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Intracellular Synthesis, Processing, and Transport of Proteins Encoded by ORFs 5 to 7 of Porcine Reproductive and Respiratory Syndrome Virus

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Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), a small enveloped virus containing a positive-strand RNA genome, possesses at least three major structural proteins designated N, M, and E. The N protein is considered as the major component of the nucleocapsid, whereas M and E are membrane-associated. Previous studies using peptide-specific antibodies assigned these proteins to ORFs 7, 6, and 5, respectively. In the present report, monospecific antisera raised against *Escherichia coli*-expressed ORFs 5, 6, and 7 products were used to study the synthesis and processing of PRRSV structural proteins in the highly permissive MARC-145 cell line. Treatment of viral proteins with various glycosidases showed that only E was modified by N-linked glycans. Pulse–chase experiments revealed that intracellular transport of the major envelope glycoprotein was delayed in the premedial Golgi compartment. During the first 30 min of chase, E undergoes a gradual downward shift of its apparent molecular weight, thought to result from trimming of the mannose-rich glycan structures. Once E is transported to the medial Golgi or proximal elements, some molecules undergo complete processing of all their high-mannose N-linked oligosaccharides to complex type, while in other molecules only a fraction of N-linked glycans are terminally glycosylated. These two differentially glycosylated forms of E were found to be incorporated into extracellular virions. In cells and virions, both M and E were shown to occur in heterodimeric complexes linked by disulfide bonds. The oligomerization process, as analyzed from pulse–chase experiments, showed that M and E are incorporated into M-E complexes with different kinetics and efficiencies, in a fashion similar to their counterparts in equine arteritis virus. Apparently, all steps of E protein N-glycans processing proceed after its association with M which occurs in the endoplasmic reticulum (ER). In the infected cells, E and M appear highly membrane-associated, while N is predominantly cytosolic.

INTRODUCTION

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) is the causative agent of a new disease in swine that results in considerable economic losses due to severe reproductive failure in sows of any parities and respiratory signs in unweaned and growing pigs (Benfield *et al.,* 1992; Collins *et al.,* 1992; Dea *et al.,* 1992; Wensvoort *et al.,* 1991). The virus was shown to be morphologically, structurally, and genomically similar to members of the *Arterivirus* genus (Conzelmann *et al.,* 1993; Meulenberg *et al.,* 1993a and 1993b; Benfield *et al.,* 1992; Dea *et al.,* 1992; Wensvoort *et al.,* 1992), which further includes equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV). The PRRSV genome is a positive single-strand polyadenylated RNA molecule, approximately 15 kb in length, containing eight open reading frames (ORFs) which are expressed as a nested set of subgenomic RNAs (Conzelmann *et al.,* 1993; Meulenberg *et al.,* 1993a). ORF1a and ORF1b (at the 5′ end) contain several motifs characteristic of proteins involved in the replication process, whereas ORFs 2 to 6 (from 5′ to 3′ end) are predicted to encode for membrane-associated proteins, and ORF 7 for a highly basic protein. The virion contains three major structural proteins: a nucleocapsid protein of 15 kDa (N), an unglycosylated membrane protein of 18–19 kDa (M), and a glycosylated membrane protein of approximately 25 kDa (E), which are encoded by ORFs 7, 6, and 5, respectively (Mardassi *et al.,* 1995; Meulenberg *et al.,* 1995; Nelson *et al.,* 1993). In the case of EAV, four structural proteins (N, M, G_L, and G_S) have been identified and shown to be encoded by ORFs 7, 6, 5, and 2, respectively (De Vries *et al.,* 1992). The N, M, and G_L of EAV correspond to the products of PRRSV ORFs 5, 6, and 7, respectively (Mardassi *et al.,* 1995; Meulenberg *et al.,* 1995). The protein profile displayed by LDV is similar to that of EAV. However, as for PRRSV, the counterpart of the G_S protein has not yet been identified in purified virus preparations (Faaberg and Plagemann, 1995). Four major structural proteins have been reported for SHFV, and with the exception of the p15 and p20 species, which have been shown to be encoded by ORFs 7 and 6, respectively, the gene assignment for the other proteins is still unknown (Godeny *et al.,* 1995).

Recently, the synthesis and processing of EAV structural proteins have been investigated. Gel electrophoresis under nonreducing conditions revealed that G_S occurs in the infected cells in three distinct monomers and

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as a disulfide-linked dimer (De Vries *et al.,* 1995a) and that M and G_L are engaged into disulfide-linked heterodimers (De Vries *et al.,* 1995b). Such association between the two major envelope proteins of the virions has been also demonstrated in the case of LDV and was claimed to be essential for viral infectivity (Faaberg *et al.,* 1995).

In the present study, we have confirmed the gene assignment of the three major structural proteins of PRRSV using monospecific antisera raised against *Escherichia coli*-expressed ORFs 5, 6, and 7 products. Then, we analyzed the intracellular synthesis, processing, and transport of the three major viral structural proteins and demonstrated that the two putative membrane-associated proteins fold into disulfide-linked heterodimers, as their counterparts in EAV and LDV. Finally, we discussed the implications of our findings for virus assembly.

MATERIALS AND METHODS

Cells and viruses

The Québec cytopathic strain IAF-Klop of PRRSV (Mardassi *et al.,* 1994a) was propagated in MARC-145 cells (Kim *et al.,* 1993), a clone of MA-104 cells highly permissive to PRRSV, kindly provided to us by J. Kwang (U.S. Meat Animal Research Center, Clay Center, NE). Infectivity titers were determined by titration of clarified tissue culture medium using an endpoint dilution procedure and calculation of 50% tissue culture infective doses ($TCID_{50}$) per milliliter (Dea *et al.,* 1992). For the present study, the virus was plaque-purified twice and then passaged at low multiplicity of infection (m.o.i.; 0.01 $TCID_{50}$/ml).

Recombinant DNA clones

All constructs were made by using standard methods (Sambrook *et al.,* 1989). The coding sequence of PRRSV ORFs 5 and 6 were inserted into pBS SK+ plasmid vector (Stratagene) to yield pBS5 and pBS6, as previously described (Mardassi *et al.,* 1995). Both genes were then subcloned from their respective plasmids, following digestion with *Eco*RI and *Not*I endonucleases, within the appropriate sites of the procaryotic expression vector pGEX-4T1 (Pharmacia), in frame with the glutathione *S*-transferase (GST) gene to form pGEX-5 and pGEX-6, respectively. The ORF7 gene was amplified by RT-PCR using the primer pair SRRP-ENS/SRRP-NI, as previously described (Mardassi *et al.,* 1994b). Sequences of the oligonucleotide primers were derived from the 3′ end genomic region of the Québec IAF-exp91 reference strain (EMBL/GenBank Accession No. L40898); their sequence and position were SRRP-ENS (5′-CTAAATATGCCAAATAACAAC-3′; 2307–2327) and SRRP-NI (5′-CTCAAGAATGCCAGCTCA-3′; 2700–2683). For directional cloning, *Eco*RI and *Bam*HI recognition sites were added at the 5′ end of these primers, respectively. The amplified product was cloned into *Eco*RI–*Bam*HI-digested pMAL-c2 procaryotic expression vector (New England Biolabs Ltd, Mississauga, Canada), in frame with the maltose-binding protein (MBP) gene to form pMAL-7.

Production of GST-ORF5, GST-ORF6, and MBP-ORF7 fusion proteins

Plasmids pGEX-5 and pGEX-6 were introduced into BL21(DE3) (Novagen)-competent *E. coli* cells, according to standard methods (Sambrook *et al.,* 1989). The recombinant bacteria were grown in 2× YT medium containing 2% D-Glucose and 100 μg/ml of ampicillin. To prepare GST-ORF5 and GST-ORF6 fusion proteins, an overnight culture of the recombinant bacteria was diluted 1:10 in 2× YT medium lacking glucose and allowed to grow at 37° to an optical density at 600 nm of 1.0. Protein expression was induced by the addition of 0.1 m*M* IPTG to the culture medium. After 4 hr at 37°, the cells were collected by centrifugation, resuspended in Laemmli sample buffer at 1:30 of the original culture volume, and subjected to 10% SDS–PAGE. The bands corresponding to GST-ORF5 and GST-ORF6 fusion proteins were electroeluted from the gel at 45 V for 6 hr in electrophoresis buffer (25 m*M* Tris, 192 m*M* glycine, 0.1% SDS, pH 8.3) and then for 1 hr in the same buffer but lacking SDS, using a Bio-Rad Electro-Eluter (Richmond, CA). Expression of MBP-ORF7 from TB1 strain of *E. coli* (New England Biolabs) transformed with pMAL-7 was induced by IPTG (Mounir *et al.,* 1995). The fusion protein was purified by affinity using an amylose column and an elution buffer that contains 10 m*M* of maltose, as recommended by the manufacturer (New England Biolabs).

Generation of antisera specific to PRRSV ORFs 5, 6, and 7 products

New-Zealand albino rabbits were inoculated subcutaneously with 250 to 300 μg of the eluted GST-ORF5 and GST-ORF6 fusion proteins emulsified with complete Freund's adjuvant (GIBCO BRL). The rabbits were boosted four times with the same amount of fusion protein given intramuscularly at 2-weeks intervals. In the case of ORF7, the fusion protein MBP-ORF7 was subjected to digestion with factor Xa, electrophoresed, and the band corresponding to ORF7 product was electroeluted. Immunization of rabbits with the unfused ORF7 product was carried out as described above. Reactivity of the antisera was assessed by indirect immunofluorescence assay (Mardassi *et al.,* 1994a) using IAF-Klop-infected MARC-145 cells. Antisera to GST-ORF5, GST-ORF6, and to unfused ORF7 product were designated α5, α6, and α7, respectively. A porcine hyperimmune serum raised against IAF-Klop isolate was used as positive control (Mardassi *et al.,* 1995).

SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting

Supernatants of PRRSV-infected MARC-145 cells displaying 75% cytopathic effect were clarified at 8000*g* for

30 min, and then extracellular virions were concentrated and purified by isopycnic ultracentrifugation on continuous 30 to 50% sucrose gradients prepared in TNE buffer (50 m*M* Tris–HCl, pH 8.0, 150 m*M* NaCl, 1 m*M* EDTA), as previously described (Dea *et al.,* 1989). Replicas of viral proteins, separated by electrophoresis through 12% SDS–polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes, were also prepared as previously described (Mardassi *et al.,* 1994a). After saturation with 5% blotto (skim milk powder in 50 m*M* Tris-buffered saline (TBS) solution), nitrocellulose membranes were incubated for 2 hr at room temperature with porcine convalescent anti-PRRSV serum, or rabbit antisera α5, α6, or α7, diluted 1:200 in blocking buffer. After washing in TBS containing 0.05% Tween 20, the membranes were further incubated in the presence of 1:1500 dilution of either alkaline phosphatase-conjugated anti-porcine or anti-rabbit IgG (Boerhinger Mannheim) for 45 min. The immune complexes were revealed using a commercial alkaline phosphatase conjugate substrate kit (Bio-Rad) containing nitroblue tetrazolium and 5-bromo-4 chloro-3-indolyl phosphate in TBS.

Metabolic labeling of PRRSV proteins

Confluent monolayers of MARC-145 cells in 25-cm^2 tissue culture flasks (Falcon) were infected with PRRSV at a m.o.i. of 1. At 24 hr postinfection (p.i.), infected cultures were rinsed twice with PBS and incubated in methionine-free DMEM for 1 hr at 37°. Following this starvation period, [^{35}S]methionine (sp act of 1,120 Ci/mmol; Amersham Searle Co., Oakville, Ontario) was added at a final concentration of 100 μCi/ml. Incubation was continued for 2 hr, and then cells were washed twice in cold PBS and solubilized in either LB-1 lysis buffer (100 m*M* NaCl, 20 m*M* Tris–HCl, pH 7.5, 1 m*M* Na EDTA, 1% Triton X-100, 2 m*M* phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml of aprotinin) or LB-2 lysis buffer (20 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, 5 m*M* EDTA, 0.5% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 2 m*M* PMSF, 1 μg/ml of aprotinin). Wherever indicated, 20 m*M* *N*-ethylmaleimide (NEM) or 50 m*M* iodoacetamide was added to PBS and lysis buffer. Cell lysates were clarified at 10,000*g* for 30 min and used for immunoprecipitation. Cell lysates from uninfected MARC-145 cells were prepared similarly in parallel. Alternatively, extracellular virions, from the supernatant of PRSSV-infected cultures isotopically labeled for a period of 12 hr, were gradient-purified, as described above. For pulse–chase analyses, infected cells were labeled for 15 min at 24 hr p.i. with 300 μCi/ml of [^{35}S]-methionine (pulse), washed twice with DMEM containing 5 m*M* unlabeled methionine, and incubated thereafter in the same medium for different time periods (chase). Supernatants were removed at various intervals, and cells were washed and lysed, as described above. Extracellular virions released in the culture fluid of chased cells were pelleted by centrifugation at 100,000*g* for 2 hr, and virus proteins were finally solubilized in LB-2 lysis buffer prior to immunoprecipitation.

Subcellular localization of PRRSV proteins

MARC-145 cell monolayers were infected with PRRSV at a m.o.i. of 1. At 24 hr p.i., cells were isotopically labeled for 2 hr, as mentioned above. The monolayers were rinsed twice and scraped into PBS, and cells were pelleted by centrifugation at 8,000*g* for 5 min. Cell pellets were resuspended in a hypotonic buffer containing 20 m*M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid at pH 7.4, 10 m*M* NaCl, and 1 m*M* $MgCl_2$ and disrupted by passage 25 times through a 27-gauge hypodermic needle. The nuclear fraction was obtained by pelleting disrupted cells by centrifugation at 1,000*g* for 5 min. The supernatant was recentrifuged at 30,000*g* for 30 min to yield a microsomal fraction (pellet) and a cytosolic fraction (supernatant). All steps were performed at 4°. The various fractions were adjusted to 1× in LB-1 lysis buffer, passed several times through a 27-gauge needle, and finally clarified before immunoprecipitation with a pool of α5, α6, and α7 antisera.

Immunoprecipitation of viral proteins and endoglycosidases treatment

Aliquots of PRRSV-labeled proteins from purified virus preparations or infected-cell lysates were adjusted with LB-1 or LB-2 lysis buffer to a final volume of 0.5 ml and incubated overnight at 4° with 5 μl of the porcine anti-PRRSV hyperimmune serum or rabbit antisera to the recombinant viral proteins. The immune complexes were then adsorbed to protein A–Sepharose beads (Pharmacia), washed, and dissolved in electrophoresis sample buffer in the presence or absence of 5% (v/v) β-mercaptoethanol (β-ME), as previously described (Dea and Tijssen, 1989). For treatments with endoglycosidases, immune complexes were resuspended in 200 μl of either endo-β-*N*-acetylglucosaminidase H (endo H) (Boehringer Mannheim) buffer (50 m*M* sodium acetate, pH 6, 2 m*M* PMSF, 1 μg/ml of aprotinin), endoglycosidase F/N-glycosidase F (glyco F) (Boehringer Mannheim) buffer (50 m*M* sodium acetate, pH 6, 10 m*M* EDTA, 1% (v/v) β-ME, 2 m*M* PMSF, 1 μg/ml of aprotinin), or endo-β-galactosidase (endo β) (Boehringer Mannheim) buffer (50 m*M* sodium acetate, pH 5.8, 200 μg/ml bovine serum albumin, 2 m*M* PMSF, 1 μg/ml of aprotinin), and digested overnight at 37° with 4, 200, and 3 mU of enzyme, respectively. After incubation, immunoprecipitates were washed in 20 m*M* Tris–HCl at pH 7.6 and analyzed by SDS–PAGE, as described above. Specific conditions for each immunoprecipitation experiment are detailed in the figure legends.

RESULTS

Identification of PRRSV ORFs 5-, 6-, and 7-encoded proteins and related products in virions and infected cells

To identify and analyze in detail the proteins specified by PRRSV ORFs 5, 6, and 7, their entire coding sequences were expressed in *E. coli* in order to raise monospecific antisera. The latters were first employed in immunoblotting and immunoprecipitation experiments to identify the products of ORFs 5 to 7 derived from virus-infected MARC-145 cells.

Figure 1A shows the reactivity profiles of antisera α5, α6, and α7 when tested by Western immunoblotting against sucrose gradient-purified disrupted virions. Antiserum α5 (anti-ORF5 product) reacted strongly with a protein species comigrating with the viral E protein, which was also recognized by the homologous porcine anti-PRRSV (αV) hyperimmune serum. Antiserum α6 (anti-ORF6 product) recognized a single protein which was indistinguishable from the authentic viral M protein. Finally, α7 antiserum detected principally a protein that corresponds to the viral N protein, together with a faster migrating product. The latter band appeared essentially when extracellular virus was analyzed and thus may represent a degradation product of N. Although very minor bands could be detected by these antisera, they were not constantly apparent from one experiment to another, and thereby, could not be associated with specific reactions, considering that some of them were slightly reactive with most of the preimmune sera pα5, pα6, and pα7. These results indicate that PRRSV N, M, and E proteins are encoded by ORFs 7, 6, and 5, respectively, confirming what has been previously reported using antipeptide sera (Meulenberg *et al.,* 1995).

The recognition patterns of α5, α6, and α7 antisera were also analyzed by radioimmunoprecipitation assay (RIPA). When [^{35}S]methionine-labeled purified virus was disrupted with a lysis buffer containing Triton X-100 as the only detergent (LB-1), antiserum α5 precipitated the E protein and substantial amount of M (Fig. 1B). Similarly, antiserum α6 precipitated both M and E proteins. The N protein, which was strongly precipitated by antiserum α7, was also apparent in the immunoprecipitates of α5 and α6 antisera. However, neither M nor E protein were precipitated by α7 antiserum. Under "more stringent" conditions, when detergents such as NP-40, SDS, and sodium deoxycholate were incorporated into the lysis buffer (LB-2), both M and E still coprecipitated, whereas N protein was no longer precipitated along with M and E from both extracellular virions (Fig. 1C) and infected cells (see Figs. 5A and 5C). The finding that PRRSV N protein was not precipitated by protein A–Sepharose in the absence of any specific antiserum and using LB-1 lysis buffer (data not shown) suggests a specific interaction between PRRSV envelope proteins and the viral nucleocapsid.

Collectively, these data show that the two major PRRSV envelope proteins are associated both in infected cells and in virions, despite the presence of several detergents.

PRRSV E protein incorporated into virions contains N-linked oligosaccharides of the high-mannose and complex types

To study the processing of PRRSV N, M, and E proteins, we first analyzed their sensitivity to various endoglycosidases. PRRSV proteins from purified [^{35}S]-methionine-labeled virus were precipitated using a cocktail of α5, α6, and α7 antisera and digested with either endo H, which cleaves high-mannose and hybrid oligosaccharides (Tarentino and Maley, 1974), or with glyco F, which cleaves all N-linked oligosaccharides (Tarentino *et al.,* 1985). The presence of N-linked glycans modified by poly-*N*-acetyllactosamine was checked by digestion with endo-β-galactosidase (Scudder *et al.,* 1983). Despite the presence of potential N-linked glycosylation sites, as predicted from their amino acid sequences, neither N nor M protein was sensitive to any endoglycosidase treatment, indicating that during the virus infection these proteins are not modified by addition of N-linked oligosaccharides. Unlike N and M, PRRSV E protein was completely sensitive to glyco F giving rise to a 16.5-kDa species (E_0) (Fig. 2, lane F). Interestingly, treatment with endo H resulted in the appearance of a new 21-kDa species (E_1), while E protein was still present, but in lesser amounts (Fig. 2, lane H). In contrast, the E protein was completely resistant to treatment with endo-β-galactosidase (Fig. 2, lane B). No degradation could be observed in the case of untreated proteins which have been processed similarly but without addition of endoglycosidases (Fig. 2, lane M). Both E_0 and E_1 were obtained in other independent experiments, and thus, represent truly digested products. A similar digestion pattern was obtained for N, M, and E proteins analyzed from cell lysates (data not shown). These results confirm that among PRRSV N, M, and E proteins, only the membrane-associated E protein is glycosylated. If we assume that acquisition of one N-linked carbohydrate side chain results in an approximately 2.6-kDa increase in the molecular weight (MW) of a given protein (Bergman and Kuehl, 1982), the 8-kDa difference in the apparent MW of the undigested and glyco F-treated E proteins (E vs E_0) suggests that *in vivo,* each one of the three potential N-linked glycosylation sites predicted from the IAF-Klop amino acid sequence (data not shown) bear an N-glycan moiety. Unexpectedly, aside from the presence of complex N-glycans, a considerable amount of E protein incorporated into virions con-

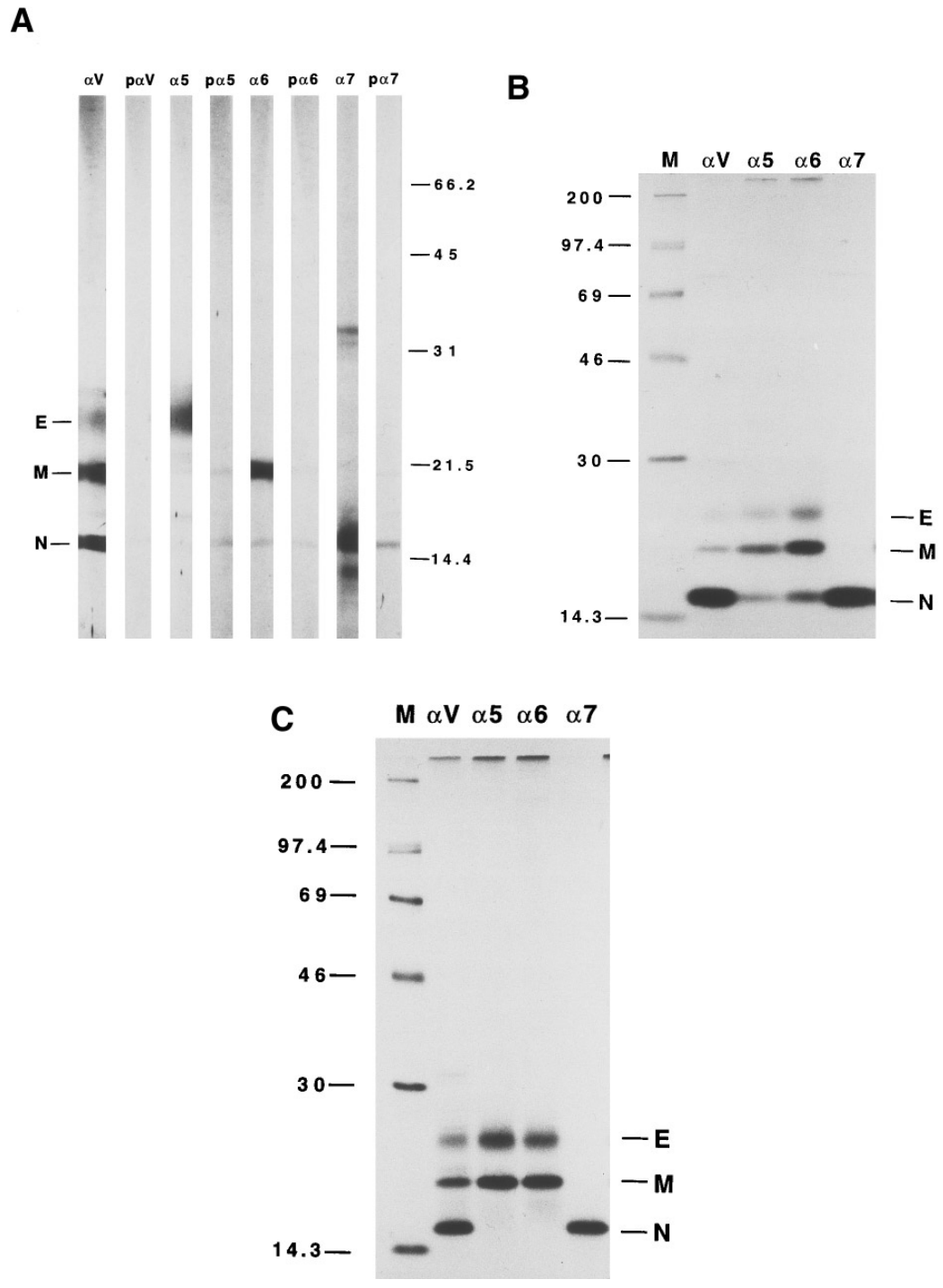


FIG. 1. Identification of proteins encoded by PRRSV ORFs 5, 6, and 7 in purified viral preparations. (A) Reactivity of monospecific antisera α5, α6, and α7 were first analyzed by Western immunoblotting assay. Proteins from sucrose-gradient purified virus were separated in denaturing 12% SDS–polyacrylamide gel and transferred to nitrocellulose membrane. Strips of nitrocellulose were probed with either α5, α6, or α7 monospecific antiserum or their corresponding preserum collected prior to immunization (pα5, pα6, or pα7, respectively). As controls, nitrocellulose strips were reacted with an hyperimmune porcine anti-PRRSV serum (αV) or its preserum (pαV). With α7 antiserum, two closely migrating bands with apparent MW slightly above 30 kDa were not constantly revealed and should be considered as nonspecific. (B) Gel analysis of PRRSV proteins immunoprecipitated from lysates of [^{35}S]methionine-labeled and sucrose gradient-purified virus. Viral proteins were solubilized with LB-1 lysis buffer containing 1% Triton X-100 followed by immunoprecipitation with αV, α5, α6, or α7 antiserum. (C) Similar to B, except that LB-2 lysis buffer was used to solubilize viral proteins. Under these conditions, the N protein was no longer coprecipitated with E and M using either α5 or α6 antiserum. The MW of [^{14}C]methylated size marker protein bands (lane M) are to the left of gels B and C, and positions of the three PRRSV major structural proteins N, M, and E, are indicated in the left of gel A and in the right of gels B and C.

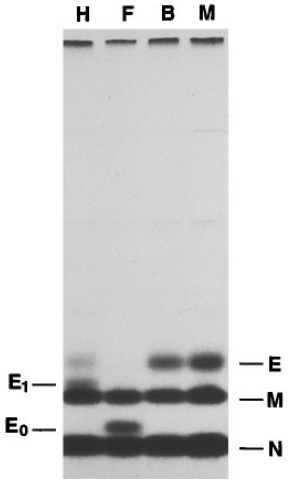


FIG. 2. Identification of PRRSV glycosylated proteins. PRRSV was labeled with [^{35}S]methionine and purified on sucrose gradient. Viral proteins were precipitated with a pool of α5, α6, and α7 antisera, treated overnight at 37° with endo H (lane H), glyco F (lane F), endoβ (lane B), or mock-treated (lane M), and finally analyzed by SDS–PAGE (12%) under reducing conditions. The glyco F-fully digested (E_0) and endo H-partially resistant (E_1) forms of E protein are indicated in the left margin, and the three major viral structural proteins N, M, and E are to the right. The various enzymatic treatments did not affect the electrophoretic mobilities of both N and M proteins.

tains N-linked oligosaccharides of the high-mannose type. The fact that after digestion with endo H, a substantial amount of endo H-resistant form of E was still present and that after glyco F treatment all the protein was converted into a single species (E_0) suggests that some E molecules may contain both types of N-linked oligosaccharides, whereas others contain only oligosaccharides which have been completely processed to complex type in the Golgi apparatus. Indeed, if all E molecules contained the two types of N-linked oligosaccharides, endo H treatment would have resulted in the complete conversion of E to E_1 species. In the case of molecules modified by both types of N-linked oligosaccharides, the reduction in the apparent MW of E after endo H treatment, which is about 3.5 kDa, correlates with the complete removal of one oligosaccharide side chain.

Intracellular processing of PRRSV N, M, and E proteins

Pulse–chase experiments were performed to examine in greater detail the processing of N, M, and E proteins. At 24 hr p.i., PRRSV-infected MARC-145 cells were pulse-labeled with [^{35}S]methionine for 15 min and processing of intracellular and extracellular viral proteins was monitored during a chase period that varied from 15 to 240 min. Viral proteins were solubilized from extracellular virions or infected cell lysates and analyzed by RIPA using a cocktail of α5, α6, and α7 monospecific antisera, as described under Materials and Methods. The immunoprecipitation pattern of intracellular N, M, and E proteins is illustrated in Fig. 3A. At 0 min of chase (following pulse), the three major viral proteins were efficiently precipitated and migrated in the gel with the expected mobility, except for E which appeared to be first synthesized as a 26-kDa protein which represents an increase of its MW of approximately 1.5 kDa. A gradual shift to faster mobility of this protein was then observed after 15 to 30 min of chase, its final MW of 24.5 kDa being obtained after 60 min of chase. No further change in E mobility was noticed until 240 min of chase, at which time the intensity of this protein dramatically decreased (Fig. 3A). Resistance of E to treatment with endo H, a property which indicates that proteins have reached the medial Golgi compartments (Dunphy and Rothman, 1985), was evident after 60 min of chase (Fig. 3B). Indeed, when immunoprecipitates were subjected to endo H digestion, E was fully sensitive and completely converted to its E_0 precursor form from 0 to 30 min of chase, indicating that it still resides in the ER, wherein trimming reactions are known to begin (Kornfeld and Kornfeld, 1985). After 60 min of chase, resistance to endo H was readily observed and was manifested by the appearance of the completely resistant form E and the partially sensitive form E_1 that have been previously identified in purified virions. After 240 min of chase, the intensity of both E and E_1 forms declined, in accordance with the pattern observed when immunoprecipitates were not digested with endo H. In the case of N and M proteins, their apparent MW, as well as their intensity, were practically unchanged during the whole chase period (Figs. 3A and 3B). These proteins were resistant to digestion with endo H during the entire chase period, confirming the results obtained with purified virions. In addition, M protein seemed to be synthesized at a higher amount than E provided that α5 and α6 were equally efficient in precipitating the corresponding proteins. Finally, when RIPA was performed with the extracellular virions of chased cells, N, M, and E proteins were precipitated all together after 60 min, at which time the intracellular form of E protein has reached its mature MW (Fig. 3C vs 3A).

In virions, PRRSV M and E proteins occured essentially in a heterodimeric form

Previous studies with LDV and EAV have revealed that their two putative membrane-associated proteins are linked via disulfide bonds; the cysteine residues which may be involved in this linkage were found to be conserved at the amino termini of all arteriviruses membrane proteins (De Vries *et al.,* 1995b; Faaberg and Plagemann, 1995). To seek for the presence of disulfide bonds between the two envelope proteins of PRRSV, gradient-purified [^{35}S]methionine-labeled extracellular virions were precipitated using the rabbit monospecific antisera and then finally analyzed by SDS–PAGE under reducing and nonreducing conditions. Figure 4A shows once

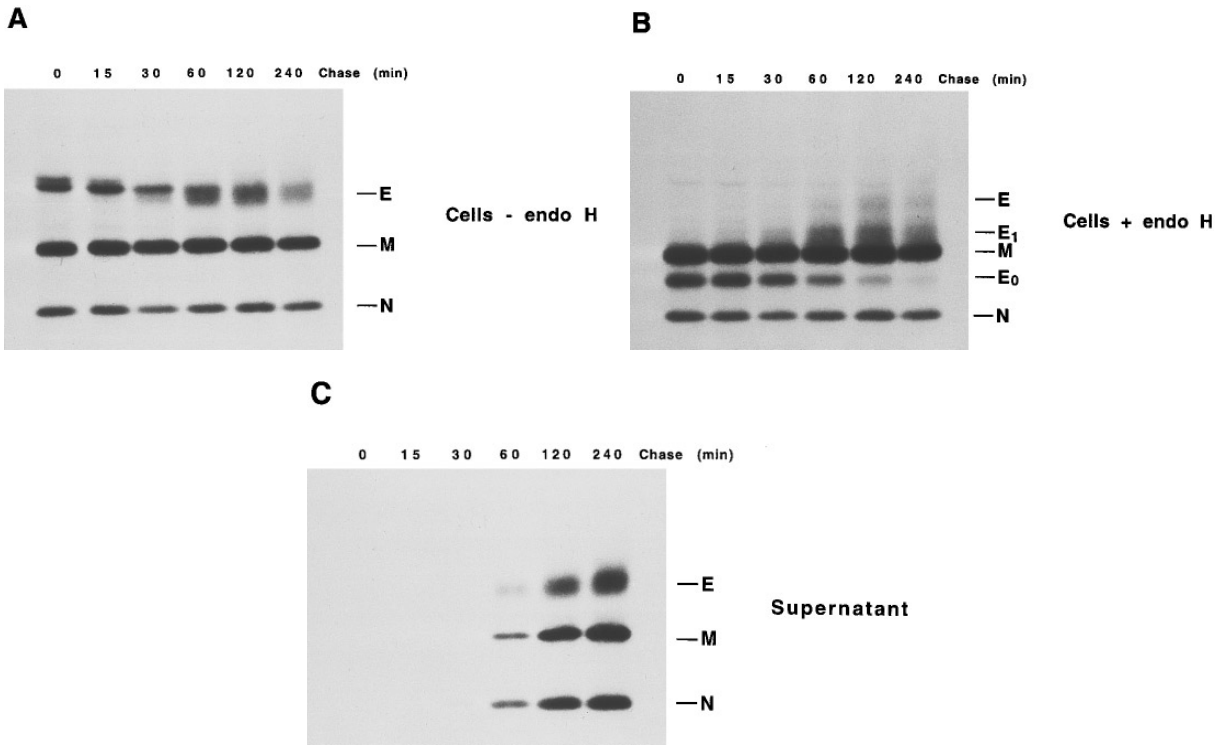


FIG. 3. Processing of PRRSV N, M, and E proteins. IAF-Klop-infected MARC-145 cells were labeled with 300 μCi/ml of [^{35}S]methionine at 24 hr p.i. for 15 min (0) and chased for various times (15, 30, 60, 120, and 240 min). Labeled proteins were solubilized from cells with a combination of ionic and nonionic detergents (LB-2 lysis buffer) and subjected to radioimmunoprecipitation with a pool of α5, α6, and α7 antisera. Immunoprecipitates were split in two equal portions and analyzed by SDS–PAGE without prior treatment (A) or after being treated with endo H (B). Released virions derived from each of the above labeling time periods were collected from supernatants upon a centrifugation of 100,000*g*. Proteins from extracellular virus were immunoprecipitated with a cocktail of α5, α6, and α7 antisera and analyzed by reducing SDS–PAGE (12%) (C). PRRSV E, M, and N proteins as well as the endo H-sensitive (E_0) and endo H-partially resistant (E_1) forms of E are indicated on the right.

again that in the presence of the reducing agent β-mercaptoethanol, both M and E are coprecipitated using either α5 or α6 antiserum. Under nonreducing conditions, M and E proteins were no longer apparent, and two new bands corresponding approximately to 40- and 87-kDa polypeptide species were precipitated using both antisera. In the case of α7 antiserum, a predominant protein of 29 kDa and a protein species migrating slightly slower than the authentic viral N protein were recognized in the absence of β-ME. The 29-kDa species was also present in the immunoprecipitates obtained with α5 and α6 antisera, presumably as a result of N protein coprecipitation. It is likely that the 40- and 87-kDa proteins represent heterooligomers of M and E proteins. To confirm this assumption, we took advantage of the fact that in Western immunoblotting assay, each monospecific antiserum recognizes only its homologous gene product. If indeed in Western immunoblotting assay under nonreducing conditions, both α5 and α6 antisera recognize simultaneously the 40- and 87-kDa proteins, this would indicate that these species are complexes of M and E proteins. Effectively, when PRRSV proteins were electrophoresed without β-ME, transferred to nitrocellulose, and reacted with either α5 or α6 antiserum, two proteins with a migration pattern similar to the 40- and 87-kDa protein species were strongly recognized by both antisera (Fig. 4B). Taken together, the data obtained suggest that in PRRSV virions, M and E proteins are associated via disulfide bonds giving rise to two heterooligomeric forms of 40 and 87 kDa. On the basis of their apparent MW, the 40-kDa protein may represent a heterodimer of M and E (M-E), whereas the slower migrating band of 87 kDa probably resulted from the association of two or three M-E heterodimers. Under nonreducing conditions, no homodimers of M or E proteins could be observed in virions. On the other hand, the N protein incorporated into virions was principally revealed as a 29-kDa species both in RIPA (Fig. 4A) and Western immunoblotting (data not shown), which likely represents a disulfide-linked homodimer of N.

PRRSV M and E proteins are recruited into heterodimeric complexes with different kinetics and efficiencies

Oligomerization of PRRSV M and E proteins was next investigated in the infected cells by analyzing the mobility shifts of pulse-labeled, chased, and *in situ*-

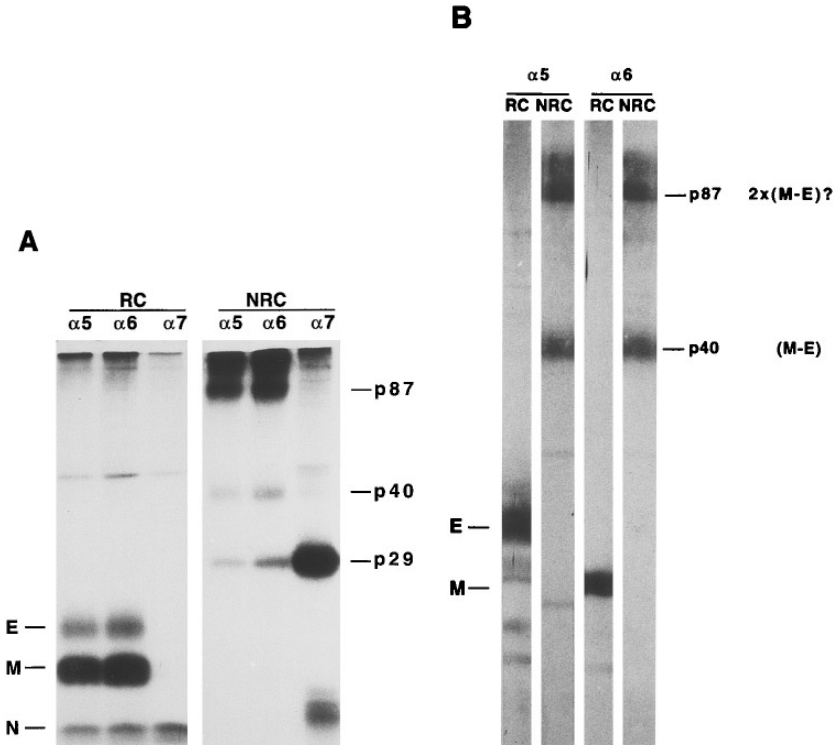


FIG. 4. Interactions between PRRSV envelope proteins via disulfide bonds. (A) Analysis of immunoprecipitated viral proteins under reducing and nonreducing conditions. Proteins from sucrose gradient-purified virus were immunoprecipitated with either anti-E (α5), anti-M (α6), or anti-N (α7) antiserum, and analyzed by 12% SDS–PAGE under reducing (RC) or nonreducing conditions (NRC). The new protein bands that arose only under nonreducing conditions are indicated on the right. The protein band of high intensity on top of the nonreducing gel precipitated by α5 and α6 antisera may represent large aggregates of proteins. The complex of proteins precipitated by α7 antiserum, which appears in the bottom of the gel, may result from a degradation process and should not be considered as the nonreduced form of N protein. Indeed, when intracellular N protein was precipitated with α7 antiserum and analyzed under nonreducing conditions, it migrated slightly faster than its reduced form (data not shown). (B) Identification of disulfide-bonded M-E complexes by Western immunoblotting assay. PRRSV proteins from sucrose gradient-purified virus were resolved by reducing (RC) or nonreducing (NRC) 12% SDS–PAGE, transferred to nitrocellulose, and finally stained with either α5 or α6 antiserum. Note that in nonreducing conditions, both p40 and p87 (indicated on the right) were recognized simultaneously by α5 and α6 antisera confirming that they represent complexes of M and E proteins. In addition, no residual monomers of M and E proteins could be observed either in radioimmunoprecipitation or in Western immunoblotting profiles. Monomers of E, M, and N are indicated on the left margin of gels A and B.

alkylated proteins. Radiolabeled PRRSV-infected cells were washed and lysed in the presence of 20 m*M* NEM to prevent the formation of nonnative disulfide bonds during or after cell lysis (Braakman *et al.,* 1992; De Vries *et al.,* 1995a). Proteins were precipitated from cell lysates using either α5 or α6 antiserum. Under reducing conditions, both M and E coprecipitated using either α5 (Fig. 5A) or α6 (Fig. 5C) antiserum. This coprecipitation was apparent upon the pulse period and once again involved all E protein intermediates which varied in their molecular weight (from 0 to 60 min of chase). As mentioned earlier, since proteins were disrupted with LB-2 lysis buffer which provides more stringent conditions for protein dissociation, the N protein was no longer precipitated with M and E following incubation with α5 or α6 antiserum. In the case where precipitation was performed using α5 antiserum and analyzed under nonreducing conditions (Fig. 5B), M-E complexes were consistently apparent upon the pulse period, indicating that association of M and E via disulfide bonds occured very shortly after their synthesis. After only 15 min of chase, practically most of the E monomers were incorporated into M-E complexes. The monomeric M protein was only coprecipitated when a fraction of E monomers was still present (Fig. 5B, lane 0), which would mean that M-E complexes are highly stable. During the chase period, M-E heterodimers accumulated in infected cells being still present at considerable amounts after 240 min. Precipitation of proteins with α6 antiserum revealed two striking features. First, unlike E, M protein was recruited much slower into M-E complexes and remains association competent during the whole period of chase. Second, antiserum α6 precipitated a protein, which on the basis of its MW is considered as a disulfide-linked dimer of M (M-M). These two features of PRRSV M protein have been previously identified for EAV M protein (De Vries *et al.,* 1995a). Finally, though

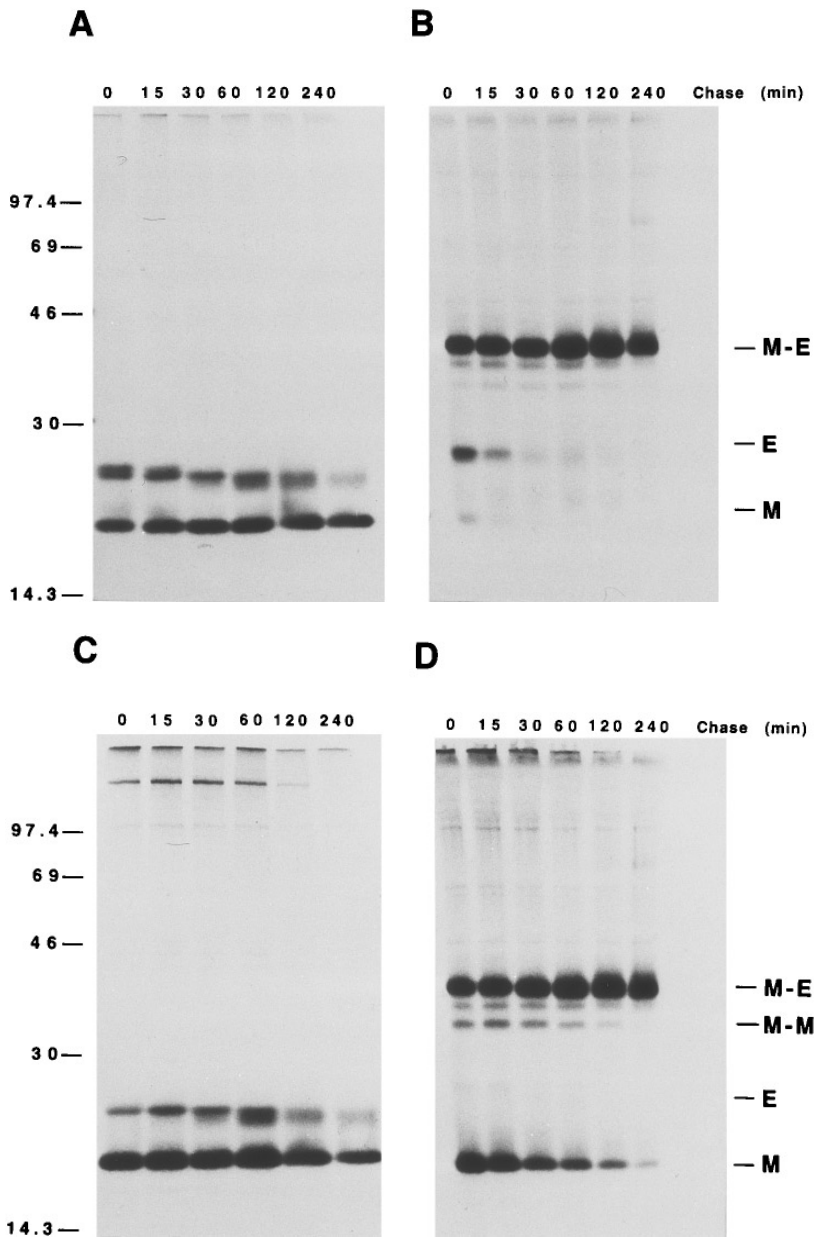


FIG. 5. Kinetics of PRRSV envelope proteins interactions. IAF-Klop-infected MARC-145 cells were pulse-labeled and chased as in the legend to Fig. 3. After each labeling period, cells were washed twice in prechilled PBS containing 20 m*M* NEM to prevent further rearrangement of disulfide bonds. Thereafter, cells were solubilized in LB-2 lysis buffer which also contained 20 m*M* NEM and proteins were immunoprecipitated with either anti-E (A and B) or anti-M (C and D) serum. Immunoprecipitates were finally analyzed in 12% SDS–polyacrylamide gels under reducing (A and C) or nonreducing (B and D) conditions. In presence of NEM, a faint band migrating closely with M-E complexes was generated. An additional band of approximately 35 kDa was particularly apparent in the immunoprecipitates of anti-M serum that was analyzed under nonreducing conditions (D). Given its apparent MW and its recognition by anti-M serum, this protein band may represent a homodimer of M protein. M and E proteins in their monomeric form or as disulfide-linked complexes are indicated on the right. Molecular sizes (in kDa) are given on the left.

abundantly precipitated from virions, neither the 87-kDa protein (Figs. 5B and 5D) nor the 29-kDa protein (data not shown) was precipitated from cell lysates that have been prepared in the presence of 20 m*M* of the alkylating agent NEM. Therefore, we concluded that the latter proteins were generated by the formation of aberrant disulfide bonds which could be prevented by alkylation of free sulfhydryl groups during preparation of cell lysates.

Collectively, these data indicate that oligomerization of PRRSV M and E proteins in infected cells resulted in the formation of heterodimers very shortly after their synthesis and that, unlike M, newly synthetized E protein molecules are rapidly and more efficiently recruited into M-E complexes.

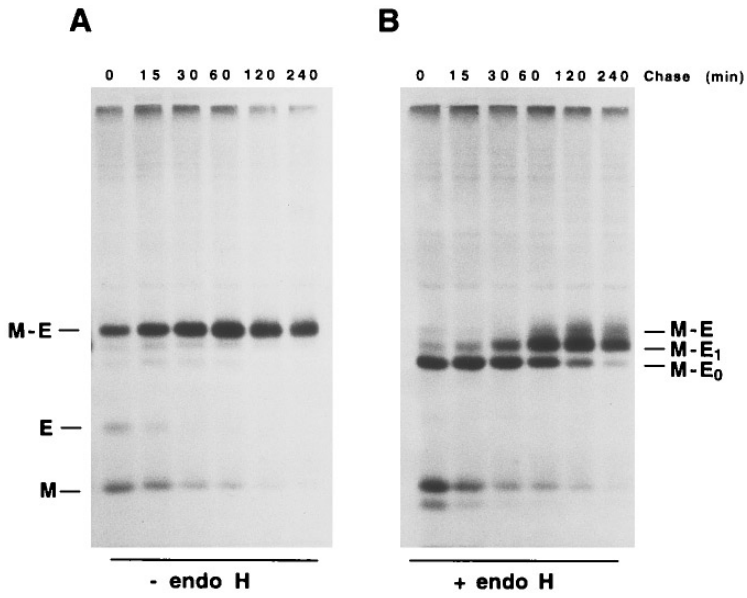


FIG. 6. Intracellular transport of PRRSV M-E complexes. Intracellular PRRSV proteins were labeled with [^{35}S]methionine and solubilized as in the legend to Fig. 5. A pool of anti-E and anti-M monospecific sera was used to immunoprecipitate the M-E complexes which were split in equal portions. One aliquot was treated with endo H (+ endo H) (B), and the other was left untreated (− endo H) (A) prior to analysis by 12% nonreducing SDS–PAGE. Heterodimers of M and E, as well as their monomeric forms, are shown on the left. The endo H-sensitive (M-E_0), endo H-partially resistant (M-E_1), and endo-resistant (M-E) forms of M-E complexes are indicated by dashes on the right.

PRRSV M protein associates to E regardless of its glycosylation state

Data obtained from pulse–chase experiments suggested that the oligomerization process of E and M is probably independent of the glycosylation state of the E protein and that the two mechanisms operate simultaneously. To confirm such assumptions, M and E were precipitated from cell lysates of the above pulse–chase experiment, using a pool of α5 and α6 antisera, treated or untreated with endo H and analyzed by SDS–PAGE under nonreducing conditions. In the absence of endo H treatment, M-E complexes were precipitated (Fig. 6A) as well as the monomeric forms of M and E. Once treated with endo H, three additional protein bands could be observed (Fig. 6B). Because their kinetics of appearence mimic that of E_0, E_1, and E proteins, we assumed that these species represent differentially glycosylated M-E complexes corresponding to M-E_0, M-E_1, and M-E heterodimers. Therefore, the data obtained are more consistent with the view that M-E heterodimers formation precedes oligosaccharide modification.

Intracellular localization of PRRSV major structural proteins

The finding that in PRRSV-infected cells M-E heterodimers formation occurs shortly after their synthesis, and presumably prior to oligosaccharide modifications of E, suggests that M protein accumulates in the ER and interacts herein with E. The fluorescence patterns obtained following incubation of PRRSV-infected cells with α6 and α5 antisera, although not identical, revealed a perinuclear fluorescence which is characteristic of proteins that localize in the ER (Figs. 7B and 7C, respectively). On the other hand, fluorescence associated with N as probed by α7 antiserum was cytoplasmic and diffuse, being more intense near the perinuclear region (Fig. 7A). Moreover, data obtained following cell fractionation experiments showed that the bulk of M and E proteins fractionated with microsomal membranes, whereas N was essentially found in the cytosolic fraction (data not shown). Treatment of the microsomal fraction with a high salt buffer (0.5 m*M* NaCl, 10 m*M* EDTA) did not result in the solubilization of E or M (data not shown), indicating that these two proteins are not loosely associated to membranes but represent true integral membrane proteins as predicted from their amino acid sequence.

DISCUSSION

In the present study, monospecific antisera raised against *E. coli*-expressed ORFs 5, 6, and 7 products were used to investigate the synthesis, processing, interactions, and transport of the major structural proteins of PRRSV propagated in the highly permissive MARC-145 cell line. The polypeptide specificities of the various monospecific antisera were confirmed by Western immunoblotting and RIPA.

In agreement with previous findings (Nelson *et al.,* 1993; Mardassi *et al.,* 1995; Meulenberg *et al.,* 1995), three major viral structural proteins, with apparent MW of 15, 19, and 24.5 kDa, could be identified from purifed

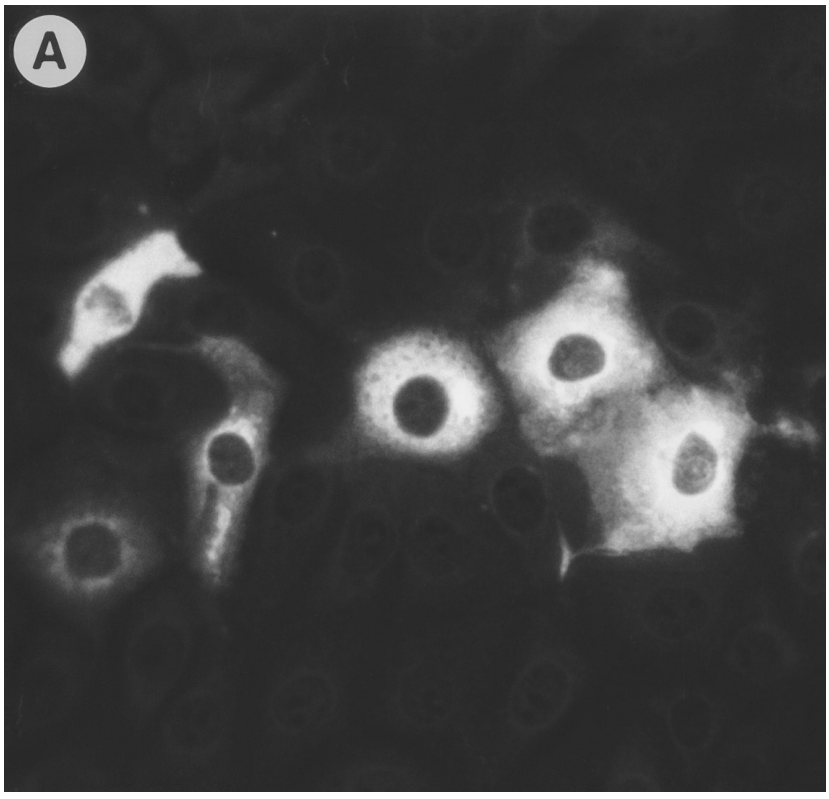
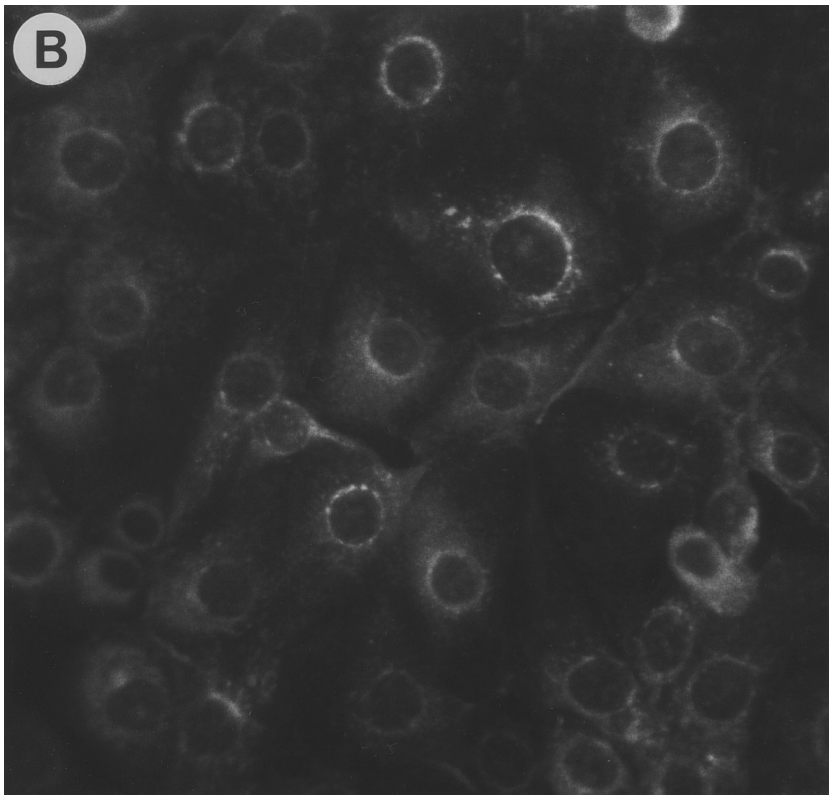


FIG. 7. Intracellular localization of the E, M, and N proteins in PRRSV-infected MARC-145 cells as revealed by indirect immunofluorecence. MARC-145 monolayers were infected with IAF-Klop strain at a m.o.i. of 1 and fixed at 24 hr p.i., as described under Materials and Methods. They were then stained with either anti-N (A), anti-M (B), or anti-E (C) serum. Note the distribution pattern of E and M proteins which tend to accumulate essentially at the perinuclear area, whereas N is mainly cytosolic.

virus preparations using convalescent anti-PRRSV sera. Virus fractionation experiments with the Lelystad virus (LV), the prototype european stain of PRRSV, demonstrated that these proteins correspond, respectively, to the nucleocapsid protein N, the unglycosylated membrane protein M, and the glycosylated membrane protein E (Meulenberg *et al.,* 1995). As expected, the α5, α6, and α7 monospecific antisera reacted specifically with the E, M, and N proteins, in agreement with earlier findings on the coding assignments of ORFs 5 to 7 of the PRRSV genome (Mardassi *et al.,* 1995; Meulenberg *et al.,* 1995). Although in the case of EAV a minor envelope protein (G_S) has been identified as an additional structural component (De Vries *et al.,* 1992), such polypeptide species from purified virus could not be identified in the case of PRRSV. The possibility that the presence of small amounts of a G_S-like protein could be missed, since no specific antiserum was available, cannot be ruled out. Similarly, three major viral proteins, within the same size range as those of EAV, have been also identified for LDV, but the presence of a counterpart to the EAV G_S protein incorporated into virions has not been unequivocally demonstrated (Faaberg and Plagemann, 1995; Faaberg *et al.,* 1995).

Among the three major PRRSV structural proteins, only E was modified by asparagine-linked oligosaccharides. Processing of PRRSV E protein N-linked glycans resulted in two stable forms, a heterogeneously glycosylated form, bearing complex type glycans as well as high-mannose oligosaccharides, and a second form containing only complex type carbohydrates. These two forms were shown to occur both in cells and in virions, the heterogeneous form being present at higher amounts. These results substantiate previous findings reported with LV (Meulenberg *et al.,* 1995). The apparent size of LV E protein propagated in the CL2621 cell line (also derived from the MA104 cell line) decreased about 1 kDa when virions were treated with endo H, whereas in the case of IAF-Klop strain such treatment resulted in a molecular weight reduction of about 3.5 kDa. Thus, unlike LV, data obtained following endo H treatment of IAF-Klop E protein incorporated into virions correlate with the removal of a complete oligosaccharide side chain bearing endo H-sensitive N-glycan moiety. Therefore, oligosaccharide side chains processing of IAF-Klop E molecules seems to be incomplete, which is consistent with the slow transport of this protein in the premedial Golgi compartment comparatively to its equivalent in EAV (De Vries *et al.,* 1992, 1995a). Indeed, consistent acquisition of endo H-resistant material occured after a chase of 30 min in the



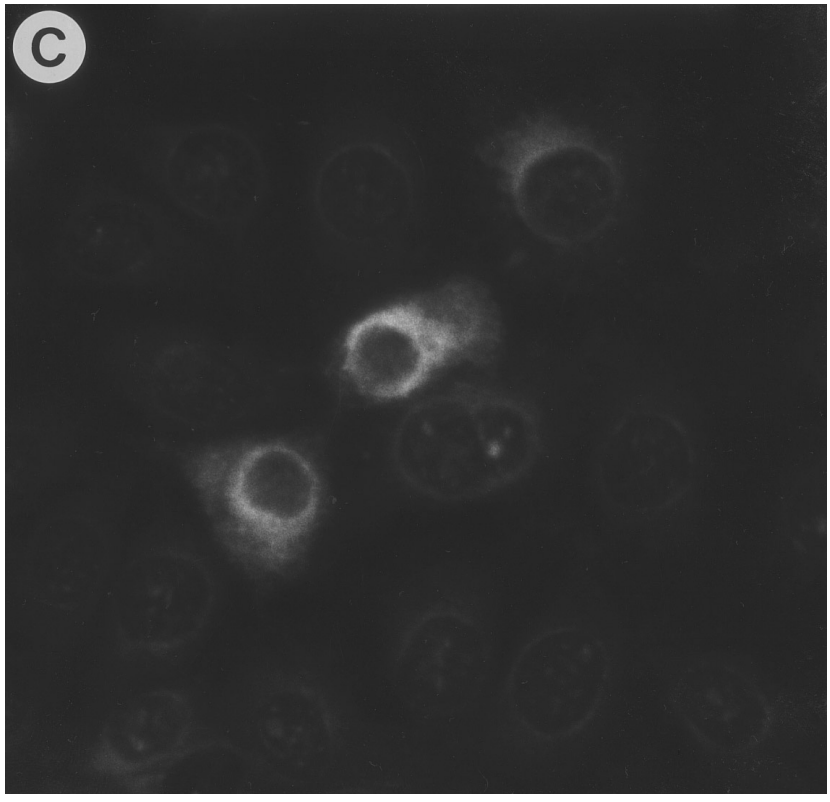


FIG. 7—*Continued*

case of E, but only after a pulse labeling of 15 min for G_L. The retardation of E in a premedial Golgi compartment is accompanied by a marked gradual decrease in its molecular weight which likely results from a very slow trimming process of the mannose-rich carbohydrate chains. In comparison to the hemagglutinin protein of

influenza virus, for which trimming is accomplished after only 10 min of chase (Copeland *et al.,* 1986), this phenomenon is particularly slow in the case of PRRSV E protein, considering the longer pulse labeling time used in our experiment. It remains to be demonstrated whether the slow processing of E protein carbohydrates is cell-type specific, the natural host of PRRSV being porcine alveolar macrophages.

Early steps in the maturation of E protein may lead to its slow processing within the ER. In this compartment, a "quality control" system ensures that transport is restricted to properly folded and assembled proteins (Doms *et al.,* 1993; Gething and Sambrook, 1992; Hurtley and Helenius, 1989; Pelham, 1989). The lumen of the ER contains a variety of enzymes and molecular chaperones which participate in the folding and oligomeric assembly of proteins (Gething and Sambrook, 1992).

Glycosylation is one of the most common modifications that occurs in the ER. It has been demonstrated that trimming of high mannose units, which results in the removal of the three terminal glucose residues, is necessary for the efficient transport of proteins from the ER to the Golgi complex (Lodish and Kong, 1984). It has been also speculated that removal of glucose confers a mature functional conformation to proteins that allows their transport (Schlesinger *et al.,* 1984; Kornfeld and Kornfeld, 1985; Hammond *et al.,* 1994). The finding that E displays essentially endo H resistance only after complete maturation, strongly suggests that its transport to the medial Golgi requires the protein to be properly folded. Since the carbohydrate trimming process is known to begin in the ER and to continue in the proximal cisternae of the Golgi complex (Kornfeld and Kornfeld, 1985), we assume that PRRSV E protein is retarded during its transit in these compartments due to a slow trimming process. Furthermore, because release of virions, as shown by concomittant precipitation of the three PRRSV structural proteins, occurs predominantly and rapidly when complete trimming has been achieved (1-hr chase), elements between the ER and the medial Golgi may represent the maturation site for PRRSV virions. These molecular data are in agreement with previous electron microscopy studies on PRRSV maturation that has been performed using either MARC-145 cells (Dea *et al.,* 1995) or porcine alveolar macrophages (Pol and Wagenaar, 1992). In both types of cells, budding and accumulation of enveloped virus particles could only be observed within smooth-walled vesicles of the ER and Golgi apparatus.

The association of M and E proteins and their incorporation into virions in the form of disulfide-linked heterodimers may suggest that they are present in virus particles in equimolar amounts. This assumption is substantiated by the fact that no residual monomeric forms of either M or E were present in radioimmunoprecipitation and Western immunoblotting profiles analyzed under nonreducing conditions. This has been previously demonstrated with EAV where roughly equal amounts of M and G_L were found on virions (De Vries *et al.,* 1992).

Pulse–chase experiments showed that E is rapidly and efficiently incorporated into M-E complexes, whereas the M protein engages into these complexes at a slower rate. Such behavior, which has been previously demonstrated for the two envelope proteins of EAV (De Vries *et al.,* 1995a), is consistent with the view that M, being synthesized at higher amounts than E, tends to accumulate at the site of oligomerization and interacts with newly synthesized E molecules.

A tendency of M protein to fold into disulfide-linked dimers in cells but not in virions was also demonstrated in the present study. In the case of EAV, it has been speculated that such dimers may act as intermediates for the formation of M-E complexes or may simply represent dead-end products (De Vries *et al.,* 1995b). In contrast to EAV M complexes, PRRSV M homodimers declined over an extended period of chase and displayed a similar kinetics pattern as M monomers. We do not know whether the fading out of M-M dimers is due to their conversion into M-E complexes or whether they are subjected to intracellular degradation.

Although most of the data accumulated so far converged for a pre-Golgi budding site for PRRSV as well as for the other *arteriviruses* (Dea *et al.,* 1995; Magnusson *et al.,* 1970; Plagemann and Moennig, 1992; Pol and Wagenaar, 1992; Snijder *et al.,* 1993), it is still premature to define a precise role for the envelope protein complexes in the virus assembly and budding processes. The data obtained in the present study suggest that the interaction between M and E precedes budding, but we cannot yet exclude that virus assembly occurs synchronously with formation of M-E complexes. However, in light of the present data and those previously reported, a general scenario for the synthesis, assembly, and budding of PRRSV virions may be anticipated. We assume that assembly of virions may start at the ER where M and E are linked via disulfide bonds and then interact with N that has already accumulated in the cytosol at essentially the perinuclear region, as shown by indirect immunofluorescence. Thereafter, vesicles containing enveloped nucleocapsids are derived from the ER and transported until the premedial or medial Golgi, where budding takes place, and some E molecules undergo terminal glycosylation, probably due to their relative proximity with the medial Golgi environment. At the end of its transit from the ER to the Golgi or proximal elements, E protein acquires its mature structure which seems to greatly influence the onset of virus release shown to occur by exocytosis (Pol and Wagenaar, 1992).

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