fig. 2B, lanes 2 and 5). Thus the protein product became integrated into the membranes during membrane-associated synthesis. Translation must have been initiated at one of the three AUG codons of ORF 1a located close together at the 5′ end of the transcript, but which one is not clear. None of the three AUGs are in favorable context for translation [18] with C rather than a purine at the -3 position, relative to the A(+1) of the AUG codon but the same is the case for the initiation codons of most of ORFs 2–7 which function efficiently and specifically in translation initiation [11]. The termination codon was provided by the vector so that the six terminal amino acids in the protein product were derived from vector sequences. Initiation at one of the three AUG codons would yield proteins with 246, 234 or 205 amino acids, respectively. Another minor product of about 45-kDa was consistently produced from the A69 transcript with properties similar to those of the about 22-kDa protein. Its nature is unknown.

The results for the translation of the 183-1 transcript, which also encodes a protein sequence with potential transmembrane segments, were similar to those described for the A69 transcript. The main product of about 36 kDa was recovered in the S fraction when synthesized in the absence of membranes, whereas it was recovered in the P fraction when synthesized in the presence of membranes (Fig. 2A, lanes 5 and 8). Incubation in carbonate buffer, pH 11.5, did not cause a significant dissociation of the protein from the membranes (Fig. 2B, lanes 8 and 9). Translation was markedly enhanced by the presence of membranes. Since the molecular weight of the product was the same whether the protein was synthesized in the absence or in the presence of membranes, none of three potential N-glycosylation sites in the predicted protein (see Fig. 1) became

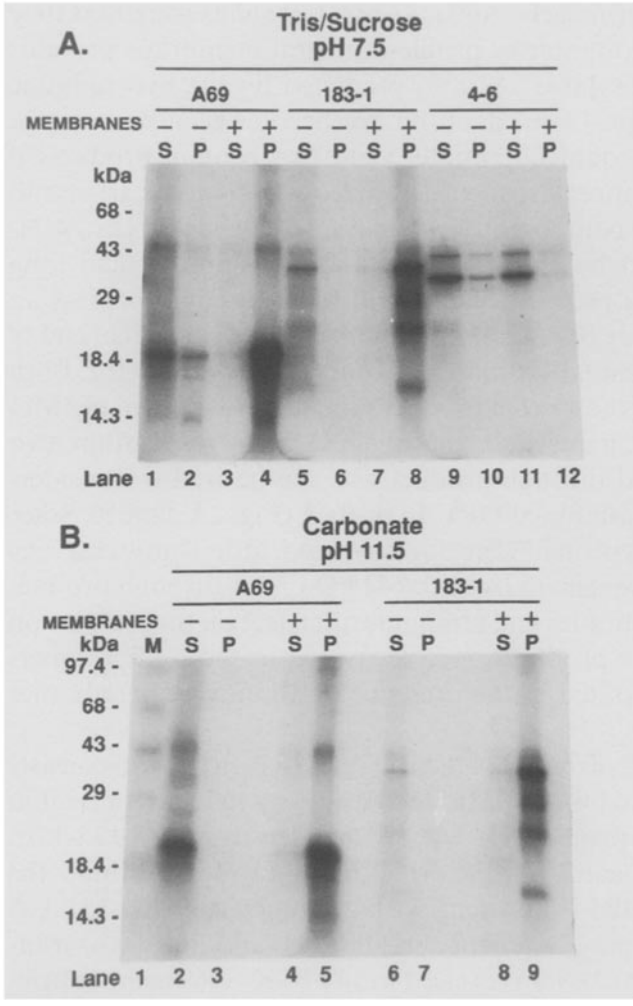

Fig. 2. Analysis of the membrane association of the in vitro translated proteins A69, 183-1, and 4–6. The transcripts of the appropriate plasmids were in vitro translated in the absence and presence of pancreatic membranes (− or +, respectively). The reaction mixtures were further incubated in Tris-sucrose, pH 7.5 (**A**), or carbonate buffer, pH 11.5 (**B**) and centrifuged. The proteins in the supernatant (*S*) and the pellet (*P*) were analyzed by tricine SDS-PAGE using 16.5%T: 3% gels

glycosylated. In contrast, we have shown previously [11, 12], that under the same experimental conditions the ORF 2–5 glycoproteins of LDV-P become N-glycosylated in vitro when synthesized in the presence of membranes.

There were also membrane-associated proteins of about 28, 24, and 16 kDa synthesized from the 183-1 transcript (Fig. 2A, lane 8). Since the putative transmembrane segments fall in the middle of the expected product (see Fig. 1), the smaller products could have resulted from premature termination or initiation at more downstream AUG codons. The former was probably the case, since there is only one additional in-frame AUG codon that could yield a product with putative membrane segments and approximately the size (22 kDa) of one of the smaller products and it is in very unfavorable context for initiation. Processing by the serine proteinase could also not play a role in generating the smaller products since the potential 183-1 product does not contain the complete SP motif.

The primary findings concerning the A69 and 183-1 proteins were that they are, as predicted by their hydrophobicity profiles, integral membrane proteins and that they are not N-glycosylated. As also predicted by the hydrophobic moment analyses (Fig. 1), the pC4.6 product did not become membrane-associated. A transcript of pC4.6 could potentially yield two protein products if a frame-shift occurred at the slippery sequence at nt 6765–6771 (see earlier and Fig. 1). One protein would be composed of 321 amino acids (35.2 kDa), 4 N-terminal amino acids encoded by the vector plus the 317 amino acid long C-terminal end of the ORF 1a protein. The second protein would possess an additional 80 amino acids (about 10 kDa), 76 representing the N-terminal end of the ORF 1b protein plus C-terminal 4 amino acids encoded by the vector. Both products were generated. Since the 44 kDa product possessed twice as many Met residues as the 35 kDa protein, namely six, the level of radioactivity in the two proteins indicates that the read-through product was synthesized in considerably lower amounts than the terminated ORF 1a protein (Fig. 2A, lane 9). Since significant amounts of a 10 kDa protein were not produced, little, if any, cleavage of the 10 kDa ORF 1b protein segment from the 44 kDa read-through product occurred. The presence of membranes had no significant effect on the translation of the pC4.6 transcript and the products were recovered in the soluble supernatant fraction whether synthesized in the presence or absence of membranes (Fig. 2A, lanes 9–12).

The predicted catalytic triad of the SP of the LDV-P ORF 1a protein consists of His-1551, Asp-1576, Ser-1646 [14, 20]. His-1551 and Asp-1576 are present in the C-terminal end of the A69 protein. Ser-1646 is encoded in cDNA 183-1. In order to examine the functionality of the SP and to further examine the membrane association of the ORF 1a protein, we have joined A69 and 183-1 at a StyI site in their overlapping segment (see Fig. 1) and in vitro transcribed/translated the construct. A69 was excised from pBSK$^-$A69 using restriction endonucleases StyI and NcoI releasing a segment of 711 nt (nt 4215–4926). pBKS$^+$183-1 was cut with StyI and the A69 segment ligated to it resulting in a clone which represented LDV nts 4779–4926 followed by LDV nts 4215–5945. This clone was then digested with BamHI and NcoI (removing LDV nt 4779–4926 from the 5′ end), blunt ended and ligated. The resulting construct pBKS$^+$A69/183-1 possessed the 5′ terminal AUG codons of A69 and potentially encoded a protein of 592 amino acids (63.7 kDa), ORF 1a amino acids 1354–1930 plus 15 C-terminal amino acids encoded by the vector. It encompassed the SP-motif and putative transmembrane segments 5–11 (see Fig. 1).

Transcription of the construct yielded a transcript of the appropriate size (Fig. 3A). Translation of the transcript yielded in some experiments a protein of the expected size of about 64 kDa (see Fig. 4), but in many others, the main product had a size of about 44 kDa whether or not membranes were present (Fig. 3B, lanes 1 and 4). The reason for this difference is not known. The 44-kDa protein could have resulted from processing of the 64-kDa protein, but, if this was the case, processing must have occurred very rapidly since a time course experiment showed that maximum amounts of product were produced by 30 min

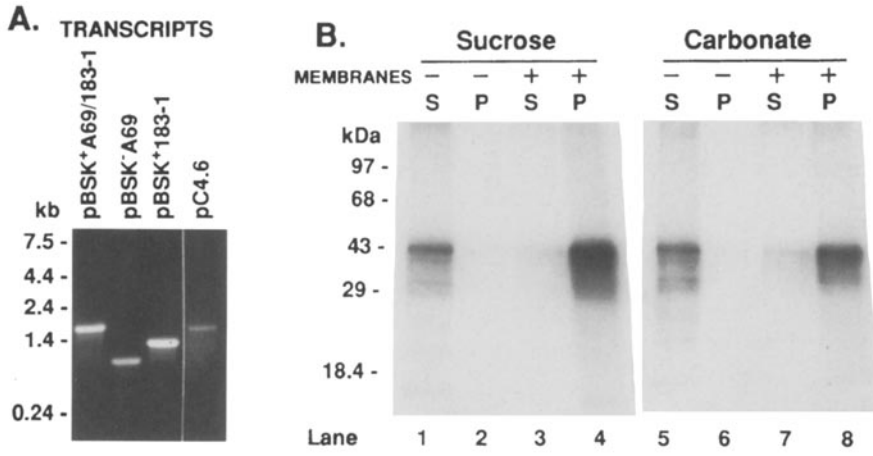

Fig. 3. Agarose gel electrophoretic analysis of the transcripts of pBKS$^+$A69/183-1, pBSK$^-$A69, pBKS$^+$183-1 and pC4.6 (**A**) and in vitro synthesis of protein A69/183-1 and analysis of its membrane association (**B**). The A69/183-1 proteins synthesized in the absence and presence of microsomal membranes were analyzed as described in the legend to Fig. 2

of incubation. The protein profile changed little, if at all, during the 15–180 min period of translation (data not shown) and there was no indication of the formation of an 18–20 kDa protein that would have been expected to be formed in such a processing step (Figs. 3B and 4). Only a minor product of 27 to 29 kDa was consistently produced (Figs. 3B and 4). Furthermore, the formation of the protein products was the same when all proteinase inhibitors (1 μg pepstatin A, 2 μg leupeptin and 2 μg aprotonin per ml, and 50 μM phenylmethylsulfonyl fluoride) were omitted from the translation reaction mixture (data not shown). Thus very little, if any, processing by the SP was observed.

Regardless, when synthesized in the absence of membranes the protein products were recovered in the supernatant fraction, whereas when synthesized in the presence of pancreatic membranes they were recovered in the membrane fraction whether the reaction products were incubated in the buffered sucrose solution or in carbonate buffer, pH 11.5, prior to centrifugation (Fig. 3B, lanes 1 and 4 and 5 and 8, respectively). The results clearly indicate that the primary protein products become membrane associated when synthesized in the presence of membranes. Furthermore, the lack of increase in size of the primary products when synthesized in the presence of membranes indicates that the proteins were not significantly glycosylated at any of three potential glycosylation sites.

In order to further investigate the membrane association of the A69/183-1 protein we determined its proteinase resistance after synthesis in the presence of microsomal membranes. Large portions of the protein seemed to be protected by membrane association from digestion by typsin, chymotrypsin, or proteinase K. The main digestion product was about 18 kDa but there were lower amounts of products with apparent molecular weights of about 29 kDa and 13–14 kDa

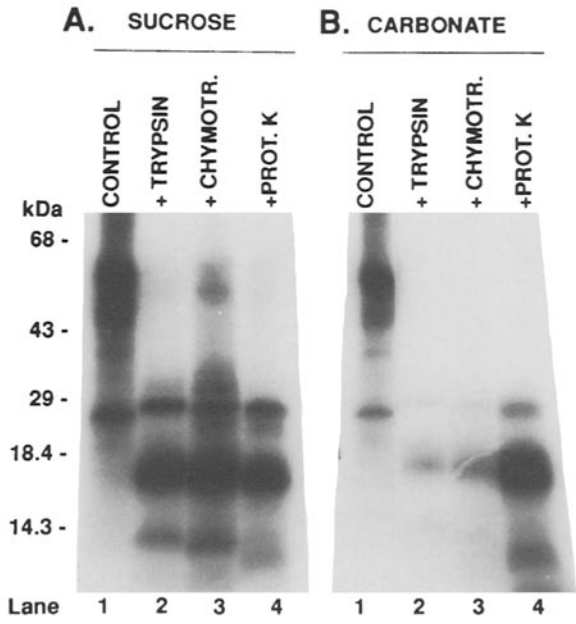

Fig. 4. Sensitivity of the membrane-associated A69/183-1 proteins to proteinase digestion. The products synthesized in vitro in the presence of membranes were incubated in Tris-sucrose, pH 7.5 (**A**), or carbonate, pH 11.5 (**B**), and then digested for 1 h on ice with trypsin, chymotrypsin or proteinase K (each at a concentration of 0.1μg/ml), or without them (control) before analysis by tricine SDS-PAGE using a 16.5%T: 3%C gel

(Fig. 4, lanes 2–4). The same products were generated by proteinase digestion after incubation of the primary product in carbonate buffer, pH 11.5 (Fig. 4B). The lower recovery of some products after the carbonate treatment does not indicate additional protein digestion since a variable loss was also frequently observed with non-proteinase-treated proteins after carbonate, pH 11.5, treatment, apparently resulting from sticking of the proteins to test tube walls [11]. The smallest proteinase digestion products varied in size depending on the proteinase used (Fig. 4); it was the smallest after proteinase K digestion.

The proteinase digestion products, except for the 13–14-kDa proteins, were larger than the segments making up the transmembrane segments 5–7 (about 11 kDa) or 8–11 (about 15 kDa). This finding indicates that portions of the A69/183-1 protein in addition to the transmembrane segments became proteinase resistant as a result of membrane association of the proteins. Similar findings have been reported for coronavirus M proteins; protein segments upstream and downstream of their transmembrane segments seem to be resistant to chymotrypsin and protein K attack [4, 24]. The same applies to the M/VP-2 and VP-3P proteins of LDV [11]. In contrast, the LDV proteins when synthesized in vitro in the absence of membranes were completely digested by the three proteinases under the same experimental conditions [11].

The authenticity of the in vitro synthesized proteins as LDV proteins was confirmed by immunoprecipitation of the products by anti-LDV antibodies. Both the about 44 kDa and 27–29 kDa products of the A69/183-1 RNA were precipitated by incubation with plasma from 5-month LDV-infected mice (IMP) but not by normal mouse plasma (NMP), or monoclonal antibodies to LDV N/VP-1 or VP-3 (Fig. 5, lanes 1–5). The same applied to the about 44 kDa and

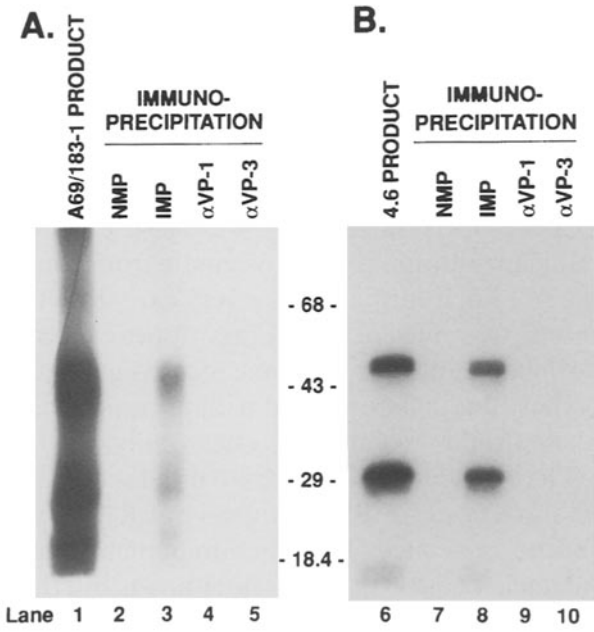

Fig. 5A, B. Immunoprecipitation of in vitro synthesized products of the A69/183-1 transcript mouse plasma (*IMP*), anti-VP-1 mAb C350201.7 or anti-VP-3P mAb 159-12 as described previously [11]

35 kDa products of the 4.6 RNA (Fig. 5, lanes 6–10). The results are also of interest in that they demonstrate that mice mount antibody responses to the C-terminal half of the ORF 1a protein.

In conclusion, the present study shows that at least a large portion of the C-terminal half of the LDV ORF 1a protein represents an integral membrane protein(s). The in vitro synthesis of the portion of the ORF 1a protein with the putative transmembrane segments is enhanced by the presence of microsomal membranes and the protein(s) synthesized in the presence of the membranes becomes inserted into the membrane in a form that is not released by disruption of the membrane vesicles in a carbonate buffer, pH 11.5, and that is largely protected from proteinase digestion. Much larger portions of the protein seem to become proteinase resistant than strictly the portions making up the putative transmembrane segments, suggesting intimate association with the membrane.

The membrane association of a large portion of the ORF 1a protein suggests that the ORF 1a protein or at least its C-terminal half is synthesized in vivo on rough ER. One scenario suggests that the synthesis of the ORF 1a protein starts on free ribosomes, that the N-terminal end is processed autocatalytically by the two PAPs releasing products of 22 and 26 kDa, (Fig. 1; nsp-1α and 1β, respectively) [7]. This might be followed by the autocatalytic release of another protein (nsp-2) by the action of cysteine protease [7]. One of the putative transmembrane segments then could function as a processed or uncleaved signal peptide so that the synthesis of the remainder of the ORF 1a protein occurs on membrane vesicles, however, in a manner that prevents N-glycosylation. In contrast, all the N-glycosylation sites in the ectodomains of the ORF 2 and ORF 5 glycoproteins become glycosylated during membrane-associated in vitro synthesis and the

oligosaccharide chains seem to become processed during traverse through Golgi membranes in vivo [11, 12].

Nothing is known about the functions of the ORF 1a protein. However, the integration of at least a large portion of the protein into ER membranes suggests one possibility, namely that this process results in the formation of unique double-membrane vesicles that are invariably associated with the replication of all arteriviruses regardless of the nature of the host cell [23, 27, 28]. Electron micrographs suggest that these double membrane vesicles originate from rough ER by protrusion and detachment [28]. Their formation is a very early event in LDV replication in macrophages and free nucleocapsids first appear among these vesicles before budding into single-membrane cisternae located generally next to Golgi membranes [23, 27]. These findings combined with the uniqueness of these structures suggests that these double membrane vesicles may play an important role in virus replication. The possibility that comes to mind is a role in the synthesis of viral genomic and subgenomic mRNAs since viral RNA synthesis in general seems to be membrane associated. The function of membranes and their associated proteins in viral nucleic acid synthesis is not known, but they might supply some organizational component to the replication complex and/or facilitate RNA folding as suggested for RNA chaperones [16]. In the case of the arteriviruses, the ORF 1b replicase protein is not an integral membrane protein and probably supplies the catalytic RNA replicase functions since it possesses helicase, replicase, and zinc binding motifs [22]. Our results as well as those for EAV [8] suggest that the ORF 1b protein is synthesized in much smaller amounts than the ORF 1a protein which is typical for ribosomal frame shifting [1]. Our results are consistent with the hypothesis that the ORF 1a and 1b proteins provide structural and catalytic functions, respectively.

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