PRELIMINARY CHARACTERIZATION OF A MONO-CLONAL ANTIBODY SPECIFIC FOR A VIRAL 27 kD GLYCOPROTEIN FAMILY SYNTHESIZED IN PORCINE EPIDEMIC DIARRHOEA VIRUS INFECTED CELLS

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ABSTRACT

We describe a new monoclonal antibody No. 204 (mcAb 204) which recognized a family of four polypeptides, consisting of a 27kD, a 24/23kD double band and a 19kD protein present within PEDV infected cell lysates. These proteins were identified by immunoprecipitation as well as by staining of immunoblots. In infected Vero cell cultures, the synthesis of the 27kD protein was initiated between 6 and 8 hours post inoculation. The 24/23kD double band and the 19kD protein were only detectable later. At least the 27 and the 24/23kD proteins were apparently glycosylated and present in purified virions.

Pulse-chase as well as solubilization experiments indicated that the faster migrating bands represented processed products of the 27kD glycoprotein. The nature of the processing is not known at present.

We suggest that the 27kD protein family may represent the integral membrane protein M of PEDV. Since this protein is highly abundant in virions as well as in infected cells, and since mcAb 204 is able to react with its antigen under various conditions, this monoclonal antibody may be useful to further studies of the M-protein of PEDV. In addition, it may provide a useful tool for routine diagnosis.

INTRODUCTION

Porcine epidemic diarrhoea virus (PEDV), a causative agent of severe diarrhoea in pigs, was identified in 1978 by Pensaert and Debouck[1] but only in 1988 could the virus be propagated in cell cultures using Vero cells and medium containing trypsin[2]. Consequently, little is known about the structural PEDV proteins synthesized in infected cell cultures. Using *in vitro* cultivated virus, the viral surface protein S and the nucleocapsid protein N have been unambiguously identified and partially characterized[3].

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Egberink *et al.*,[4] who, in contrast to the above study, used purified PEDV from intestinal perfusates of infected pigs, were able to show by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) three virion structural proteins, N, S, and a cluster of proteins with molecular weights ranging from 20 to 32kD which was believed to represent the integral membrane protein M.

In this study, we describe a monoclonal antibody which identified a family of PEDV glycoproteins with molecular weights similar to those designated M-protein by Egberink *et al.*[4] The different forms apparently originate from a single precursor. Based on evidence provided in this paper as well as on previous reports referring to other coronaviruses (reviewed in [5]), we suggest that the 27kD protein family which is highly abundant in infected Vero cells may represent the integral membrane protein M of PEDV.

METHODS

PEDV strain CV777 was propagated in Vero cells essentially as described[2,3]. Viral proteins were metabolically labelled by supplementing the medium with 0,1 MBq ^{35}S-methionine (Amersham) per ml. Hybridomas secreting monoclonal antibody No. 204 (mcAb 204) were obtained as reported previously[6]. Standard methods were used for polyacrylamide gel electrophoresis, transfer to nitrocellulose (BA85, Schleicher&Schüll), and radioimmunoprecipitation[3]. Prior to immunostaining, the blots were treated with Triton X-100 (1%, 10 min.) and trypsin (80 µg/ml, 3 min.)[7]. The reactions were visualized using rabbit-anti-mouse antibodies and protein-A peroxidase as conjugate and H_2O_2 and chloronaphtol as substrate[3]. The solubilization experiments were done in 30 mM phosphate buffer with pH values as indicated in the text. OBG (n-octyl-beta-d-glucopyranoside, Sigma) 1.7% and 1.4% Triton X-100 (Boehringer) were added as detergents. The Glycan Detection Kit (Boehringer) was used according to protocol A of the manufacturer. Immunohistochemical staining with mcAb 204 was done on formaldehyde fixed and paraffin embedded gut samples of PEDV infected pigs (kindly supplied by A. Pospischil). The sections were pretreated with protease type XXVII (0.1%, Sigma), the binding reaction was visualized using a DAKO-PAP-KIT[8].

RESULTS AND DISCUSSION

mcAb 204 reacts with a 27kD structural glycoprotein of PEDV

In order to identify and characterize the viral protein corresponding to mcAb 204, individual Vero cell cultures were infected with PEDV and mock infected. The newly synthesized proteins were metabolically pulse labelled for consecutive two hour intervals before harvesting. The cellular lysates were separated on SDS polyacrylamide gels, transferred to nitrocellulose, and immunologically stained with mcAb 204. Starting from between 6 and 8 hours post infection (hpi), a ^{35}S-methionine labelled 27kD protein, which was synthesized exclusively in the PEDV infected cells, could be observed by autoradiography. As shown in Fig. 1, the corresponding polypeptide could be immune stained with mcAb 204 (lane 2). During the following intervals, faster migrating bands were detected. Between 8 and 10 hpi a double band of 24 and 23kD (24/23, lane 3) and between 10 to 12 hpi a 19kD band became detectable (lane 4). When crudely purified virions were tested with mcAb 204 on Western blots, the 27 and the 24/23kD bands were

also visible, whereas the 19kD band remained undetectable (lane 5, arrow). In order to test wether these polypeptides represented glycoproteins, purified virion preparations were oxidized in the course of the glycan detection procedure prior to immunoprecipitation. Following polyacrylamide gel electrophoresis and transfer of these pretreated immunoprecipitates to nitrocellulose, the 27 and 24/23kD bands were identified as glycoproteins by the use of the glycan detection kit (lane 8, dots). In contrast to the autoradiograms (lane 7), the glycan detection revealed two additional bands, one of which

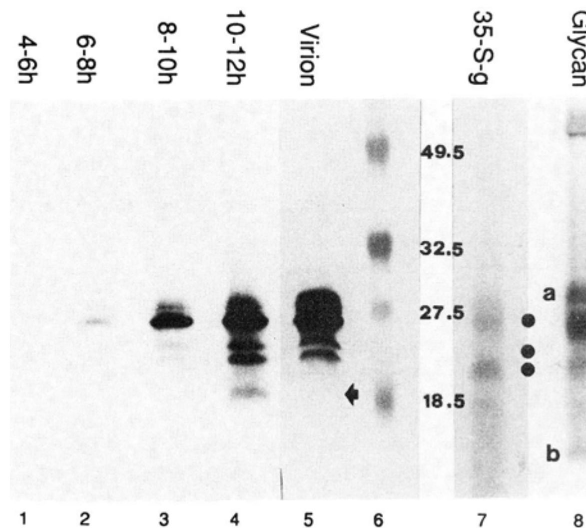

Figure 1. Western blot stainings (with mcAb 204 or with Glycan Detection Kit) and autoradiographic images are shown. Lanes 1 to 4: Immunostained PEDV infected cell lysates harvested at the time indicated at the top of each lane. Lane 5: Immunostained virion preparation. Arrow: lacking 19kD protein in virion preparation. Lane 6: Molecular weight (MW) marker. The numbers on the right indicate MW in Kilodaltons (kD). Lane 7: Autoradiographic image of ^{35}S-methionine labelled and with mcAb 204 immunoprecipitated PEDV antigen. Lane 8: Glycan detection of the material in lane 7. Dots indicate the position of the viral glycoproteins.

migrated more slowly than 27kD (a) and the other one faster than 19kD (b). Judging from mock infected controls (results not shown), band (a) apparently represented a fraction of the immunoglobulins. The nature of band (b) remained unresolved. Results obtained with immunoprecipitations of tritium-glucosamine labelled, infected cell lysates (data not shown) indicated in addition that all four members of the 27kD protein family were glycosilated.

In accordance with previous studies on PEDV structural proteins[4] as well as with reports concerning the structural proteins of coronaviruses in general[5], we conclude that the 27kD glycoprotein family may represent the integral membrane protein M of PEDV.

The 24/23 and the 19kD bands are processed products of the 27kD glycoprotein

It was not clear from the above described experiments if the faster migrating proteins were synthesized independently or if they represented processed products of the 27kD polypeptide. In order to study these faster migrating reaction partners of mcAb 204 more precisely, two sets of experiments i) pulse-chase and ii) solubilization studies were done.

i) Pulse-chase experiments. PEDV infected cells were pulse labelled for one hour intervals with ^{35}S-methionine. The radioactive precursor was then removed from the medium and, in order to prevent further translation of mRNA into protein without interfering with processive events, the cells were chased in the presence of 170 µg/ml cycloheximide for 30, 60 or 180 minutes. Finally, the cellular lysates were electrophoresed, blotted onto nitrocellulose, probed with mcAb 204 and exposed to autoradiography. The results (not shown) indicated that at least the 27 and the 23kD protein were radioactively labelled during the pulse period. In the course of the chase periods, increasing amounts of a faster migrating band (22kD) and less of the 27 and the 23kD bands could be detected by autoradiography. Interestingly, the 27kD band remained visible even after 3 hours of chase in the presence of cycloheximide, whereas the 23kD band disappeared during the same period. In contrast, the 24kD band was barely visible and the 19kD polypeptide was not at all seen by autoradiography throughout the chase period. On the other hand, by immunostaining with mcAb 204 the 27, 24, and 23kD bands could be seen immediately. In the course of the chase period, however, the 22 as well as the 19kD bands appeared, whereas the 27 and the 24kD bands grew increasingly faint. Finally, the 23 and 22kD bands merged into a single, blurry band. Such bands are typically seen with glycoproteins. From these experiments, it was clear that the 27kD glycoprotein family was processed posttranslationally. However, it was not possible to elucidate the ways and means of processing unambiguously.

ii) Solubilization studies. Using either OBG or Triton X-100 as detergents, infected cells were lysed and solubilized under variing pH conditions. The soluble and the insoluble fractions were separated by high speed centrifugation. The supernates containing the soluble proteins were removed, dialyzed, and adjusted to SDS buffer before electrophoresis. The pellets were resuspended and solubilized directly in SDS buffer. Samples of each fraction were electrophoresed and probed with mcAb 204 on Western blots before the bands were quantitated using a computing densitometer. The results of these experiments are summarized in Fig. 2. Under mildly acidic conditions (pH 5.5-6.0), the 27kD protein family appeared to be poorly soluble. With increasing pH (pH 6.6-8), however, higher amounts of 27kD polypeptide were present in the soluble supernatant fraction, until the pellet disappeared completely. Although in each quantifiable fraction the 27kD protein as well as faster migrating bands were observed, the amounts of the individual members of the protein family varied considerably with the conditions used for extraction. At pH 8, with OBG as the detergent, the 27kD polypeptide represented more than 90% of the immunostained protein. In contrast, when the cellular lysates were solubilized with Triton X-100 under otherwise identical conditions, only minor amounts of the 27kD protein could be detected, whereas the faster migrating 24/23kD double band represented more than 80% of the protein identified by mcAb 204.

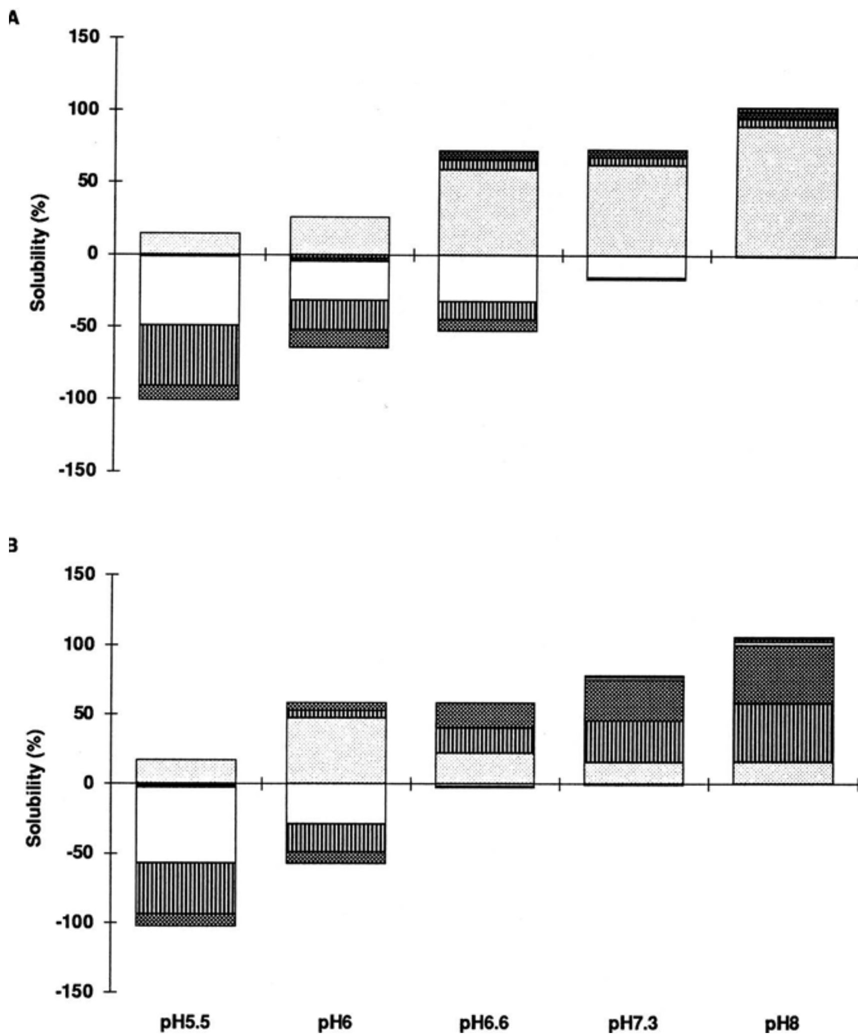

Figure 2. Solubility of 27kD protein family with OBG (A) and Triton X-100 (B). PEDV infected cell lysates were treated as described in the text. The pH values used for extraction are indicated on the x-axis. The mcAb 204 stained bands were quantitated by computing densitometry and the values were expressed in % of maximal staining (y-axis). Positive values represent proteins of the soluble fractions; negative values represent proteins contained in the insoluble pellets. 27kD protein (soluble fraction=grey; insoluble fraction=empty). 24/23kD double band (striped). 19kD protein (hatched).

We conclude that the 27kD protein family was processed to faster migrating forms, by both posttranslational processing and chemical breakdown during sample preparation. Although the various forms of the 27kD protein family were observed in abundant amounts when the infected cells were extracted directly with SDS buffer, we have insufficient evidence to suggest unambiguously that the faster migrating proteins represent naturally occuring polypeptides which are processed within infected cells from the 27kD precursor. Multiple forms of the integral membrane protein of other coronaviruses have been reported by others[5]. It has been suggested that dispersely migrating integral membrane glycoproteins may arise from different degrees of glycosylation or

heterogeneity of the attached oligosaccharide chains[4]. On the other hand, the influence of sample treatment on producing various polypeptide patterns has been reported for a number of coronavirus M-proteins (reviewed in [5]). Thus, it is possible that the extraction at pH 8 with OBG as detergent somehow protected the 27kD protein from being artificially processed. In any event, the means of processing are not known at present. Therefore both post translational processing and chemical breakdown have to be considered. Studies employing specific glycohydrolases, proteases and protease inhibitors as well nucleotide sequence analyses are in progress to provide further insight into these unanswered questions.

Potential applications for mcAb 204

The monoclonal antibody described in this study may provide a versatile tool to further study the integral membrane protein of PEDV, since it was able to immunoprecipitate the corresponding proteins as well as to stain its antigen on immunoblots. It should be mentioned that the latter technique required pretreatment of the transferred proteins with proteinase. Methanol or formaldehyde fixation as well as paraffin embedding did not interfere with binding of mcAb 204 to PEDV antigens in tissue sections (not shown). This monoclonal antibody may therefore be applied for routine diagnosis and for retrospective investigations[8].

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