Identification of the Membrane Protein of Porcine Epidemic Diarrhea Virus

ANNA UTIGER, KURT TOBLER, ANNE BRIDGEN, & MATHIAS ACKERMANN

Institute of Virology, Faculty of Veterinary Medicine, University of Zurich, CH-8057 Zurich, Switzerland

Requests for reprints should be addressed to Mathias Ackermann, Institute of Virology, Faculty of Veterinary Medicine, University of Zurich, CH-8057 Zurich, Switzerland

Abstract. Sequence information on the genome of porcine epidemic diarrhea virus (PEDV) has only recently been determined. In contrast, very little is known about the viral proteins. In the present report we have identified the membrane glycoprotein (M) of PEDV by use of rabbit anti-peptide sera and transient expression of the cloned M gene in Vero cells and by expression in the baculovirus system. The native M protein of PEDV is incorporated into virions, is N-glycosylated, and migrates with a relative mobility (Mr) of 27 k in polyacrylamide gels. In contrast, the M protein synthesized by recombinant baculoviruses migrates with a Mr of 23 k, that is, with identical mobility as the deglycosylated product of PEDV. Thus, it appears that M protein specified by the recombinant baculovirus is poorly, if at all, glycosylated. Using monoclonal antibodies and rabbit antipeptide sera specific for the N and C termini of the M protein, we were able to show that a 19 k band detected in PEDV-infected cells but not in virions represented a fragment of M from which the C terminus had been cleaved off. Finally, by electron microscopy and immunogold labelling, the relative orientation of M within the virion envelope was determined as NexoCcyt. In conclusion, all of these data strongly support the hypothesis that PEDV should be classified with the group I coronaviruses.

Key words: coronavirus, porcine epidemic diarrhea, PEDV, membrane protein

Introduction

Porcine epidemic diarrhea virus (PEDV) causes diarrhea in pigs, particularly in neonates. The disease has been recognized for approximately 20 years, but the causative virus was only identified in 1978 (1), and another 10 years elapsed before a method was developed for propagation of the virus in cell culture (2). Recently, the sequence of approximately 8000 nucleotides located nearest the genome 3' end was determined (3–5). In Fig. 1 (lines a and b) an overview of the 3' end of the PEDV genome is shown.

The predicted amino acid sequences of PEDV suggest that this virus belongs to the same genetic subset as transmissible gastroenteritis virus (TGEV), feline infectious peritonitis virus (FIPV), and human respiratory coronavirus 229E (HCV229E). Interestingly, detection of antibodies to PEDV in feline and human sera has been reported only recently (6). This observation indicates that PEDV or a closely related virus may circulate in species other than pigs. The further characterization of PEDV is therefore an important prerequisite for the identification of such new viruses.

It has been suggested that the immune reaction to the membrane (M) protein of coronaviruses may play an important role in the induction of protection (7) and in mediating the course of

Anne Bridgen's present address is Institute of Virology, University of Glasgow, Church Street, Glasgow G11 5JR.

the disease (8). Furthermore, the M proteins of these viruses provide an interesting opportunity for studying the taxonomic position of coronaviruses because of a number of unifying and distinguishing features of the amino acid sequences themselves and of their cotranslational and post-translational processing. According to the literature (9,10), M protein is largely buried within the virus membrane with an extraviral NH_2 terminus and an intraviral COOH tail, thus adopting a NexoCcyt orientation. The typical M protein has a molecular weight of 17–30 kD, it spans the membrane three times, and its extraviral domain may carry O-linked oligosaccharides, as do some members of antigenic group II coronaviruses, for example, human coronavirus OC43 (HCVOC43) (11), bovine coronavirus (BCV), and mouse hepatitis virus (MHV) (10). Alternatively, the extraviral domain may be N-glycosylated, as has been suggested for most coronaviruses of antigenic group I, for example, HCV229E (12) and TGEV (13). According to the published sequences (4), a schematic representation of the typical domains of the putative PEDV membrane protein is depicted in Fig. 1d. Notably, three potential N-glycosylation sites (shown by asterisks; only one of which is actually expected to be functional) and three transmembrane (TM) regions (shaded) have been anticipated. The fourth hydrophobic domain (hatched) is not predicted to have a helical structure. It thus probably represents a membrane-associated domain rather than an additional membrane-spanning segment. In contrast to TGEV, no putative signal sequence has been predicted for the membrane protein of PEDV.

In this report we identify for the first time a gene product of PEDV. Antisera against synthetic peptides and both transiently expressed and recombinant baculovirus expressed M protein were used in these experiments. The sequences and relative map locations of the peptides used for immunization of rabbits are given in Fig. 1 (lines e and f). We demonstrate by immunoprecipitation and western blot analysis that these antipeptide sera reacted with the same family of viral proteins as monoclonal antibody 204. It has previously been suggested that this antibody recognizes the M protein of PEDV (14). Furthermore, we show that the M protein of

PEDV is N-glycosylated and migrates with a relative mobility (Mr) of 27 k in polyacrylamide gels. The deglycosylated and unglycosylated forms of M migrate with an Mr of 23 k. In the virion, M protein adopts a typical NexoCcyt orientation. A carboxy-terminally cleaved fragment of M is not incorporated into the virions.
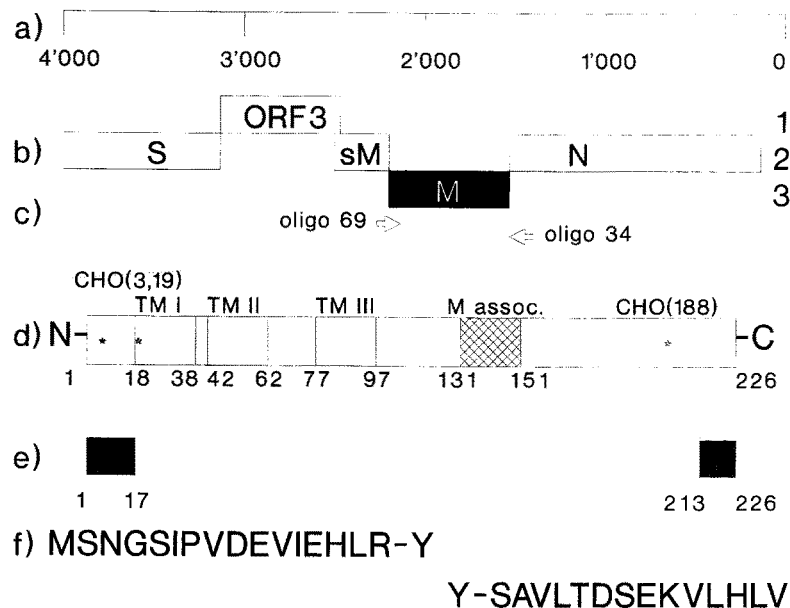
Materials and Methods

Antipeptide Sera, Monoclonal Antibody, and Conjugates

Oligopeptides representing the termini of the putative M protein were synthesized and coupled to ovalbumin by a commercial supplier (Neosystem S.A., Strasbourg, France). A tyrosine was added during synthesis of the peptides (Fig. 1, lane f) in order to facilitate coupling to ovalbumin by bisdiazobenzidine. New Zealand White rabbits were subcutaneously injected with the peptide-carrier conjugate in Freund's complete or incomplete adjuvant essentially as described previously (15). The rabbits received booster injections every 2 weeks. Rabbit sera were collected 10–14 days after each injection. Rabbit sera R34 were collected from an animal that had been immunized with the carboxy-terminal peptide, while rabbit sera R52 originated from an animal that had been immunized with the amino-terminal peptide.

Monoclonal antibody 204 has been described previously (14). Goat anti-rabbit FITC and unlabelled rabbit anti-mouse IgG (heavy and light chain) were obtained from Nordic Immunological Laboratories (Tilburg, The Netherlands). Goat anti-rabbit peroxidase was kindly provided by Mark Suter. Peroxidase labelled protein-A conjugate was supplied by Bio-Rad Laboratories (Richmond, CA). Goat anti-mouse Ig and goat anti-rabbit Ig, each conjugated to 6 nm gold particles, were obtained from Aurion (Wageningen, The Netherlands). The conjugates were used as suggested by the manufacturers.

Virus and Cells

PEDV strain CV777 was propagated in Vero cells 1587 (ATCC) essentially as described previ-

a)
```
4'000        3'000        2'000        1'000         0
```

b)
```
              ORF3                                    1
    S                sM              N                2
```

c)
```
                    M                                 3
        oligo 69 ⇨        ⇦ oligo 34
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d)
```
    CHO(3,19)
      TM I   TM II   TM III   M assoc.   CHO(188)
  N- * *  │   │    │    │    ▨▨▨▨                  -C
    1  18 38 42  62  77  97  131  151              226
```

e)
```
    ■                              ■
    1   17                    213   226
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f) MSNGSIPVDEVIEHLR-Y

Y-SAVLTDSEKVLHLV

Fig. 1. On the top of the figure is shown a schematic representation of the 4000 nucleotides nearest to the poly(A) tail of the PEDV genome (*b*) and a scale (*a*). The reading frames are indicated on the right and the M gene is highlighted in black. *c* indicates the relative map location of the oligonucleotide primers oligo 69 (sense) and oligo 34 (anti-sense) used for pcr amplification of the M gene. *d* represents some of the predicted features of the putative M protein, including the amino terminus (N), the carboxy terminus (C), and the amino acid (aa) number (total of 226 aa). Three potential glycosylation sites (CHO) are indicated with asterisks at aa 3, 19, and 188. The three putative transmembrane regions (TM I, TM II, TM III) are indicated in grey. The aa number of the predicted starting and end points of the TM regions are indicated at the bottom. A potentially membrane-associated region is crosshatched (M assoc.). *e* indicates two peptides, one at the amino terminus (aa 1–17) and the other at the carboxy terminus (aa 213–226), which were used to raise antisera to synthetic peptides in rabbits. *f* gives the aa sequence of the synthetic peptides. Tyrosines (Y) were added to the peptides at the N and C terminus of the C- and N-terminal peptides, respectively, in order to facilitate coupling of the peptides to ovalbumin prior to immunization of the rabbits.

ously (2,16). Briefly, cell growth and virus infection media consisted of Eagle's minimal essential medium (EMEM) buffered with HEPES and bicarbonate. This was supplemented with 5% (v/v) fetal bovine serum for the growth medium or 0.3% (w/v) tryptose phosphate broth and 10 μg/ml trypsin for virus infection. In addition, nonessential amino acids (Amimed, Muttenz, Switzerland) were reconstituted from a 100 × stock solution. Incubation of cell cultures and infected cells was at 37°C and 5% CO_2.

Preparation and Treatment of Infected Cell Lysates and Virions

Two-day-old monolayers of Vero cells were washed with phosphate buffered saline (PBS) and infected with PEDV at a multiplicity of infection (moi) of 0.2 or mock infected. After 2 hr of adsorption at 4°C, the inoculum was replaced by fresh virus infection medium. The infected cultures were incubated for 14 hr at 37°C in 5% CO_2. When the cytopathic effect (cpe) reached 100%, the cells were harvested together with the supernatant and centrifuged at 12,000 rpm (Sorval HB-4 rotor) for 30 min. The pellet was lysed in $NaDodSO_4$ buffer as described previously (14). For partial purification of virions, the clarified supernatants were centrifuged in a Beckmann SW28 rotor at 24,000 rpm for 2 hr. The pelleted virions were resuspended in a buffer appropriate for the planned use: for PAGE $10^{7.5}$ cell equivalents were resuspended in 100 μl of $NaDodSO_4$ buffer, for N-glycosidase F treatment double glass-distilled water was used, and for electron microscopy TNE buffer (consisting of Tris-HCl, 10 mM; NaCl, 100 mM; EDTA, 1 mM) was used.

Radiolabeling of Viral Polypeptides

Infected and mock-infected cell proteins were metabolically labeled by supplementing normal EMEM with either 0.1 Mbq ^{35}S-methionine/ml (Amersham) and 90% EMEM without L-methionine (GIBCO-041-01900) or 0.02 Mbq L-[U-^{14}C] amino acid mixture/ml (Amersham) and 70% EMEM without Arg, Cys, Leu, Met, inositol, glucose, and glutamine (Gibco-51091-015; former cat. no. 041-01091) and were supplemented with 100× L-methionine (Gibco); 1.15 Mbq N acetyl-D-[1-^{14}C] glucosamine/ml (Amersham) 50% EMEM (Gibco-51091-015) for each purpose.

Tunicamycin Treatment

Two-day-old monolayers of Vero cells were washed with PBS before fresh EMEM supplemented with different concentrations (7, 20, 60, and 210 ng/ml, respectively) of tunicamycin (Boehringer, Mannheim, Germany) was added. The cultures were incubated at 37°C for an additional 24 hr. The medium was then removed and the cultures were infected as usual but with the addition of tunicamycin at the same concentration as during the pretreatment. When the control PEDV-infected culture (without tunicamycin treatment) showed 100% cpe, the cells were harvested for PAGE and immunoblotting.

Treatment of Virions with N-Glycosidase F

Virions were prepared as described earlier. Following the addition of a final concentration of 0.5% NaDodSO$_4$, the suspension was incubated in a boiling water bath for 2 min. Recombinant N-glycosidase F was obtained from Boehringer (Mannheim, Germany) and was used as recommended by the manufacturer. N-glycosidase F incubation buffer (sodium phosphate, 20 mmol/l, pH 7.2; NaN$_3$, 10 mmol/l; EDTA, 50 mmol/l; Nonidet P40, 0.5%, v/v) was added before the sample was boiled again for 2 min. Thereafter, an estimated amount of 5 units of N-glycosidase F/mg of total protein was added and deglycosylation was carried out overnight in a 37°C water bath. Aliquots were sampled at each step of the assay. The samples were solubilized in Na-DodSO$_4$ buffer, separated by PAGE, transferred to nitrocellulose, and analyzed by immunoblotting.

Polyacrylamide Gel Electrophoresis, Immunoblotting, Immunoprecipitation, and Autoradiography

NaDodSO$_4$ PAGE was done according to conventional protocols in 12% acrylamide gels crosslinked with bis-acrylamide. The samples were electrophoresed for 4 hr at 35 mA before being transferred electrically to nitrocellulose sheets (BA85, Schleicher & Schüll, Dassel, Germany) overnight at 2 V/cm^2. For immunoblotting, the nitrocellulose sheets were incubated in Tris-buffered saline (TBS) containing 10% skimmed milk (v/v) for 4 hr at 37°C. Then they were pretreated with PBS containing 0.5% Triton X-100 for 15 min before being washed with PBS and were further incubated for 5 min with PBS containing 80 μg/ml trypsin. After washing with PBS, the blots were submerged overnight at 4°C with hybridoma supernatant containing monoclonal antibody 204 or rabbit sera (diluted 1 : 160 in TBS) as indicated in Results. After washing several times in TBS, binding of the antibodies was visualized either with goat anti-rabbit peroxidase conjugate or with unlabeled rabbit anti-mouse IgG and protein-A peroxidase, followed by incubation in a substrate containing chloronapthol and peroxide.

For radioimmunoprecipitation (RIP), the samples were solubilized in RIP buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 1% 1-O-n-Octyl-β-D-glucopyranoside (Boehringer, Germany)) for 30 min on ice and were kept at -20°C before further use. Following centrifugation for 30 min at 12,000 ×g, the soluble antigens were incubated overnight with antibodies as specified for each experiment. Immune complexes were adsorbed to protein-A sepharose CL-4B (Pharmacia, Uppsala, Sweden) for 1 hr at ambient temperature. After washing with RIP buffer, the samples were solubilized in NaDodSO$_4$ buffer and subjected to PAGE. Radiolabelled proteins on the nitrocellulose sheets were visualized either by conventional autoradiography or by exposure to a phosphor screen and scanning with a PhosphoImager (Molecular Dynamics) as described previously (15).

Electron Microscopy

Virus particles were purified by ultracentrifugation and resuspended in TNE buffer as described earlier. For electron microscopy virions were adsorbed to carbon-coated copper grids before being washed with PBS containing 0.05% bovine serum albumin (BSA). Primary antibodies at a dilution of 1:100 (monoclonal antibody 204) or 1:1000 (rabbit antipeptide sera) were added for 5 hr before further washing. Gold (6 nm)-labeled anti-mouse or anti-rabbit conjugate (1:25) was then overlaid for 45 min. The grids were washed consecutively in PBS-containing BSA and in double-distilled water before negative staining with 1% potassium phosphotungstate (pH 6.6) and electron microscopy in a Phillips CM12 microscope.

Plasmid Constructions

The open reading frame (ORF) encoding the M protein of PEDV was amplified by polymerase chain reaction (pcr) using the oligos 34 (5′ GAG-GATCCTGAAAGCTGACAG 3′) and 69 (5′ CT-ATAAAT<u>ATG</u>TCTAACGG 3′) (Fig. 1c). The pcr product initially amplified from the viral cDNA was blunt-end cloned into the *Eco*RV site of the plasmid vector pBluescript® KS+ (4). This clone, designated pBS-M, was sequenced completely and, then was used as the template for further amplification using fewer amplification cycles (25 as opposed to the original 39 cycles) to produce a product for cloning into the *Pst*I-*Bam*HI sites of the baculovirus transfer vector pAcDSM (17). The pcr reactions were performed in an end volume of 30 μl containing 2.5 units *Pfu* polymerase (Stratagene GmbH, Zürich, Switzerland) dNTPs (200 μM each), 3 μl 10× *Pfu* polymerase reaction buffer #1 (Stratagene) and 1.33 μM of each primer. Amplification was performed in a first cycle of 95°C for 5 min, 45°C for 5 min, and 74°C for 3.5 min, followed by 38 or 24 cycles of 95°C for 50 sec, 47°C for 50 sec, and 74°C for 50 sec.

For directional cloning into the vector pAcDSM, the pcr product was treated with T4 DNA polymerase to form blunt ends, phosphorylated with polynucleotide kinase (New England Biolabs, Beverly, MA), and digested with

*Bam*HI. It was then cloned into the transfer plasmid pAcDSM, which had been cut with *Pst*I, blunt end repaired with T4 DNA polymerase (New England Biolabs), *Bam*HI digested, and dephosphorylated with calf intestine phosphatase (Pharmacia Biotech AG, Dübendorf, Switzerland). The use of oligo 69 in the pcr and the cloning into the *Pst*I site of pAcDSM resulted in restoration of the entire original polyhedrin promoter of AcMNPV immediately 5′ to the M gene. This transfer plasmid was termed pAc-DSM-M.

To construct a transient expression vector, the M gene was excised from pBS-M using *Hin*dIII and *Bam*HI, and was ligated into pSCT GAL X-556 (18) that had been cut with *Hin*dIII and *Bgl*II, and dephosphorylated with calf intestine phosphatase (Pharmacia). This resulted in plasmid pSCT-M, with the M gene under the control of the CMV immediate early promoter.

Transient Expression of M Protein in Vero Cells and Immunofluorescence

A total of 10^5 Vero cells in Dulbecco's MEM (DMEM; Gibco BRL, Gaithersburg, MD) supplemented with 2% FCS were seeded in a Lab-Tek® chamber/slide with four chambers per slide (Nunc, Naperville, IL) and incubated at 37°C and 5% CO_2. After 12 hr 5 μg DNA were dissolved in 13.5 μl H_2O and 1.5 μl of 2.5M $CaCl_2$ and 15 μl of 2× concentrated HEBS (50 mM Hepes, 280 mM NaCl, 75 mM Na_2HPO_4, 75 mM NaH_2PO_4, pH 7.13) were added. The mixture was incubated for 30 min at room temperature before being added to the cells. After 24 hr the cells were washed with TBS, shocked with 20% glycerol in TBS for 2 min, then washed twice with TBS. Finally, fresh DMEM supplemented with 2% FCS was added. The transfected cells were incubated for a further 12 hr at 37°C and 5% CO_2. They were then rinsed with PBS and subsequently fixed for 30 min with methanol. After washing three times with PBS for 10 min, the slides were incubated for 2 hr at 37°C with rabbit serum R34 (diluted 1:200 in PBS). The slides were washed three times for 10 min with PBS and subsequently incubated for 1 hr with goat anti-rabbit FITC (diluted 1:200 in PBS). Finally, they were washed three times with PBS,

counterstained with Eriochrome black, and examined for fluorescence with a Leitz Diaplan fluorescence microscope equipped with a PL FLUOTAR 25/0.75 W objective and an exciter filter for light with a wavelength of 450–490 nm. Photographs were taken with Kodak Ektochrome 1600 ASA film.

Insertion of the M Gene into the PAK-6 Genome

Sf9 cells were cotransfected with 2 μg of pAc-DSM-M DNA and 0.5 μg of PAK6 (Clontech, ITC Biotechnology GmbH, Heidelberg, Germany) genomic DNA, which had been previously digested with an excess of Bsu36I (New England Biolabs). Progeny virus was harvested and plaque purified under an agarose overlay (0.75%). Single plaques were picked and examined for synthesis of recombinant M protein.

Results

### Identification of an Antipeptide Serum Specific for the M Protein

A rabbit antipeptide serum against the predicted COOH terminus of the putative M polypeptide (Fig. 1, e and f) was raised as described in Materials and Methods. In order to identify the product of the M gene and to demonstrate the specificity of the antipeptide serum, Vero cells were transfected with a plasmid cloned M gene under the control of the CMV immediate early promoter. Then 36 hr post transfection, the cells were fixed and incubated with the immune rabbit serum. As demonstrated by immunofluorescence (Fig. 2), the immune serum (R34) reacted specifically with transiently synthesized antigens localized in the cytoplasm and the membranes of the transfected cells. Mock-transfected cells did not fluoresce.

Reactivity with Monoclonal Antibody 204 and Glycosylation of the M Protein

Previously we have reported that monoclonal antibody 204 reacted with a family of glycoproteins

with relative mobilities (Mr) of 27, 24/23, and 19 k (14). In order to test our hypothesis that these bands represented the M protein of PEDV, Vero cells were either infected with PEDV or mock infected in the presence of a ^{14}C-amino acid mixture to label the viral proteins metabolically. After harvesting, the lysates were immunoprecipitated with monoclonal antibody 204 or rabbit anti-peptide serum R34, as described in Materials and Methods. Following electrophoresis and western transfer, the nitrocellulose sheets were exposed to autoradiography. As shown in Fig. 3A, both the immune rabbit serum and monoclonal antibody 204 precipitated the same labeled protein bands with relative mobilities of 27 k and 24/23 k. Unfortunately, the corresponding bands could not be identified unambiguously on the western blots because the light chains of the antibodies comigrated with the membrane protein of PEDV and caused a strong concurrent signal (not shown).

In order to show that the M protein was glycosylated and to further document that the above-mentioned antibodies recognized the same polypeptide, infected and mock-infected cell cultures were incubated with ^{14}C-glucosamine to label the glycoproteins metabolically. After harvesting, virus particles were purified from one part of the sample and solubilized NaDodSO$_4$ buffer, whereas the remaining part was solubilized in RIP buffer before being immunoprecipitated with monoclonal antibody 204. After electrophoresis and transfer to nitrocellulose, the sheets were exposed to a phosphor screen and scanned with a phosphorimager. The virion preparation contained at least three virus-specific glycoprotein bands (indicated by open arrows on lane 2 of Fig. 3B). Specifically, the Mr 180/200 k glycoprotein band most probably represented the surface glycoprotein S, whereas the nature of the 20 k band remains to be determined. The band migrating with an Mr of approximately 27 k most probably represented the membrane glycoprotein M. The same band could be visualized following immunoprecipitation of the PEDV-infected cell lysate with monoclonal antibody 204 (lane 4). The immune rabbit serum R34 precipitated the same band as monoclonal antibody 204 (not shown). These results indicated that rabbit serum R34 as well as monoclonal antibody 204 were specific
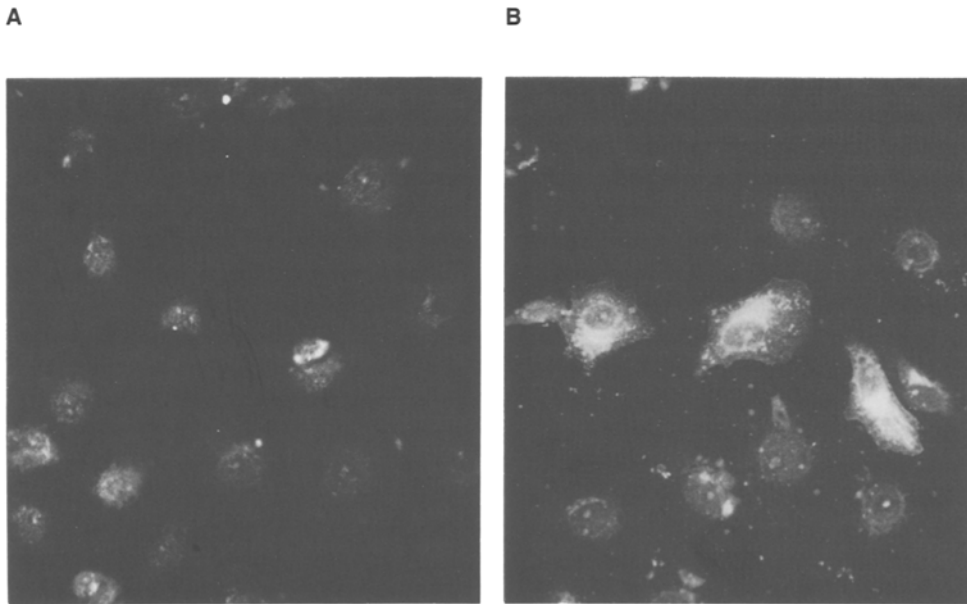
Fig. 2. Immunofluorescence images of mock transfected (*A*) and transfected (*B*) Vero cells are shown. For transfection a plasmid containing the M gene under the control of the CMV immediate early promoter was used. The cells were fixed and stained with rabbit serum R34 and an FITC-labeled goat anti-rabbit conjugate before being photographed under a fluorescent light microscope.

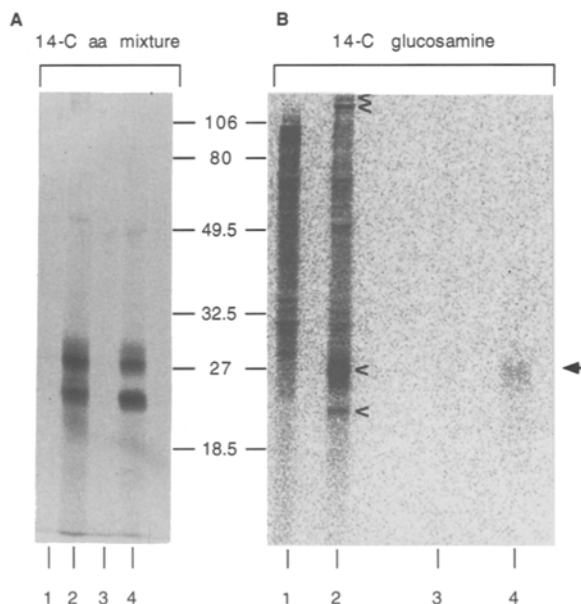for the M protein of PEDV and that M was glycosylated and incorporated into virions.

Comparison of the M Protein in PEDV Particles with the Recombinant M Protein Synthesized by Recombinant Baculovirus in Infected Sf9 Cells

Several recombinant baculoviruses were selected for their ability to produce the M protein in Sf9 cells. In order to compare the M gene product synthesized in heterologous insect cells to the M protein of PED virions, lysates of Sf9 cells infected with one of the recombinant baculoviruses, designated PAK-BM-4, and mock-infected Sf9 cell lysates, as well as partially purified PED virions, were separated on polyacrylamide gels and blotted to nitrocellulose before being probed with monoclonal antibody 204. As shown in Fig. 4A, the recombinant baculovirus-infected cells revealed a single band of Mr 23 k, whereas a family of proteins, including slower migrating members, was seen in the lane containing the PED virions. It was hypothesized that the recombinant M protein, in contrast to the authentic product, was only poorly, if at all, gly-

cosylated. As reported previously (14), the 19 k band was absent from the virion preparation that had been immunostained with the monoclonal antibody.

N-Glycosylation of the PEDV M Protein

Typically, the membrane glycoproteins of the members of the antigenic group 1 of coronaviruses are N-glycosylated (10). N-glycosidase F and tunicamycin were used to test whether this was also true of the M protein of PEDV. N-glycosidase was used to remove N-linked carbohydrates from purified virions, whereas tunicamycin was used to test whether glycosylation could be inhibited. Vero cells were infected with PEDV and incubated in the presence of various concentrations and in the absence of tunicamycin. The cellular lysates as well as untreated virions and N-glycosidase F–treated virion proteins were electrophoretically separated, transferred to nitrocellulose, and immune stained with monoclonal antibody 204. Figure 4B shows that only a single band of Mr 23 k was immunologically detected in the lysates of cells that had been incubated in the presence of tunicamycin.

A

14-C aa mixture

B

14-C glucosamine

— 106 —

— 80 —

— 49.5 —

— 32.5 —

— 27 —

— 18.5 —

| | | | | | | |

1 2 3 4   1   2   3   4

Fig. 3. Autoradiograms (*A*) and a printout of the phosphorimager scan (*B*) are shown. Numbers between the two panels indicate the relative mobility (Mr × 1000) of the proteins. *A:* PEDV-infected and mock-infected cells were metabolically labeled with the ^{14}C-amino acid mixture before being harvested and lysed in RIP buffer as described in Materials and Methods. Replicate samples of mock-infected lysates (lanes 1 and 3) and PEDV-infected lysates (lanes 2 and 4) were immunoprecipitated with either monoclonal antibody 204 (lanes 1 and 2) or immune rabbit serum R34, which was specific for the C terminus of the PEDV M protein. The samples were electrophoresed in polyacrylamide gels, transferred to nitrocellulose, and exposed to autoradiography. *B:* PEDV-infected and mock-infected cells were metabolically labeled with ^{14}C-glucosamine as described in Materials and Methods. One fraction of each of the samples was ultracentrifuged as described for the preparation of virions before polyacrylamide gel electrophoresis and transfer to nitrocellulose (lane 1, mock infected; lane 2, PEDV infected). The remaining fractions were lysed in RIP buffer and immunoprecipitated with monoclonal antibody 204 (lane 3, mock infected; lane 4, PEDV infected) before electrophoresis and western transfer. The nitrocellulose was exposed to a phosphor screen for 5 days before being scanned with a phosphorimager. The arrow on the right points to a glycoprotein band of Mr 27 k that was precipitated by monoclonal antibody 204. Open arrows point to viral glycoproteins detected in PEDV virion preparations.

The same band was visible in glycosidase-treated virions. In contrast, the conventional cell lysates and the untreated virions revealed the expected family of glycoprotein bands. The intensity of the 23 k band decreased with decreasing concentrations of tunicamycin (lanes 7–11). In compensa-

tion, traces of the slower migrating bands could be detected at a concentration of 7 ng tunicamycin/ml (lane 11). These results indicate that the PEDV membrane protein is N-glycosylated in PEDV-infected Vero cells. The observation that the unglycosylated and the deglycosylated bands migrated at the same position as the polypeptide specified by the recombinant baculovirus (PAK-BM-4) in infected Sf9 cells further substantiated our hypothesis that the recombinant M protein was not glycosylated in the baculovirus system.

Carboxy-Terminal Cleavage of M

Previously, it had been observed that the fastest migrating band (19 k) of the M protein family was absent from purified virions and that the putative M protein was degraded when incubated in the presence of Triton X-100 (14). It was hypothesized that this 19 k band represented a soluble fragment of M. In order to test this hypothesis, an additional antipeptide serum specific for the N terminus (see Fig. 1, lanes e and f) of the M protein was raised and characterized as described for the antiserum against the C terminus of M (not shown). Equal amounts of virions were solubilized either directly in NaDodSO$_4$ buffer or were incubated in phosphate buffered saline containing Triton X-100 before NaDodSO$_4$ was added. Triplicate samples were separated side by side in polyacrylamide gels, transferred to nitrocellulose, and stained separately with the immune rabbit sera or monoclonal antibody 204. As shown in Fig. 5, with all of the samples that had been treated immediately with NaDodSO$_4$, the typical pattern of the M-glycoprotein family, with the exception of the 19 k band, was visualized (lanes 1, 4, and 7). In contrast, with the samples that had been treated with Triton X-100, the band that was expected to migrate with a relative mobility of 27 k was almost not discernible by immune staining (lanes 2, 5, and 8). To compensate, the 19 k band appeared following immune staining with the rabbit serum specific for the N terminus of M (lane 2) as well as with monoclonal antibody 204 (lane 8). Triton X-100–treated virions showed a different pattern of immune staining with the C-terminal serum (lane 5). The original 27 k band and some of the 24/23
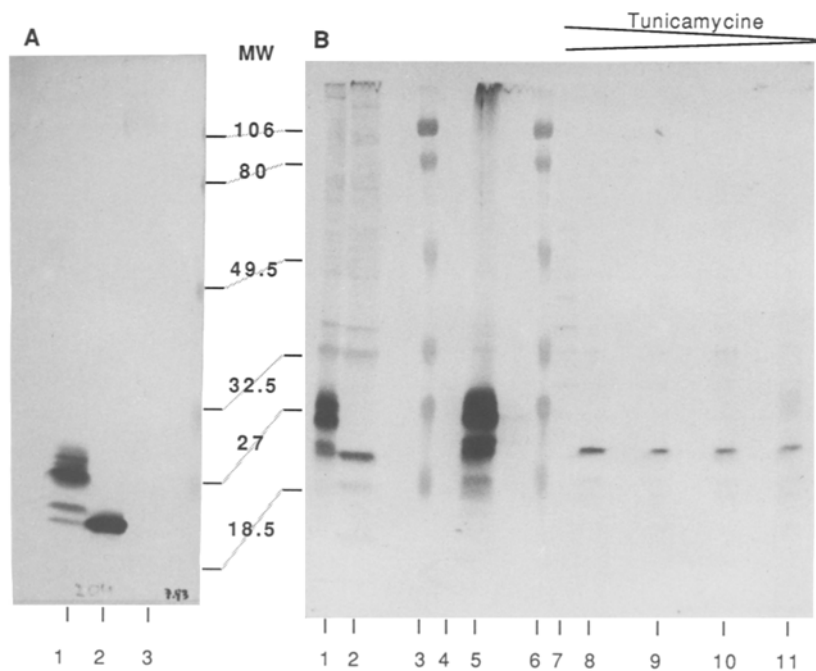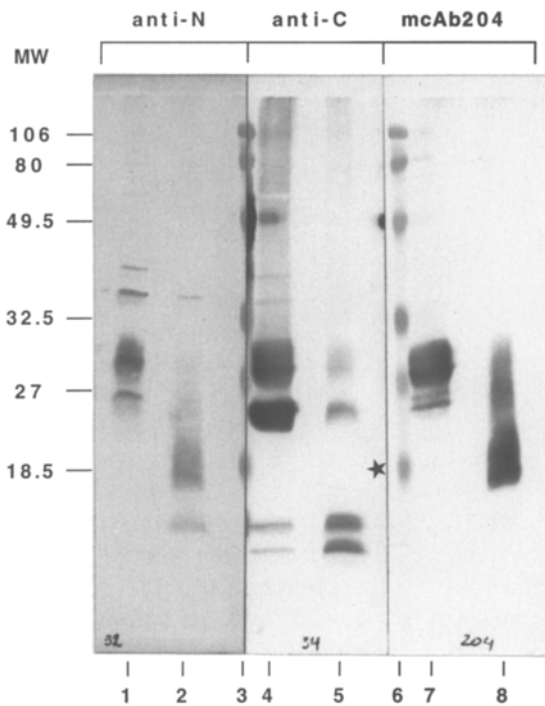
Fig. 4. Immunoblots of electrophoretically separated cell lysates and virion preparations are shown. Vero cells and Sf9 cells were infected or mock infected as described in Materials and Methods. The samples, treated as indicated for each lane separately, were electrophoresed in polyacrylamide gels and transferred to nitrocellulose before being immune stained with monoclonal antibody 204 and peroxidase-conjugated rabbit–anti-mouse IgG. The immune reaction was visualized by the incubation of the nitrocellulose sheet in a substrate containing chloronapthol and peroxide. *A:* Purified PED virions (lane 1). Lysates of Sf9 cells infected with recombinant baculovirus (PAK-BM-4; lane 2) and wild type baculovirus (PAK 6; lane 3), respectively. *B:* Untreated (lane 1) and N-glycosidase F treated (lane 2) purified PED virions and lysates of PEDV-infected (lanes 5,8–11) and mock-infected Vero cells (lanes 4 and 7). The cell cultures used for the preparation of samples in lanes 7–11 had been incubated in the presence of tunicamycin. Lanes 7 and 8 were incubated with 210 ng tunicamycin/ml, lane 9 with 60 ng tunicamycin/ml, lane 10 with 20 ng tunicamycin/ml, and lane 11 with 7 ng tunicamycin/ml. A molecular weight marker was run in lanes 3 and 6.

k double band were stained faintly, and at least two faster migrating bands of Mr 8 k and 10 k were apparent. Although this lane had been loaded with high amounts of protein, absolutely no traces of the 19 k band could be visualized (asterisk). Both antipeptide sera also immune stained an intermediate band of Mr 14 (open arrow), which was almost not recognized by the monoclonal antibody. In conclusion, these observations indicate that the 19 k band represented an N-terminal fragment of the M protein from which a C-terminal piece had been cleaved off. Furthermore, the epitope recognized by monoclonal antibody 204 could be mapped to the N-terminal fragment of M. Because the C terminus of M is possibly involved in protein-protein interaction with N and is required for assembly of virions, it is conceivable that such a truncated form of M protein is not incorporated into virions.

M Protein of PEDV Is a Virion Component and Adopts a NexoCcyt Orientation

In order to demonstrate that the M protein was a structural component of the virus particle, purified virions were incubated with monoclonal antibody 204 and the antipeptide sera, respectively. In order to visualize this reaction, the samples were further incubated in the presence of gold-labeled conjugates before negative staining and electron microscopy. Figure 6 shows that the outside of coronavirus-like virions were stained by the gold-labeled antibodies provided that they had been incubated with monoclonal antibody 204 (A) or the N-terminal peptide serum (B). The

Fig. 5. Immunoblots of electrophoretically separated virion preparations are shown. Purified PED virions were prepared as described in Materials and Methods, and were divided into two equal portions. One of the portions was solubilized directly in NaDodSO$_4$ buffer, whereas the remaining fraction was mixed with water supplemented with Triton X-100 at a final concentration of 0.1%. The sample containing Triton was kept for 15 min on ice before NaDodSO$_4$ was added. Equal amounts of the Triton treated (lanes 2, 5, 8) and untreated (lanes 1, 4, 7) samples were loaded, separated by polyacrylamide gel electrophoresis, and transferred to nitrocellulose. Molecular weight markers were included in lanes 3 and 6. The nitrocellulose was cut within lane 3 and in between lanes 5 and 6. The three strips were immune stained separately, as indicated on the top of the figure. The first strip, containing lanes 1, 2, and half of lane 3, was stained with rabbit serum R52, which is specific for the N terminus of the M protein. The second strip, containing half of lane 3, lane 4, and lane 5, was stained with rabbit serum R34, which is specific for the C terminus of M. The third strip, containing lanes 6–8, was immune stained with monoclonal antibody 204. The immune reactions were visualized by species–specific peroxidase conjugates and chloronapthol substrate, as described in Materials and Methods.
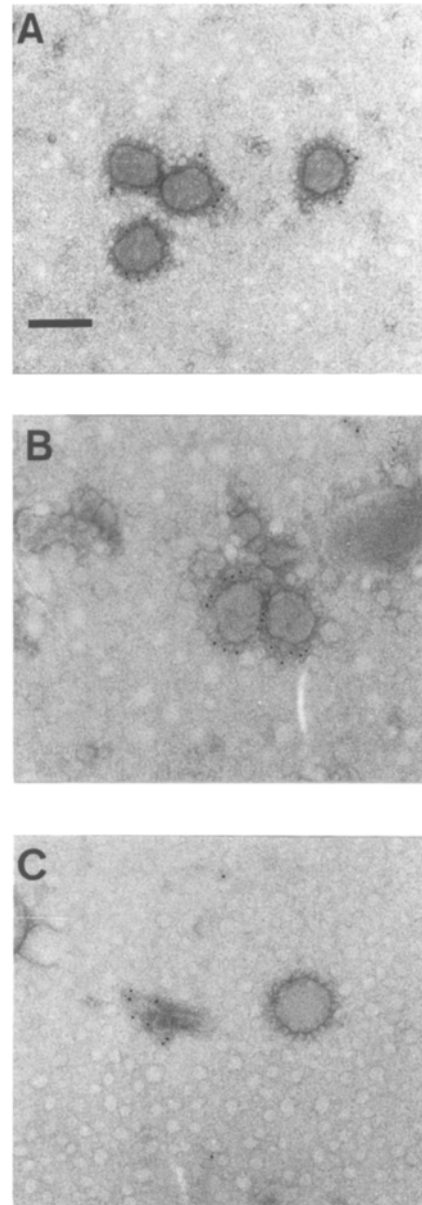
Fig. 6. Electron micrographs of immunogold labeled PED virions. Virus particles were incubated with (*A*) monoclonal antibody 204, (*B*) rabbit serum 51 (specific of the N terminus of M), and (*C*) rabbit serum 34 (specific for the C terminus of M). Visualization of A by gold-labeled goat anti-mouse IgG, and of B and C by gold-labeled goat anti-rabbit conjugate is described in Materials and Methods. Bar: 100 nm.

C-terminal rabbit serum did not yield gold labeling of the intact virions, indicating that the corresponding epitope was not accessible under the applied conditions. Interestingly, some of the gold label could be detected in conjunction with degraded particles (C). These observations indicated that the N terminus of M protein was accessible at the outside of the virion, whereas the C-terminal end was hidden at the inside. The mapping of the epitope recognized by monoclonal antibody 204 within the N-terminal fragment of the M protein could be confirmed by these experiments.

Discussion

In this article a gene product of the porcine epidemic diarrhea virus (PEDV) has been conclusively identified for the first time. The salient features of our observations are as follows: (a) The membrane protein M of PEDV migrated with a relative mobility (Mr) of 27 k in polyacrylamide gels. Several faster migrating bands of M represented predominantly cleavage and degradation products. These observations are in good agreement with the apparent molecular weights reported for M of other coronaviruses (10). According to the predicted amino acid sequences, the M proteins specified by Transmissible Gastroenteritis virus (TGEV) and human coronavirus 229E (HCV229E) are most closely related to that of PEDV (4). Specifically, 119 identical amino acids (aa) are shared between M of TGEV and M of PEDV. For TGEV, an Mr of 27.8 k has been calculated for the unglycosylated form of M (19). In contrast, the M protein of HCV229E shares 129 identical aa with M of PEDV (20). A relative mobility of 19 k has been described for the *in vitro* translated M protein of HCV229E (12), whereas we report that the deglycosylated and unglycosylated forms of M of PEDV migrated with an Mr of 23 k. The differences of the relative mobilities between M of PEDV (226 aa) and M of HCV229E (225 aa), on the one hand, and M of TGEV (262 aa), on the other hand, may be explained by the absence of the signal sequences in the M proteins of PEDV and HCV229E. Although 104 aa are identical between TGEV and HCV229E, only 80 aa are com-

mon to all three virus proteins, indicating that the M protein of PEDV occupies an interesting position in between those of TGEV and HCV229E.

(b) The M protein was cotranslationally N-glycosylated, as demonstrated by inhibition of glycosylation by the addition of tunicamycin to the growth medium and by cleavage of the carbohydrates by N-glycosidase F. This finding clearly implied that PEDV should not be grouped with the coronaviruses of antigenic group II, which are, for unknown reasons, predominantly O-glycosylated (10). Interestingly, M of PEDV appeared to be poorly if at all glycosylated when expressed by a recombinant baculovirus. Poor glycosylation of coronavirus (21) and influenza virus (22) glycoproteins by the baculovirus expression system has also been observed by others. In order to be glycosylated, the M protein has to be integrated in the ER. In the absence of a cleavable signal sequence, this may be achieved through each one of the three membrane-spanning domains common to all of the M proteins of coronaviruses (10). Thus, provided that the construct inserted into the recombinant baculovirus did not contain undetected sequence errors, it may be concluded that the Sf9 insect cells did not accept the signal provided by the putative transmembrane domains of PEDV M protein. It should be very interesting to address this question in the future by the expression of M in other eukaryotic vector systems such as recombinant vaccinia or herpes viruses.

(c) As demonstrated by immunogold labeling and electron microscopy, the M protein of PEDV was incorporated into virions with the NH_2 terminus facing towards the outside of the virus particle and the COOH terminus towards the inside, thus adopting the NexoCcyt orientation that appears to be typical for the membrane proteins of coronaviruses (23). According to the lack of a predicted signal sequence, it could be expected that the M protein of PEDV does not undergo N-terminal proteolytic cleavage as do the M proteins of TGEV (19) and feline infectious peritonitis virus (FIPV) (7). In infected cells, however, we observed an approximately 19 kD fragment of M that was not incorporated into virions. The fragment could be reconstituted from virions following incubation with Triton X-100. The frag-

ment reacted with a rabbit anti-peptide serum specific for the N terminus of M and with monoclonal antibody 204 but not with the C-terminal antiserum. This N-terminal fragment represented approximately two thirds of the M protein. We hypothesize for this reason that incubation with the detergent made a protease cleavage site accessible to the enzyme and the cytoplasmic tail of M was cleaved off. Others have postulated that the cytoplasmic fraction of M mediates interaction with the nucleocapsid protein and is required for particle formation (10). This may explain the fact that the 19 kD band was not incorporated into the virions.

The results presented in this communication are important prerequisites for further characterization of PEDV M protein. As outlined by others (24,25), more precise information on glycosylation, the intracellular localization of M, and its role in virion formation remain to be determined. For this, however, the construction of recombinant PEDV would be highly advantageous, but to date no infectious clone has been constructed for any coronavirus. The immunological tools and expression vectors described in this study may be highly useful for the identification of so far unknown coronaviruses in species other than porcines. Only recently has the detection of antibodies to a PED-like virus in human and feline sera been reported (6).

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