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Biochemical characterization of porcine enteric calicivirus: analysis of structural and nonstructural viral proteins

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Summary. In this report, the molecular weight and antigenicity of the proteins of a porcine enteric calicivirus (PEC) were characterized. The PEC virions were purified from intestinal contents of infected pigs and from infected cell culture lysates. The average buoyant density of the purified virus was 1.37 gm/cm^3 in cesium chloride. One major structural protein with a molecular weight of approximately 58 k was found in the gut and cell culture-passaged PEC using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Using immunoblotting techniques only one immunoreactive protein (58 k) ws identified. The PEC and a prototype calicivirus, feline calicivirus (FCV) were propagated in pig kidney and feline kidney (Crandell) cell lines, respectively and intrinsically labeled using [^{35}S]methionine at various times post-inoculation (PI). SDS-PAGE of the radiolabeled proteins indicated the presence of the major structural protein (58 k) and one probable nonstructural protein (28 k) synthesized in the PEC-infected cell lysates by 12 h PI. Other minor protein bands were also evident by 24 h PI (32 k and 82 k). Only the 58 k major protein was detected by radioimmunoprecipitation (RIP) analysis using hyperimmune anti-PEC serum. SDS-PAGE and RIP analysis of FCV-infected cell lysates using hyperimmune anti-FCV serum identified a single major protein of approximately 64 k. No antigenic relationship between PEC and FCV proteins was detected by RIP analysis. The single major structural protein of PEC, the morphological appearance and size of the virus, and its average density of 1.37 gm/cm^3 in cesium chloride are consistent with properties of other members of the family *Caliciviridae*.

Introduction

Caliciviruses are small (35–40 nm) single-stranded RNA viruses possessing only one structural protein with a size range of 60 k to 71 k. This property differentiates the caliciviruses from picornaviruses, which have 4 structural proteins

[14]. The characterized prototype members of *Caliciviridae* include vesicular-exenthema of swine virus (VESV), San Miguel sea lion virus (SMSV), and feline calicivirus (FCV) [11].

The diarrheic feces of many species have yielded virus particles which possess biochemical and morphological characteristics that are similar to the caliciviruses. Only a few of these candidate caliciviruses have been biochemically characterized, since most attempts to grow them in vitro have met with little or no success [1]. One of the candidate caliciviruses, the Norwalk virus is a major cause of acute epidemic gastroenteritis in humans. In recent years, the Norwalk virus has been tentatively classified as a calicivirus, since it is similar to the caliciviruses in its morphology, size, buoyant density and possession of a single major structural protein of 59 k [5]. A single major structural protein of 62 k and 58 k was identified in human and canine candidate caliciviruses, respectively [13, 15]. In future studies of other small enteric viruses, information on the polypeptide and nucleic acid composition of the viruses would be useful for their identification and classification.

A porcine enteric calicivirus (PEC) was first reported in association with diarrhea in piglets in 1980 [9]. The PEC is considered a probable member of the family *Caliciviridae* because it is morphologically similar to caliciviruses. It has been serially propagated in primary porcine kidney cell cultures by incorporating an intestinal contents preparation into the cell culture medium [2]. Another study by Flynn et al. [3] has shown that PEC produces diarrhea and intestinal lesions after oral inoculation of pigs.

In an effort to further study PEC, we have purified the pig-passaged PEC from intestinal contents of infected gnotobiotic pigs and analyzed the structural proteins by SDS-PAGE and Western blotting (immunoblotting) techniques. We have also adapted the PEC to growth in a procine kidney cell line (LLC-PK-2) (Parwani AV et al. unpubl., 1989), and similarly characterized the viral proteins of the cell culture-passaged virus. Infected cell culture lysates, intrinsically labeled with [^{35}S]methionine at various times post-infection were analyzed using SDS-PAGE and RIP techniques to characterize the viral structural and nonstructural proteins and compare them with those of FCV. The immunoprecipitation of radiolabeled cell lysates was used to examine the serological cross-reactions between the viral proteins of PEC and FCV.

Materials and methods

Viruses and cells

The PEC specimen used for cell culture inoculation had been previously passaged 19 times in primary pig kidney cells [2] and was at the ninth passage level in a porcine kidney cell line (LLC-PK-2). The pig-passaged PEC specimen was obtained from the large intestinal contents of a gnotobiotic pig passage of the original isolate as described previously [3, 9]. The vaccine strain of FCV (F-9) was kindly provided by Dr. Louis Harris (Boehringer Ingelheim Animal Health, St. Joseph, Mo.). The porcine kidney cell line (LLC-PK-2) was obtained from the American Type Culture Collection, (Rockville, Md.). The Crandell feline kidney cell line (CrFK) was kindly provided by Dr. Roger Woods (NADC, Ames, Iowa).

Cell culture

The FCV was propagated in CrFK cells which were seeded into six-well tissue culture plates (Corning Glass works, Corning, N.Y.) and grown to confluency in Eagle's minimal essential medium (EMEM, Gibco Laboratories, Grand Island, N.Y.) supplemented with 100 units/ml penicillin, 100 units/ml streptomycin, 5 units/ml mycostatin, and 10% fetal bovine serum (Gibco). The PEC was adapted to the LLC-PK cell line (Parwani and Saif, unpubl. 1989). The LLC-PK cells were seeded into six-well tissue culture plates and grown to confluency in the EMEM supplemented with antibiotics, as described for CrFK cells. Virus inoculation of these cell cultures is described in the radiolabeling section.

Antisera

A hyperimmune gnotobiotic pig anti-PEC serum produced against purified pig-passaged PEC as described previously was used [3]. As an antibody negative control, serum was also obtained from an uninfected gnotobiotic pig. A rabbit anti-FCV hyperimmune serum (virus neutralization titer of 1:4,096) and a FCV antibody negative rabbit serum (virus neutralization titer of $< 1:8$) were kindly provided by Dr. Roger Johnson (University of Guelph, Guelph, Ontario, Canada). These antisera were used in the immunoblotting and the RIP experiments.

Virus purification

For analysis of proteins of the pig-passaged virus, PEC was purified from intestinal contents of infected gnotobiotic pigs. As a control, PEC-negative intestinal contents from uninfected gnotobiotic pigs were also subjected to a similar purification scheme. The intestinal contents (PEC-infected and PEC-negative) were diluted 1:2 in Tris-$CaCl_2$ buffer (0.05 M Tris-HCl, 0.1 M NaCl, and 1 mM $CaCl_2$), pH 7.5 and sonicated (BIOSONIK III, Bronwill, Rochester, N.Y.) on ice twice, 30 sec each time. The suspensions were clarified by centrifugation at 8,700 × **g** for 30 min. The supernatants were centrifuged at 72,600 × **g** for 2 h at 4 °C. The pellets were resuspended in Tris-$CaCl_2$ buffer, and the suspension was layered on a 40% sucrose cushion and centrifuged at 72,660 × **g** for 2 h at 4 °C. The pellets were resuspended in Tris-$CaCl_2$ buffer and layered on preformed CsCl gradients with a density of 1.32 to 1.41 gm/cm^3. The samples were centrifuged to isopycnic equilibrium at 72,660 × **g** for 16 h at 4 °C. Fractions, approximately 0.5 ml each, were collected by using a density gradient fractionator. The refractive index of each fraction was measured and the corresponding density in CsCl was determined. Fractions with similar densities were pooled. Each pooled fraction was assayed for virus by immune electron microscopy (IEM) as described previously [8]. The PEC was purified from infected cell culture lysates and fractions were assayed in a similar manner. As a control, mock-infected cells were subjected to a similar purification scheme. SDS-PAGE and immunoblotting techniques were used to characterize the proteins of PEC in the major fraction containing intact PEC particles as determined by IEM.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting

For protein analysis, the purified virus samples were subjected to SDS-PAGE. Stacking gels were 3% while separating gels ranging in concentration from 10% to 12% were used. The samples were heated for 5 min at 95 °C in Laemmli's sample buffer with or without 2-mercaptoethanol [6], and electrophoresed in Laemmli's electrophoresis buffer [6]. Molecular weight standards used were myosin (200 k), β-galactosidase (116.25 k), rabbit muscle phosphorylase (97 k), bovine serum albumin (66.2 k), hen egg white ovalbumin (42.699 k), bovine carbonic anhydrase (31 k), soybean trypsin inhibitor (21.5 k) and hen egg white lysozyme (14.4 k) (Bio-Rad Laboratories, Richmond, Calif.). Duplicate gels were run, with one gel being subjected to immunoblotting treatment while the other was stained either

with 0.125% Coomasie Blue R-250 (Bio-Rad) or silver-stained according to procedures outlined by Sammons et al. [10].

For immunoblotting, the electrophoresed PEC proteins were electrotransfered to nitrocellulose (NC) membranes in transfer buffer, pH 8.6 (25 mM Tris, 0.192 M glycine, 20% methanol, 0.01% SDS) for 4 h at 50 V using a Hoeffer Transblot apparatus (Hoeffer, San Francisco, Calif.). The standard proteins were transfered to a separate NC membrane and were detected by staining with amino black (Sigma Chemical Co., St. Louis, Mo.). Following electrotransfer, the blots were blocked by incubation with 2% nonfat dry milk for 2 h at room temperature (RT). The blots were washed with wash buffer (10 mM Tris, 160 mM NaCl containing 0.2% Tween 20) three times, 5 min each time, and incubated for 2 h at RT with the diluted (1 : 100) hyperimmune gnotobiotic pig anti-PEC serum or the antibody-negative serum. The blots were washed 3 times with wash buffer and incubated for 2 h at RT with a rabbit antiserum prepared against porcine IgG [7]. The washes were repeated and the blots were incubated for 1 h at RT in horseradish peroxidase-conjugated goat anti-rabbit IgG (BioRad). The blots were washed and developed for 10–15min at RT using a color developing reagent containing 4-chloro-1-napthol (Bio-Rad Laboratories, Calif.).

Radiolabeling and radioimmunoprecipitation experiments

Radiolabeling experiments were conducted using modifications of procedures described by Welch and Saif [16]. Briefly, confluent monolayers of LLC-PK cells in 6-well plates were washed and incubated in serum-free EMEM for 3 to 4 h. Immediately before inoculation, the medium was decanted and the cells were inoculated with 0.5 ml of the cell-culture adapted PEC or mock-infected with serum-free EMEM. The plates were incubated at 37 °C for 1 h and 2.5 mls of serum-free EMEM was added to each well. To the mock-infected and PEC-infected cells, 250 µl of diluted (1 : 8) intestinal contents preparation (ICP) was added. The ICP was prepared as described previously [2]. At 4 h PI, the medium was replaced with methionine-free medium, supplemented with 250 µl ICP and 5 µg/ml actinomycin D (Sigma Chemical Co., St. Louis, Mo.). At 5 h PI, 1.5 ml of the medium was removed and the cells were labeled with 50 µCi/well of [^{35}S]methionine (NEN Research Products, Boston, Mass.) for 1 h. After labeling, the monolayers were gently rinsed 2 to 3 times with ice-cold phosphate buffered saline (PBS; pH 7.4) containing 1 mM phenylmethylsulphonyl fluoride (PMSF; Sigma Chemical Co., St. Louis, Mo.). The cells were removed using a rubber policeman, placed into Eppendorf tubes and centrifuged. The cells were lysed with RIP buffer (0.15 M NaCl, 0.01 M Tris-HCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, and 1% aprotinin; pH 8.0) and stored at −70 °C, until further analysis.

Confluent monolayers of CrFK cells in six-well plates were inoculated with FCV at a low MOI (<0.1 pfu/ml) or mock infected with the maintenance medium. The cells were maintained and labeled wtih [^{35}S]methionine as described above except ICP was omitted from the maintenance medium. FCV-infected and mock-infected CrFK cells were pulsed with [^{35}S]methionine for 1 h at 15 and 20 h PI. For the analysis of the kinetics of PEC protein synthesis, PEC-infected cells were pulsed with [^{35}S]methionine for 1 h at 6-h intervals, begining at 6 h PI and continuing through 30 h PI. Mock infected cells were pulsed for 1 h at 18 h PI.

The RIP assay was performed using modifications of methods described by Welch and Saif [16]. The PEC and FCV cell lysates were pretreated with 10% formalin-fixed *Staphylococcus aureus*-protein A suspension (Zysorbin; Zymed Laboratories, Inc., South San Francisco, Calif.) for 1 h at RT. Undiluted PEC or FCV antisera or antibody-negative sera (20 µl) were incubated with 100 µl of 10% formalin-fixed *Staph*-protein A suspension at 37 °C. After 1 h, 100 µl of pretreated cell lysates were added and incubated for 2 h at RT, or at 4 °C overnight. The suspension was centrifuged at 8,000 × **g** for 5 min in a microfuge (Beckman Instruments Inc., Palo Alto, Calif.) and the pellets were washed once with RIP

buffer A (1.0 M NaCl, 0.01 M Tris-HCl, 0.1% NP-40; pH 7.2), once with RIP buffer B (0.1 M NaCl, 1 mM EDTA, 0.01 M Tris-HCl, 0.1% NP-40 and 0.3% SDS; pH 7.2) and once with RIP buffer C (0.01 M Tris-HCl, 0.1% NP-40; pH 7.2). The pellets were dissolved in 60 µl of Laemmli's sample buffer and heated for 5 min at 95 °C. However, 2-mercaptoethanol was excluded from the sample buffer so that the antibody molecule remained undissociated (to prevent comigration in SDS-PAGE of the viral major protein with the antibody heavy chain). The suspensions were centrifuged at 8,000 × **g** for 5 min and the supernatants were subjected to SDS-PAGE in 12% gels as described previously in this report.

The resulting gels were treated with EN^{3}HANCER (New England Nuclear, Boston, Mass.), dried and exposed to Kodak X-OMAT AR X-ray films (Eastman Kodak Co., Rochester, N.Y.) at −70 °C for 1 to 7 days. The molecular weight standards used were [^{14}C]methylated myosin (205 k), β-galactosidase (116 k), phosphorylase b (97.4 k), bovine serum albumin (66 k), chicken egg albumin (45 k), and carbonic anhydrase (29 k) (Sigma Chemical Co., St. Louis, Mo.). A different set of molecular weight standards was used in some experiments: [^{14}C]methylated myosin (200 k), phosphorylase b (92.5 k), bovine serum albumin (69 k), chicken egg albumin (46 k), and carbonic anhydrase (30 k) and lysozyme (14 k) (Amersham, Arlington Heights, Ill.).

Results

Virion purification

The PEC was purified from fecal samples of infected pigs and infected cell culture lysates. The purification scheme yielded intact virus as determined by IEM, for analysis by SDS-PAGE and immunoblotting. When fractions with a similar density were pooled and examined by IEM, intact PEC particles were most numerous in fractions with an average density of 1.37 g/cm^3. The morphology of purified PEC was similar to that previously reported [2, 9], consisting of viral particles with ten surface projections and 6-pointed hollow stars. The average size of the particles ranged from 33 to 35 nm in diameter (Fig. 1). No

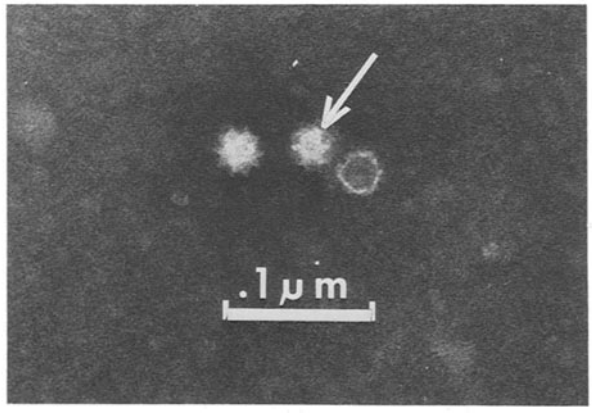

Fig. 1. Morphology of PEC particles purified from intestinal contents of infected gnotobiotic pigs and detected by immune electron microscopy using a high dilution of anti-PEC serum (1 : 1,000). The arrow points to a particle showing a hollow 6-pointed star appearance. The particle on the left shows the ten surface projections also typical of caliciviruses. The particle on the right appears empty (penetrated by the negative serum)

virus particles were observed in fractions purified similarly from intestinal samples of uninfected pigs or mock-infected cell cultures.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting analysis of the purified PEC

The proteins of PEC purified from intestinal samples of infected gnotobiotic pigs and infected cell cultures were examined by SDS-PAGE and their molecular weights were estimated by comparison with standard proteins. One major protein with a relative molecular weight of 58 k was consistently identified (data not shown). Immunoblotting analysis of the viral structural proteins was conducted in order to determine their reactivity with anti-PEC serum or serum negative for PEC antibodies (Fig. 2). The uninfected control and PEC fractions with an average density of 1.37 g/cm^3 were subjected to SDS-PAGE and electrotransferred onto nitrocellulose membranes. The transfered proteins were probed with hyperimmune anti-PEC serum or the antibody-negative serum. In Fig. 2, one major protein, 58 k, was detected when the PEC sample was incubated with the anti-PEC serum (lane B). No proteins were detected in the control

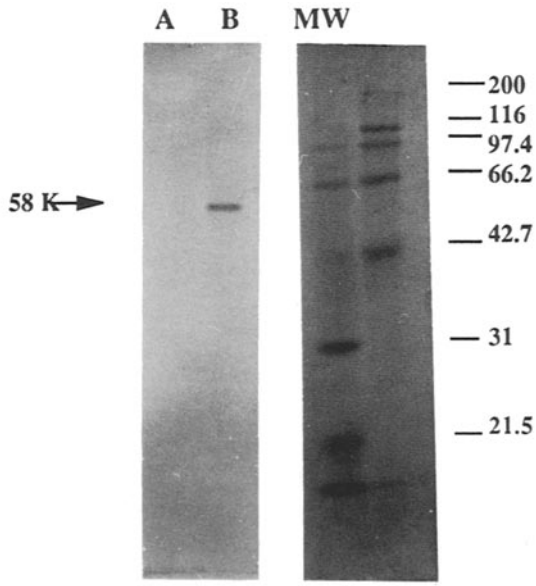

Fig. 2. Immunoblot analysis of the proteins of PEC purified from intestinal contents of infected gnotobiotic pigs. Samples were run in a 10% SDS-polyacrylamide gel and electrotransfered onto a nitrocellulose membrane. The membrane was probed with hyperimmune anti-PEC serum, as described in the text. The estimated molecular weight of the PEC protein is indicated (arrow). *A* Control sample purified from feces of uninfected pigs; *B* sample purified from feces of pigs infected with PEC. Low and high molecular weight standards ($\times 10^{-3}$) shown on the right were run in the same gel, transfered onto separate nitrocellulose membranes, and stained with amido black

sample (Fig. 2, lane A). A duplicate blot was incubated with antibody-negative serum under the same conditions. No proteins were detected (data not shown).

Time course of PEC protein synthesis in infected cells

The LLC-PK cell monolayers were infected with PEC and viral protein synthesis was examined by pulse labeling with [^{35}S]methionine at various times after infection. In PEC-infected cells, virus specific proteins (not seen in mock-infected cells; Fig. 3, lane A) were first detected at 12 h PI (Fig. 3, lane C). The major 58 k protein was detected at this time, and at subsequent time intervals (Fig. 3,

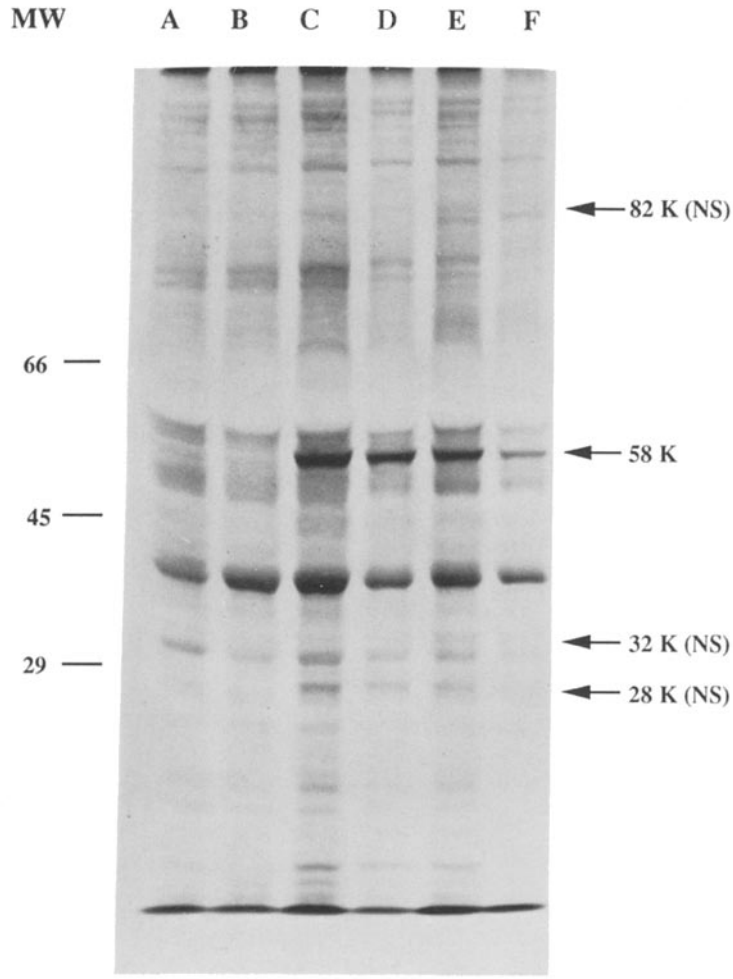

Fig. 3. Time course of protein synthesis of PEC. [^{35}S]methionine labeled cell lysates from infected and mock-infected LLC-PK cells were prepared, run in a 12% SDS-polyacrylamide gel and autoradiographed as described in the text. The estimated molecular weights of the viral proteins are indicated on the figure (right arrows; *NS* nonstructural). *A* Mock-infected LLC-PK cells 18 h PI; *B–F* PEC-infected LLC-PK cells labeled at 6 ,12, 18, 24, and 30 h PI, sequentially. Molecular weight ($\times 10^{-3}$) marker positions are indicated on the left

lanes C-F). A second protein, with an approximate molecular weight of 28 k, was visualized from 12 h PI through 24 h PI. Other minor PEC protein bands were also evident as shown in Fig. 3, with estimated molecular weights of 82 k and 32 k (Fig. 3, lanes E and F). The major and minor proteins were absent in the 18-h mock-infected (Fig. 3, lane A) and 6-h PI samples (Fig. 3, lane B).

Radioimmunoprecipitation analysis of cell lysates

The RIP experiments using anti-PEC and anti-FCV sera were conducted to further define the structural viral proteins reactive with each antisera and examine serological cross-reactions between the proteins of the two viruses (Figs. 4 and 5). Both infected and mock-infected cell lysates were analyzed. Each lysate was tested with antibody negative and hyperimmune serum. In PEC-infected

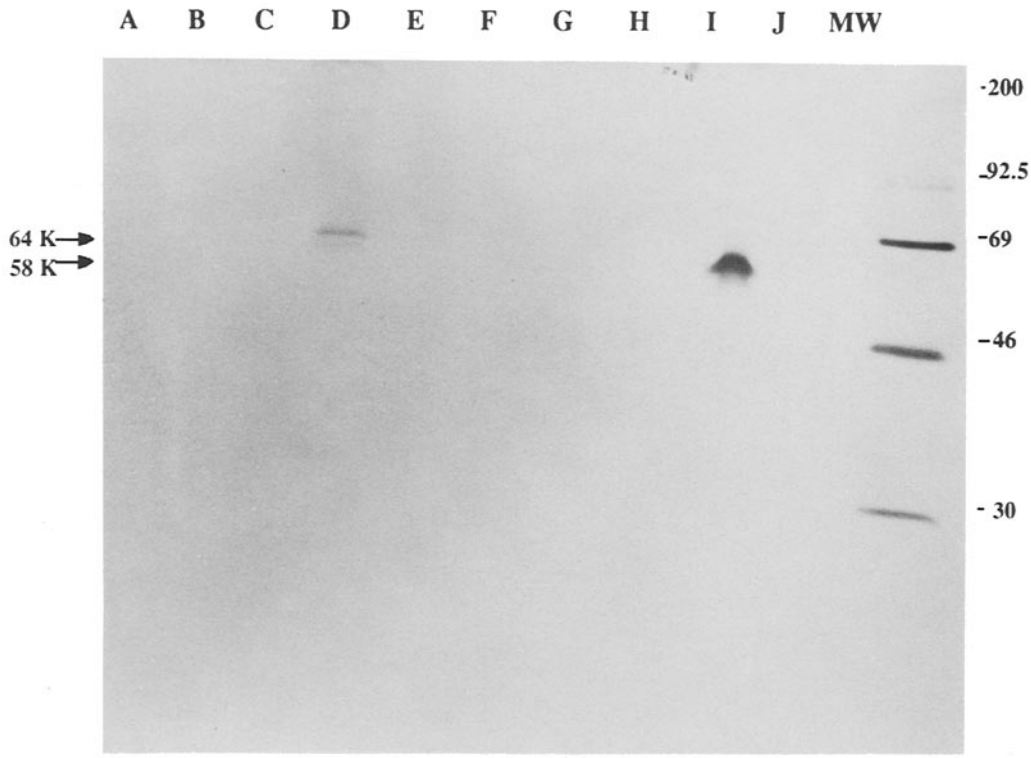

Fig. 4. Radioimmunoprecipitation analysis of [^{35}S]methionine labeled cell lysates from PEC-infected and mock-infected cells. The lysates were immunoprecipitated, run on 12% SDS-polyacrylamide gels and the gels subjected to autoradiography as described in the text. The estimated molecular weight of the viral protein is indicated (arrow). Mock-infected cell lysates at 18 h PI (*A, B*); PEC-infected cell lysates at 6 h PI (*C, D*); PEC-infected cell lysates at 12 h PI (*E, F*); PEC-infected cell lysates at 18 h PI (*G, H*), and PEC-infected cell lysates at 24 h PI (*I, J*). Molecular weight standards ($\times 10^{-3}$) are shown on the right. *A, C, E, G, I* Samples were reacted with 20 µl of undiluted antibody-negative serum; *B, D, F, H, J* samples were reacted with 20 µl of undiluted hyperimmune anti-PEC serum

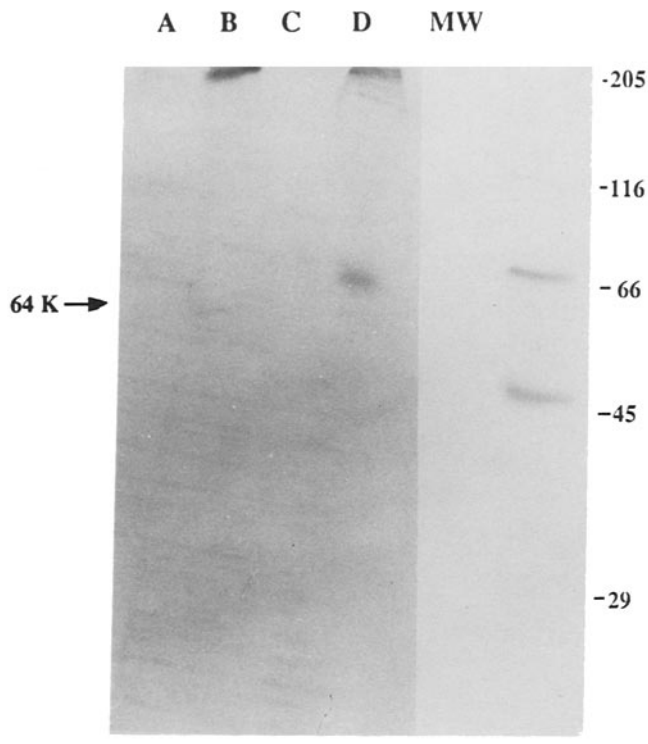

Fig. 5. Radioimmunoprecipitation analysis of [^{35}S]methionine-labeled CrFK cell lysates from FCV-infected and mock-infected cells. The lysates were immunoprecipitated, run on 12% SDS-polyacrylamide gels and the gels subjected to autoradiography as described in the text. The estimated molecular weight of the viral protein is indicated (arrow). Mock-infected cell lysates at 15 h PI (*A, B*) and FCV-infected cell lysates at 15 h PI (*C, D*). Molecular weight standards ($\times 10^{-3}$) are shown on the right. *A, C* Samples were reacted with 20 µl of undiluted antibody-negative serum; *B, D* samples were reacted with 20 µl of undiluted hyperimmune anti-FCV serum

cell lysates, the major viral protein of 58 k was detected in lysates from infected cells tested with anti-PEC serum (Fig. 4, lanes F, H, and J). None of the minor proteins were immunoprecipitated. No proteins were detected in mock-infected cell lysates (Fig. 4, lanes A and B), in lysates incubated with antibody negative serum (Fig. 4, lanes E, G, and I), or in cell lysates from 6 h PI samples (Fig. 4, lanes C and D). A single protein of 64 k was detected in FCV-infected cell lysates (Fig. 5, lane D). This protein was not present in mock-infected cell lysates (Fig. 5, lanes A and B). No proteins were detected when FCV antibody-negative serum was used as a probe (Fig. 5, lane C).

Both FCV-infected and PEC-infected cell lysates were incubated with anti-FCV and anti-PEC serum in the same RIP experiment (Fig. 6). No cross-reactivity was observed when PEC and FCV samples were probed with anti-FCV and anti-PEC sera, respectively (Fig. 6, lanes E and J). The 58 k PEC-specific protein was detected with anti-PEC serum in PEC-infected cell lysates

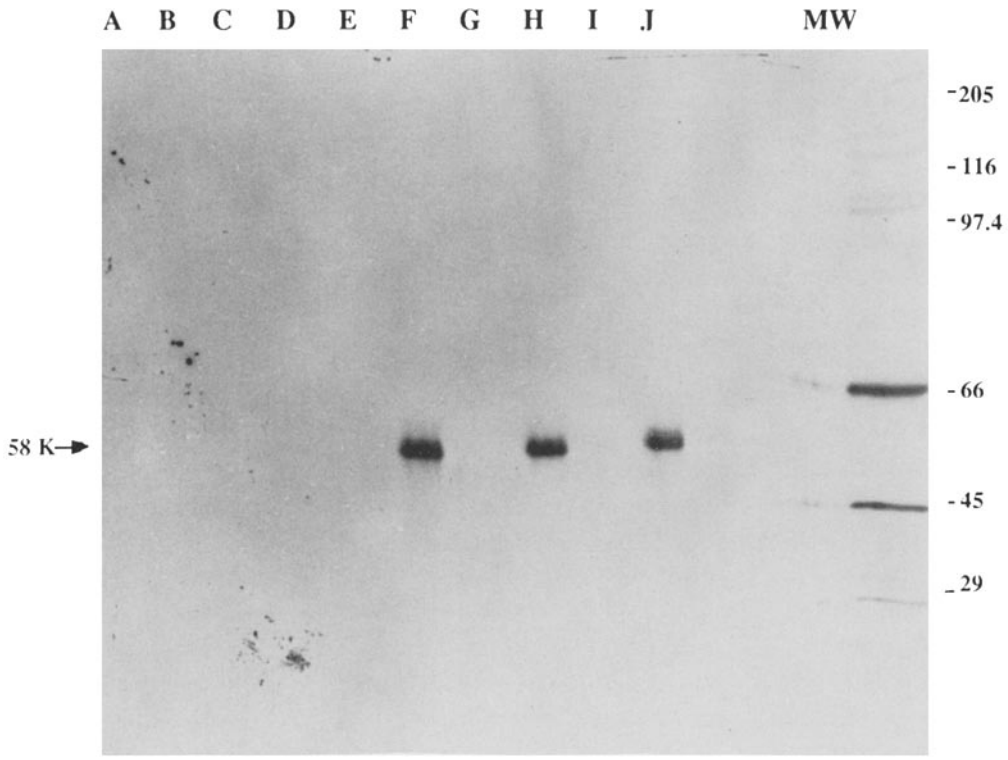

Fig. 6. Comparative analysis of the viral proteins of PEC and FCV by radioimmunoprecipitation analysis of [^{35}S]methionine labeled cell lysates from FCV-infected, PEC-infected and mock-infected cells. The lysates were immunoprecipitated, run on 12% SDS-polyacrylamide gels and the gels subjected to autoradiography as described in the text. The estimated molecular weights of the viral proteins are indicated (arrow). *A–E* CrFK cells; mock-infected cell lysates at 20 h PI (*A, B*) and FCV-infected cell lysates at 20 h PI (*C–E*). *F–J* LLC-PK cells; mock-infected cell lysates at 20 h PI (*F, G*) and PEC-infected cell lysates at 20 h PI (*H–J*). Molecular weight standards ($\times 10^{-3}$) are shown on the right. *A, C* Samples were reacted with 20 µl of undiluted FCV-antibody negative serum; *B, D, J* samples were reacted with 20 µl of undiluted anti-FCV serum; *F, H* samples were reacted with 20 µl of PEC-antibody negative serum; *E, G, I* samples were reacted with 20 µl of anti-PEC serum

(Fig. 6, lane I) and the 64 k FCV-specific protein was observed when reacted with anti-FCV serum (lane D). No bands were observed in mock-infected cell lysates (Fig. 6, lanes A, B, F, and G).

Discussion

Electron microscopy has led to the discovery of calicivirus-like particles in the feces of many species. Some of these viral particles have been associated with gastroenteritis in humans (candidate human enteric calicivirus, Norwalk virus), dogs (canine calicivirus), pigs (PEC) and chickens (avian calicivirus) [1]. Prototype caliciviruses are reported to have a buoyant density of 1.36 to 1.39 g/cm^3 in cesium chloride [12]. The candidate human enteric calicivirus (HCV)

has a density of 1.37 to 1.38 g/cm^3 in cesium chloride [15] and the reported density for the Norwalk virus is 1.38 to 1.40 g/cm^3 in cesium chloride [5]. Our studies indicate that the purified PEC has an average buoyant density of 1.37 g/cm^3 in cesium chloride. The average size (33 to 35 nm in diameter) and morphology of the PEC is also comparable to other caliciviruses [9]. These data indicate that PEC is a candidate calicivirus.

Caliciviruses are unique among vertebrate viruses in that they possess only one major structural protein with a molecular weight range of 60 k to 71 k [11]. This single major structural protein could be considered as a unique biochemical characteristic of all caliciviruses. It has been found that this protein is the main product of protein synthesis in cells infected with the prototype calicivirus, vesicular exanthema of swine virus [14], although five viral non-structural proteins have also been identified in cells infected with SMSV [4].

A few candidate caliciviruses have been characterized and found to possess a single major protein [1]. These include the Norwalk virus (59 k), human candidate calicivirus (62 k) and canine calicivirus (58 k). Possession of a single major protein (58 k) provides additional evidence that PEC is closely related to prototype and other candidate enteric caliciviruses. The 58 k protein was recognized by hyperimmune serum in both our immunoblotting and RIP experiments. This suggests that this major viral protein is highly immunogenic, or present in levels needed to evoke good antibody responses. None of the minor proteins were recognized by hyperimmune serum in either immunoblotting or RIP experiments. These findings suggest that the minor proteins are nonstructural proteins, are present in low concentrations or are poorly immunogenic in nature.

We have labeled and immunoprecipitated FCV in the same manner as the PEC. The single major protein of PEC (58 k) was found to be smaller than FCV (64 k) when coelectrophoresed on the same gel (Fig. 6). The size of the major PEC protein is comparable to that reported for other candidate caliciviruses such as Norwalk virus (59 k) and canine calicivirus (58 k). The three minor proteins of PEC (82 k, 32 k, and 28 k) are similar in molecular weight to some of the nonstructural proteins identified in SMSV-infected cells, with approximate molecualr weights of 115 k, 80 k, 40 k, 35 k, and 29 k [4]. The 28 k minor protein in PEC is similar in size to the 30 k soluble protein identified in the Norwalk virus [5], and closely parallels the molecular weight (29 k) of a nonstructural protein in SMSV-infected cells [4]. Further evidence for these minor proteins of PEC being of a possible nonstructural nature, was the failure to detect their presence in SDS-PAGE analysis of purified PEC, which revealed only the major 58 k protein.

The PEC appears to be closer to the candidate caliciviruses, canine calicivirus and particularly the Norwalk virus in its polypeptide composition. In an earlier study, PEC was found to be antigenically unrelated to FCV and SMSV [9]. In another study of a canine calicivirus, anti-PEC serum showed no reaction when tested with the canine calicivirus [13]. Using the RIP test, we have

confirmed that the viral proteins of PEC are serologically unrelated to those of a prototype calicivirus, FCV (Fig. 6). However, no studies on the serological relatedness of PEC and Norwalk virus have been published to date.

We have found that if 2-mercaptoethanol is included in the sample buffer for SDS-PAGE analysis of the radioimmunoprecipitated protein of PEC, the result is the comigration of the major protein of PEC with the heavy chain protein of the immunoglobulin molecule, since both these protein molecules have similar molecular weights (58 k and 55 k to 60 k, respectively). This resulted in the appearance of distorted bands, leading to an inaccurate estimation of the molecular weights (data not shown). This problem of comigration was avoided by excluding 2-mercaptoethanol from the sample buffer, resulting in appearance of sharp PEC protein bands. In these experiments we also found that there is little or no difference in the molecular weight estimate of the proteins of PEC with or without the addition of 2-mercaptoethanol in the sample buffer for SDS-PAGE analysis.

The three candidate caliciviruses, PEC, canine calicivirus and the Norwalk virus appear to share at least one biochemical characteristic with other prototype caliciviruses, the possession of a single major structural protein. However, the major protein in these candidate caliciviruses is smaller than the reported molecular weight range of 60 k to 71 k for the prototype caliciviruses. The present findings suggest that PEC is a probable member of the calicivirus group and strongly resembles some other candidate caliciviruses in the composition of its structural and nonstructural proteins.

Future studies will be directed towards understanding the nature of the minor proteins of PEC and determining their significance in the infectivity of PEC. Another important area of focus will be the serological relatedness of PEC to other candidate enteric caliciviruses. The ability to cultivate PEC in cell culture should permit other useful biochemical and molecular analyses of this enteric calicivirus not feasible with the noncultivable human enteric caliciviruses, and allow PEC to serve as a useful model for these candidate caliciviruses.

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