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Preliminary Evidence that *Clostridium perfringens* Type A Enterotoxin Is Present in a 160,000-M_r Complex in Mammalian Membranes

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***Clostridium perfringens* type A ^{125}I-enterotoxin (^{125}I-CPE) was bound to rabbit intestinal brush border membranes (BBMs) or Vero cells and then solubilized with 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS). Solubilized radioactivity was analyzed by gel filtration chromatography on a Sepharose 4B column or by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) without sample boiling and autoradiography. Specifically bound ^{125}I-CPE extracted from either BBMs or Vero cells was primarily associated with a complex of approximately 160,000 M_r. The CPE complex was partially purified by gel filtration or SDS-PAGE without sample boiling. SDS-PAGE analysis with sample boiling of the partially purified ^{125}I-CPE complex from Vero cells or BBMs suggested that CPE complex contains both a 50,000-M_r protein and a 70,000-M_r protein in approximately equimolar amounts. This result is supported by affinity chromatography with CPE immobilized on Sepharose 4B, which showed the specific interaction of similar size proteins with CPE. The simplest explanation for these results is that CPE (M_r 35,000) interacts with 50,000-M_r and 70,000-M_r eucaryotic proteins to form a membrane-dependent complex of approximately 160,000 M_r. These results suggest that the receptor or target site(s) or both for CPE are similar in both BBMs and Vero cells. The significance of these findings in terms of CPE binding, insertion, and biologic action is discussed.**

Clostridium perfringens type A produces a protein enterotoxin (CPE) responsible (16) for the characteristic symptoms (diarrhea and abdominal cramps) of *C. perfringens* food poisoning. This food poisoning is usually mild, but the lethal potential of *C. perfringens* food poisoning should be recognized, especially for elderly or debilitated individuals (20). Recent reports suggest that CPE is also involved in some cases of infectious or antibiotic-associated diarrhea, infantile diarrhea, and sudden infant death syndrome (see reference 11 for a review).

CPE is a single polypeptide of approximately 35,000 M_r. CPE has a unique mode of action distinguishable from those of other bacterial enterotoxins (11). The initial step in CPE action is specific binding of CPE to membrane receptors (17, 26). The enterotoxin receptor is protease sensitive (13, 15, 25), indicating that it is, at least partly, proteinaceous. No involvement of lipids in CPE binding has been detected (25). A 50,000-M_r protein in rabbit intestinal brush border membranes (BBMs) was shown by affinity chromatography to specifically bind CPE (24). This protein may be all or part of the CPE receptor in BBMs. Specific binding of CPE is essentially irreversible since little or no CPE dissociation is observed even with long incubations of bound CPE in the presence of chaotropic salts (13, 15, 25). Specifically bound ^{125}I-CPE becomes increasingly insensitive to protease degradation with time (13), suggesting a rapid insertion of CPE into membranes following binding.

CPE is believed to act directly by effects on the plasma membrane (11, 16). At present, there is no evidence supporting CPE internalization into the cytoplasm of cells. Subcellular localization studies (22) indicate that CPE associates only with the plasma membrane. No CPE was detectable in any other subcellular fraction. Lysosomotrophic agents which prevent acidification of endocytic vesicles do not protect Vero cells from CPE action (10). Microinjected cytoplasmic anti-CPE antibodies were unable to protect Vero cells from CPE (19).

Direct experimental support exists for a plasma membrane action for CPE. Permeability alterations, such as ion and small-molecule fluxes (3, 6, 9, 10, 13), occur rapidly after CPE binding and insertion in Vero cells. These early permeability alterations then presumably lead to secondary CPE-induced effects including disruption of the cellular osmotic equilibrium, inhibition of macromolecular synthesis and energy metabolism, morphologic damage, and, eventually, cell death (11, 16).

The molecular mechanism by which CPE induces permeability alterations in plasma membranes remains unclear. A recent study (13) from our laboratory employed sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with sample boiling and suggested that specifically-bound ^{125}I-CPE exists as a complex in BBMs. Dreyfus and Robertson (1) have used SDS-PAGE without sample boiling to study the interaction of *Escherichia coli* heat-stable enterotoxin with rat BBMs. We used this approach to further clarify the interaction of ^{125}I-CPE with eucaryotic membranes and to provide stronger evidence for CPE complexes in membranes. Our studies suggest that the CPE complexes consist of CPE and two membrane proteins.

MATERIALS AND METHODS

Purification of CPE. CPE was prepared and purified and its biologic activity was assayed as described previously (18).

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Purified CPE was radiolabeled enzymatically as described previously (13) with lactoperoxidase-glucose oxidase (Bio-Rad Laboratories, Richmond, Calif.) and $Na^{125}I$ (ICN Radiochemicals, Irvine, Calif.). The radioactive specific activity of the ^{125}I-CPE was 1 to 3 mCi/mg of protein. ^{125}I-CPE preparations maintained biologic and serologic activity (13) following the radioiodination procedure, indicating that the radiolabeling conditions did not grossly inactivate CPE. Also, under iodination conditions yielding at least one iodine molecule substituted per CPE molecule (produced by radioiodination with a radioactive and nonradioactive iodine excess), there was no significant decrease in CPE bioactivity. Evidence supporting the biologic relevance of ^{125}I-CPE specific binding has been presented previously (13).

Preparation of intestinal BBMs. BBMs were prepared from the small intestine of female New Zealand White rabbits (2 to 4 kg each) by the method of Sigrist et al. (21).

Binding of ^{125}I-CPE to BBMs. Specific binding of ^{125}I-CPE (5 μg) to rabbit intestinal BBMs (200 μg) at 24°C was determined as described previously (13). Binding solutions for BBMs contained Dulbecco phosphate-buffered saline without or with $CaCl_2$ (0.9 mM) and $MgCl_2$ (0.5 mM) as specified. Specific binding was calculated by subtracting the counts associated with BBMs cotreated with a 50-fold or greater excess of unlabeled enterotoxin (nonspecific binding) from the counts associated with membranes treated only with ^{125}I-CPE. Specific binding was approximately 2×10^5 cpm/200 μg of BBM protein, or about 70% of total binding.

Binding of ^{125}I-CPE to Vero cells. Vero cells were grown to confluency and harvested as described previously (14). Binding solutions for Vero cells contained Hanks balanced salt solution without or with $CaCl_2$ (1.7 mM) and $MgCl_2$ (1.8 mM) as specified. ^{125}I-CPE (5 μg) was added to 2×10^6 cells in 0.5 ml of binding solution in the presence or absence of a 50-fold or greater excess of unlabeled CPE. After gentle shaking for 10 min at 24°C, the cells were washed with 0.5 ml of binding solution, pelleted, and resuspended in 180 μl of the appropriate binding solution without ^{125}I-CPE. The radioactivity in a sample was counted, and specific binding was determined as described above for BBMs. Specific binding represented about 8×10^4 cpm/2×10^6 cells, or approximately 30% of total binding.

Identification of BBM and Vero cell ^{125}I-CPE complexes by SDS-PAGE without sample boiling. For most experiments, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS) (Sigma Chemical Co., St. Louis, Mo.) in H_2O was added to a final concentration of 1% to the ^{125}I-CPE binding mixture containing BBMs or Vero cells. After 15 min at 24°C, the CHAPS extractions were microcentrifuged for 3 min to remove insoluble material. Approximately 60% of membrane-bound radioactivity was solubilized by the CHAPS extraction. Additional extractions released most of the remaining radioactivity from the initial insoluble pellet. This material contained complex in amounts proportional to that in the primary extraction. In specified experiments, SDS was used to solubilize membrane-bound radioactivity with similar results. Samples (30 μl) of the supernatants were added to an equal volume of SDS-PAGE sample buffer (0.0625 M Tris hydrochloride [pH 6.8], 2% SDS, 10% glycerol, 0.001% bromophenol blue) with or without β-mercaptoethanol (5% final concentration) as noted for each sample, and samples were analyzed on 16-cm SDS-PAGE slab gels containing 5, 6, 7, or 8% acrylamide with the buffer system of Laemmli (7). Molecular weight standards were purchased from Sigma (myosin, 200,000; β-galactosidase, 116,000; phosphorylase *b*, 97,000; bovine serum albumin, 68,000; ovalbumin, 43,000). After electrophoresis at 5 W of constant power until the tracking dye reached 1 cm from the bottom of the gel, the gels were dried and autoradiographed with intensifying screens (Cronex Hi-Plus) at −70°C for 2 to 4 days.

Gel filtration chromatography of ^{125}I-CPE complex from BBMs. Bound radioactivity was solubilized with CHAPS as described above and diluted to 1 ml with Dulbecco phosphate-buffered saline without Ca^{2+} or Mg^{2+}. These samples were loaded onto a Sepharose 4B column (76 by 1.5 cm; Pharmacia, Uppsala, Sweden) equilibrated with Dulbecco phosphate-buffered saline without Ca^{2+} or Mg^{2+}. Chromatography was performed at 4°C, and 80-drop fractions (about 2.5 ml) were collected at a flow rate of 0.5 ml/min. Samples of the fractions were counted in a gamma counter, and the fractions corresponding to ^{125}I-CPE complex (as determined by SDS-PAGE without sample boiling) were combined. The complex material was then concentrated by ultrafiltration with a membrane (PM-10; nominal molecular weight cutoff, 10,000; Amicon Corp., Lexington, Mass.) and analyzed by SDS-PAGE with sample boiling.

CPE complex molecular weight was determined by the method of Whitaker (23) with CPE (35,000), bovine hemoglobin (64,500), bovine serum albumin (68,000), mouse immunoglobulin G (158,000), and horse spleen ferritin (440,000).

Partial purification of ^{125}I-CPE complex from BBMs and Vero cells. ^{125}I-CPE complex was partially purified by SDS-PAGE without β-mercaptoethanol or sample boiling. ^{125}I-CPE (50 μg) was bound to BBMs (2.0 mg of protein) or Vero cells (2×10^7 cells) and then extracted with CHAPS and diluted (1:1) with 1 ml of SDS-PAGE sample buffer. Following electrophoresis on 6% acrylamide gels as described above, the gels were immediately placed on X-ray film and autoradiographed overnight at 4°C to determine the migration of ^{125}I-CPE complex. Sections of the gels corresponding to ^{125}I-CPE complex were cut out. The ^{125}I-CPE complex material was electroeluted from the gel slices into SDS-PAGE buffer overnight at 4°C. The electroeluted complex material was dialyzed against sterile distilled H_2O overnight at 4°C, lyophilized, and resuspended in 100 μl of SDS-PAGE sample buffer. Approximately 50 to 100 μg of protein (20,000 to 60,000 cpm) was recovered (total amounts from four gels that were combined at the electroelution step).

Affinity chromatography. CPE-coupled and control affinity columns were prepared with CNBr-activated Sepharose 4B (Pharmacia) as described previously (24). Either Tergitol NP-40 (Sigma) extracts of BBMs (24) or CHAPS extracts of BBMs were chromatographed (24) on both columns. BBMs (5 to 10 mg of protein) were solubilized with 1% CHAPS prior to chromatography.

Analysis of complex composition by SDS-PAGE with sample boiling. Samples received β-mercaptoethanol (5% final concentration) and were placed in a boiling water bath for 3 min. SDS-PAGE was performed with 10-cm slab gels as described previously (13). Gels were stained with either Coomassie brilliant blue R-250 (Bio-Rad) or silver stain (Bio-Rad) as specified prior to drying and autoradiography. Molecular weights were determined with standards from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.).

Other methods. Protein determinations were by the method of Lowry et al. (8) with bovine serum albumin as the standard. Radiolabeled samples were counted with a Packard gamma spectrophotometer. Protease digestion of ^{125}I-CPE was done with Pronase and trypsin as described previously (13). Densitometric scans and peak area integrations

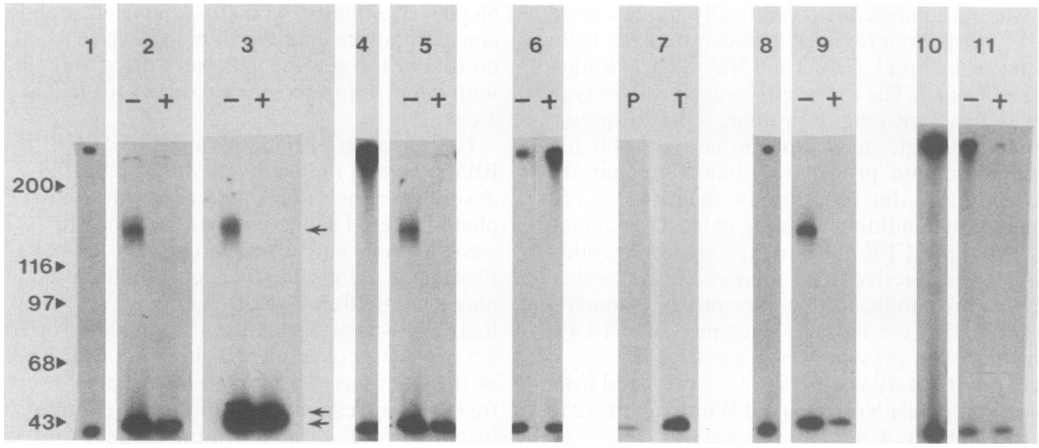

FIG. 1. Autoradiographs showing CPE complex analyzed by SDS-PAGE on 6% acrylamide gels. Samples were free ^{125}I-CPE in solution or radioactivity bound to BBMs (200 μg of protein) or Vero cells (2×10^6 cells) in the absence (−) or presence (+) of an excess of unlabeled CPE. All samples received an equal volume of SDS-PAGE sample buffer prior to electrophoresis. Samples were treated (see Materials and Methods) as follows: (1) free CPE treated with CHAPS for 15 min; (2) CPE bound to BBMs extracted with CHAPS; (3) CPE bound to Vero cells extracted with CHAPS; (4) free CPE treated with SDS for 15 min; (5) CPE bound to BBMs extracted with SDS; (6) free CPE incubated for 15 min with CHAPS extractions of BBMs (200 μg of protein); (7) free CPE treated with CHAPS for 15 min and then with Pronase (P) or trypsin (T) for 5 min; (8) free CPE treated with CHAPS for 15 min and then with 5% β-mercaptoethanol for 5 min; (9) CPE bound to BBMs extracted with CHAPS and then with 5% β-mercaptoethanol for 5 min; (10 and 11) treated same as samples 8 and 9, respectively, and then placed in a boiling water bath for 3 min. CPE complex (single arrow) and free CPE (double arrow) migrated as indicated. Positions of molecular size markers (in kilodaltons) are indicated on left side of figure. Samples showing ^{125}I-CPE bound to BBMs or Vero cells in the absence of unlabeled CPE (lanes −) contained about 3×10^4 cpm or 1/10th the total radioactivity per sample (see Materials and Methods). Corresponding free ^{125}I-CPE controls contained approximately the same amount of radioactivity. Similarly, 3×10^5 cpm of ^{125}I-CPE were added to BBM extracts (sample 6) and were treated with proteases (sample 7).

were performed on a video densitometer (model 620) with the 1-D Analyst program (Bio-Rad).

RESULTS AND DISCUSSION

Solubilization and partial characterization of ^{125}I-CPE complexes. Recently, we have reported (13) some evidence suggesting that CPE forms high-M_r complexes after binding to BBMs. To further determine whether CPE forms complexes in membranes, we used the system of Dreyfus and Robertson (1). In their assay system, membrane-bound ^{125}I-labeled *E. coli* heat-stable enterotoxin was CHAPS extracted and the solubilized radioactivity was then analyzed by SDS-PAGE without sample boiling and autoradiography. Using this method, they showed that ^{125}I-labeled *E. coli* heat-stable enterotoxin specifically binds to a single BBM component with a molecular weight of 100,000.

When similar experiments were performed with membrane-bound ^{125}I-CPE, a significant amount of radioactivity was associated with a high-molecular-weight material (Fig. 1, sample 2, lane −), which is referred to as CPE complex. The association of radioactivity with CPE complex was completely blocked when binding of ^{125}I-CPE was performed in the presence of an excess of unlabeled CPE (Fig. 1, sample 2, lane +), strongly suggesting that the formation of CPE complex was due to specific binding of ^{125}I-CPE to BBMs. CPE complex represented 60 to 80% of specifically bound radioactivity. The migration of CPE complex on SDS-PAGE corresponded to a molecular weight of about 160,000 (Fig. 1, sample 2, lane −). Similar results were obtained whether the ^{125}I-CPE complex was analyzed on 5, 7, or 8% acrylamide gels (data not shown).

In this SDS-PAGE system with 6% acrylamide gels, free ^{125}I-CPE incubated first in 1% CHAPS followed by SDS-PAGE sample buffer migrated at the dye front (Fig. 1, sample 1), with some aggregation at the top of the gel. Previous studies (2, 4, 13) indicated that SDS promotes CPE aggregation during SDS-PAGE. Therefore, material at the top of the gel in the CPE samples without membranes represents aggregated CPE (13). The difference in migration of free ^{125}I-CPE in solution versus membrane-bound ^{125}I-CPE after identical detergent treatment strongly suggests that formation of CPE complex results from the interaction between membranes and specifically bound CPE. Since both membrane-bound CPE and control CPE were incubated with 1% CHAPS prior to the addition of SDS-PAGE sample buffer, CPE complex formation is not due to the presence of CHAPS (see below).

The amount of radioactivity migrating at the dye front in the total binding lane for BBMs (Fig. 1, sample 2, lane −) appears to be somewhat greater than the amount of radioactivity migrating at the dye front in those lanes representing nonspecific binding (i.e., binding in the presence of an excess of unlabeled CPE; Fig. 1, sample 2, lane +). Approximately 40% of this dye front radioactivity represents specifically bound ^{125}I-CPE, as determined by densitometry. This difference in the amount of radioactivity may support a previous suggestion (13) that there are two forms of specifically bound CPE in membranes. The specifically bound material at the migration front may represent noncomplexed, specifically bound ^{125}I-CPE in the membranes, suggesting the existence of both noncomplexed and complexed specifically bound CPE in membranes. Alternatively, this result may indicate that some of the complex dissociates during electrophoresis. SDS-PAGE analysis of chromatographically partially purified ^{125}I-CPE complex showed less than 10% of the total radioactivity migrating at the dye front (data not shown), demonstrating that some dissociation of ^{125}I-

CPE from the complex can occur during SDS-PAGE. However, this amount of dissociated radioactivity appears insufficient to account for the amount of specifically bound ^{125}I-CPE migrating at the dye front (about 25% of total specifically bound ^{125}I-CPE migrates at the dye front). Also, previous studies (13) have shown that radioactivity remains associated with intact specifically bound ^{125}I-CPE for at least 1 h. This previous result indicates that specifically bound radioactivity at the dye front on the 6% gels does not represent degradation products in this experiment.

Interestingly, specifically bound radioactivity in Vero cells also appeared to be associated with a high-molecular-weight complex (Fig. 1, sample 3, lane −). The CPE complex in Vero cells was similar in size to that in BBMs. The relative ratio of radioactivity assocated with complexed versus total noncomplexed (i.e., specifically and nonspecifically bound low-M_r radioactivity) toxin was less in Vero cells than in BBMs. This may be explained by the higher level of nonspecific CPE binding in Vero cells compared with that in BBMs (see Materials and Methods). For example, most of the total noncomplexed radioactivity associated with Vero cells was nonspecifically bound (Fig. 1, sample 3, lanes − and +).

The formation of CPE complex was not dependent on the presence of CHAPS, since a similar radioactive complex was observed (Fig. 1, sample 5, lane −) following SDS extraction (i.e., no CHAPS present) of membrane-bound ^{125}I-CPE. Note again the difference in gel patterns between free ^{125}I-CPE (Fig. 1, sample 4) and ^{125}I-CPE complex (Fig. 1, sample 5, lane −), both of which were incubated in SDS only. These results with SDS extraction of CPE complex further support the hypothesis that membranes have an effect (i.e., complex formation) on CPE migration on SDS gels.

CPE complex formation was not discernible when ^{125}I-CPE was added to CHAPS extraction of BBM proteins in the absence or presence of an excess of unlabeled CPE (Fig. 1, sample 6, lane − and +, respectively). This result indicates that CPE complexes do not form following membrane extraction and strongly suggests that complex formation is membrane dependent.

When analysis by SDS-PAGE without sample boiling was used, high-M_r ^{125}I-CPE aggregation appeared to be diminished after CPE interacted with BBMs or Vero cells compared with aggregation of free ^{125}I-CPE in solution. It could be argued that CPE complex results simply from proteolytic degradation of CPE aggregates. However, specifically bound ^{125}I-CPE is not degraded in BBMs for at least 1 h (13). Further, when ^{125}I-CPE was treated with Pronase or trypsin (Fig. 1, sample 7, lanes P and T, respectively) in the presence of CHAPS, no complex formation was observed, providing additional evidence that the complex is not an artifact of protease degradation.

The stability of the ^{125}I-CPE complex was investigated. The CPE complex was stable in the presence of β-mercaptoethanol (Fig. 1, sample 9, lane −). However, when the CHAPS-extracted material was treated with β-mercaptoethanol and then boiled prior to electrophoresis (Fig. 1, sample 11, lane −), the amount of CPE complex was always significantly reduced, although the extent of the reduction showed some variability between experiments. Under these conditions, most of the radioactivity migrated similarly to free ^{125}I-CPE in solution (Fig. 1, sample 10) subjected to SDS-PAGE. These results indicate that CPE complex is heat sensitive and suggest that CPE in the complex is not covalently cross-linked to itself or other proteins.

When CHAPS-extracted ^{125}I-CPE complex was chromatographed on Sepharose 4B (Fig. 2A), a molecular weight of 170,000 was determined by the method of Whitaker (23). Note that CPE complex is relatively stable and remains intact during Sepharose 4B chromatography even though no CHAPS or SDS is present during this procedure (i.e., the column buffer does not contain any detergent). Since gel filtration is often used to remove detergents from protein samples, this result suggests that CPE complex is stable even in the absence or reduced presence of detergents and, therefore, is not the result of mixed micelle formation. Also, CPE complex analyzed by chromatography was never treated with SDS, indicating that complex formation is not SDS dependent or an SDS-induced artifact. The elution profile of nonspecifically bound CPE (Fig. 2B) is consistent with CPE complex formation depending on the specific binding of ^{125}I-CPE to BBMs. Similar to the results shown in Fig. 1, CPE complex formation was not detectable by gel filtration with CHAPS-treated ^{125}I-CPE in solution alone (Fig. 2C) or when ^{125}I-CPE was added to CHAPS extractions of BBMs (Fig. 2D).

Kinetics and divalent-cation requirements for CPE complex formation. Recent studies (13) have shown that CPE binding to either BBMs or Vero cells is concentration dependent, rapid, and irreversible. Early effects of CPE on membrane permeability occur almost simultaneously with binding of CPE to Vero cells (10, 13). Recent studies (5, 6, 9, 13) have demonstrated both Ca^{2+}-independent and Ca^{2+}-dependent steps in CPE action. Based on current knowledge, early events in CPE action are thought to be Ca^{2+} independent, while later steps are believed to be Ca^{2+} dependent (11). The kinetics and divalent-cation (Ca^{2+} and Mg^{2+}) requirements of CPE complex formation were studied to determine the correlation with specific binding. CPE complex formation in BBMs appeared to be rapid (formation was detectable within 15 s of the addition of ^{125}I-CPE to BBMs) and increased progressively with time (Fig. 3), correlating with the rate and level of specific binding (13). Also, formation of CPE complex did not require the presence of Ca^{2+} or Mg^{2+} during BBM treatment with ^{125}I-CPE, as would be expected for an early event in CPE action (11).

Partial purification of BBM and Vero cell ^{125}I-CPE complexes. Preliminary studies were done to examine whether CPE complex in membranes represented either oligomerized CPE or CPE associated with a eucaryotic membrane protein(s). Electrophoretically partially purified CPE complexes from both BBMs and Vero cells and the corresponding regions of control gels (no CPE present in samples loaded onto gels) were analyzed by SDS-PAGE with sample boiling. Coomassie brilliant blue staining of the gels showed a number of proteins present in the partially purified samples (Fig. 4). Only proteins with molecular weights of 50,000 and 70,000 were consistently present in all experiments in both BBM (Fig. 4, lane B) and Vero cell (Fig. 4, lane E) complex samples. These proteins appeared to be present in approximately equimolar amounts. The 50-kilodalton (kDa) band was always absent and the 70-kDa band was absent from most (six of eight repetitions) of the control gels of BBMs (Fig. 4, lane C). Both the 50- and 70-kDa bands were always absent in the control gels of Vero cells (Fig. 4, lane F). No band corresponding to the M_r of CPE (35,000) was visible on these gels following Coomassie brilliant blue staining. When these gels were autoradiographed (data not shown), radioactivity aggregation typical of previous SDS-PAGE studies (13) of ^{125}I-CPE in membranes was apparent. No radioactiv-

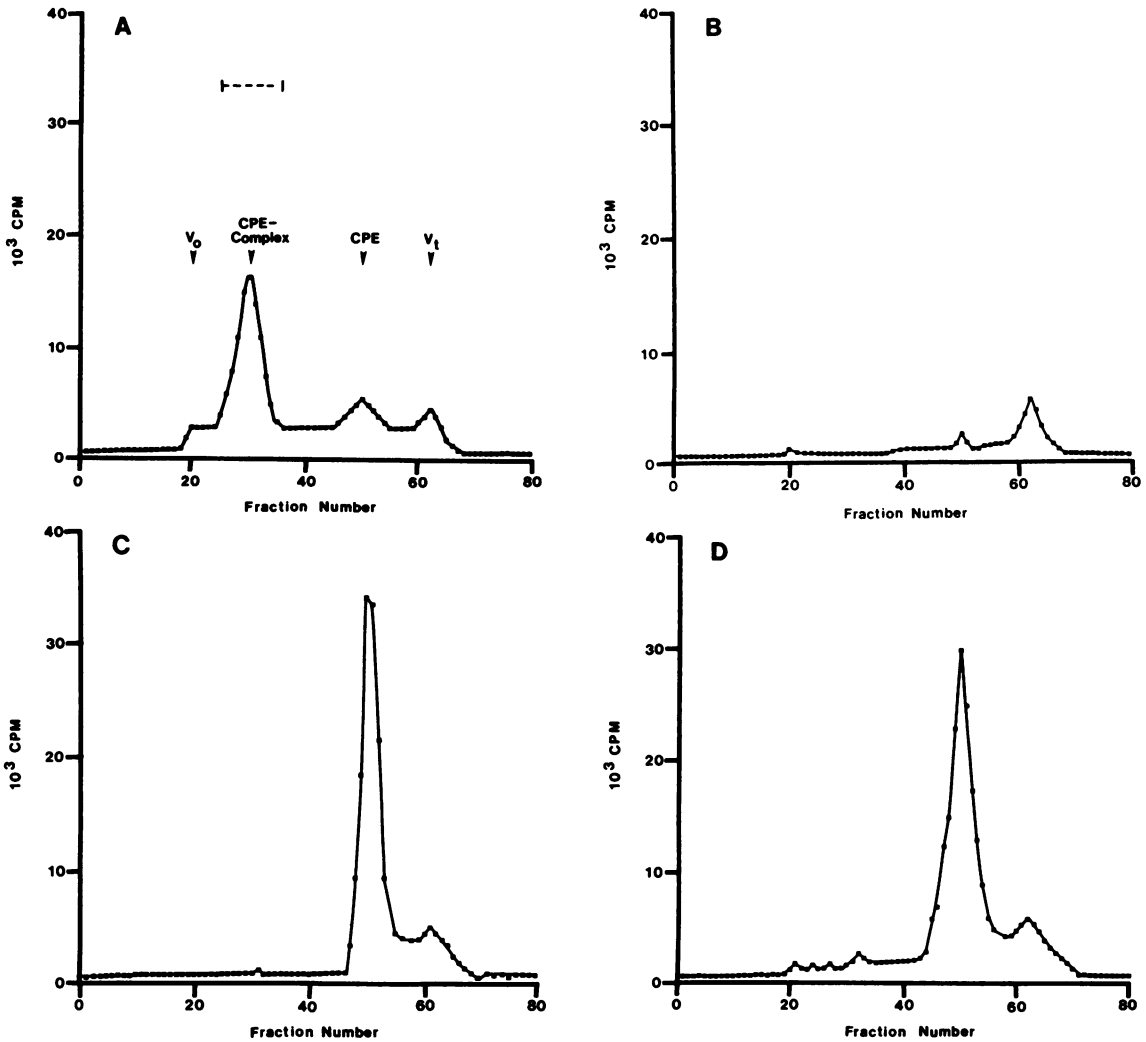

FIG. 2. Sepharose 4B column elution profiles. (A) CHAPS-extracted total ^{125}I-CPE bound to BBMs (200 μg of protein) (unlabeled CPE absent during binding); (B) CHAPS-extracted ^{125}I-CPE nonspecifically bound to BBMs (unlabeled CPE present during binding); (C) ^{125}I-CPE in solution (BBMs absent); (D) ^{125}I-CPE incubated with CHAPS extractions of BBMs (200 μg of protein) for 15 min. Radioactivity added to samples C and D was equivalent to radioactivity bound in sample A. The entire sample was then loaded onto the column: (A, C, and D) 250,000 cpm; (B) 90,000 cpm. Dashed line corresponds to area showing CPE complex when analyzed by SDS-PAGE without sample boiling. Void volume (V_0) was determined with blue dextran; total volume (V_t) was determined with free ^{125}I (see Materials and Methods).

ity was specifically present in the 50- or 70-kDa bands, indicating that these bands are not ^{125}I-CPE aggregates.

Affinity chromatography. Affinity chromatography was used to further examine the interaction of BBM proteins with CPE (Fig. 5). As shown previously (24), a 50-kDa protein from BBMs specifically binds to CPE following affinity chromatography of Tergitol NP-40 extractions (Fig. 5, lanes 4 and 5). Interestingly, when CHAPS extractions were subjected to affinity chromatography (Fig. 5, lanes 3 and 6), a specific interaction between a 70-kDa protein and CPE could be demonstrated. Depending on the extraction method used on native membranes, a specific interaction of both 50- and 70-kDa proteins with CPE can be shown (Fig. 5). These proteins comigrated identically with 50- and 70-kDa complex proteins (lanes 1 and 2), and affinity chromatography with CPE columns specifically enriched for these proteins compared with their presence in BBMs (lane 7; close inspection of the original gel indicated that 50-kDa protein in lanes 2 and 4 migrated to a position intermediate between the two heavily staining bands of approximately 50,000 M_r in lane 7). These results are consistent with both of these proteins interacting with CPE and being present in the CPE complex. Also, affinity chromatography buffers used in experiments shown in Fig. 5 did not contain detergents, again suggesting that the interaction of the 50- and 70-kDa proteins with CPE is not due to the presence of these proteins in mixed micelles during chromatography.

The observations that complex forms rapidly after CPE treatment of cells or membranes and that a CPE complex of similar size is formed in both Vero cells and BBMs support the biologic importance of complex in CPE action. Previous studies (12, 15, 22) have indicated that both Vero cells and intestinal epithelial cells are highly sensitive to CPE and have strongly suggested that CPE has a similar action on both cell types. The rapid membrane-dependent formation of a complex of similar size (and possibly containing similar eucaryotic proteins) is also consistent with CPE action being similar in both cell types.

Interestingly, Vero cells and intestinal epithelial cells specifically bind similar amounts of CPE at saturation (15),

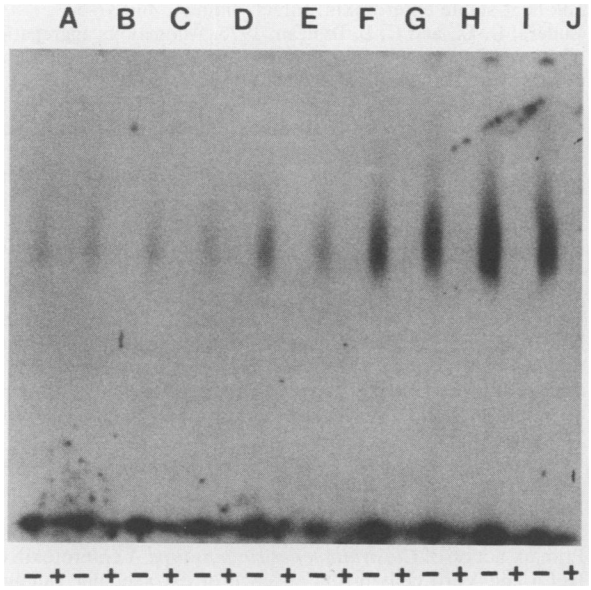

FIG. 3. Kinetics and divalent cation requirement for ^{125}I-CPE complex formation. ^{125}I-CPE (5 μg) was incubated with BBMs in Dulbecco phosphate-buffered saline (200 μg of protein) with (samples A, C, E, G, and I) or without (samples B, D, F, H, and J) Ca^{2+} and Mg^{2+} in the absence (−) or presence (+) of an excess of unlabeled CPE. At the indicated times, samples (50 μl) were pelleted, washed, treated with SDS, and then analyzed by SDS-PAGE without sample boiling on 6% acrylamide gels, followed by autoradiography. Incubation times were as follows: lanes A and B, 0.25 min; lanes C and D, 0.5 min; lanes E and F, 1.0 min; lanes G and H, 2.5 min; and lanes I and J, 10 min. ^{125}I-CPE bound to BBMs in the absence of unlabeled CPE represents about 15,000 cpm at 0.25 min and increases across the gel to approximately 35,000 cpm at 10 min. The entire sample was loaded onto the gel.

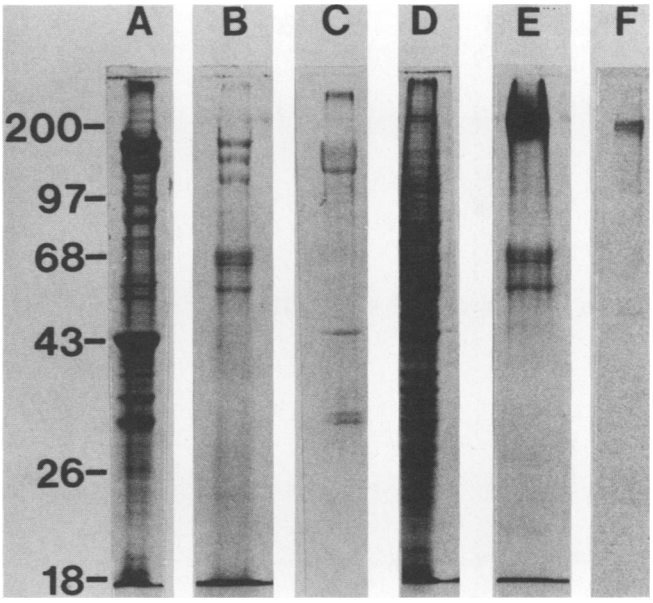

FIG. 4. Comparison of CPE complex composition from BBMs and Vero cells by SDS–10% PAGE with sample boiling. Coomassie brilliant blue-stained gel shows the following: lane A, total BBM proteins (50 μg); lane B, CPE complex from BBMs (18 μg); lane C, electroeluted control (no CPE) gel from BBMs (15 μg); lane D, total Vero cell proteins (48 μg); lane E, CPE complex from Vero cells (23 μg); and lane F, electroeluted control (no CPE) gel from Vero cells (10 μg). Positions of molecular size markers (in kilodaltons) are indicated on left side of figure.

and the CPE receptor on both Vero cells and intestinal epithelial cells is, at least partially, proteinaceous (13, 15, 25). A rabbit BBM protein of 50,000 M_r has been identified by affinity chromatography as a putative CPE receptor (24), but the receptor for Vero cells remains unknown. In our current study, two eucaryotic proteins of 70 and 50 kDa were identified which appeared to be associated with CPE complex in membranes. One interpretation of these data is that the 50-kDa complex protein is the same protein identified previously as the 50-kDa putative CPE receptor (24). This hypothesis is supported by the identical comigration of the 50-kDa complex protein and 50-kDa affinity-purified protein on the same gel (Fig. 5). If this hypothesis proves correct, it might indicate that the Vero cell CPE receptor is similar or identical to the BBM CPE receptor. Further studies are essential to examine the relationship between the 50-kDa complex protein and the 50-kDa putative CPE receptor and to directly prove a role for the 50-kDa protein in CPE binding and complex formation. It remains conceivable that the 50-kDa putative CPE receptor which binds CPE has another function in addition to (or instead of) a role in CPE binding.

Interpretation of the role or function of the 70-kDa protein is also unclear at present. Possibilities include the following: (i) the 70-kDa polypeptide is a second subunit of a large CPE receptor; (ii) the 70-kDa polypeptide is a second surface protein which interacts with CPE (or the CPE receptor) after CPE binds to the CPE receptor; or (iii) the 70-kDa protein is an integral membrane protein which interacts with CPE and the CPE receptor after CPE binding and insertion into membranes. Another possible role of the 70-kDa protein involves this protein as a second class of CPE receptor. This seems less likely since kinetic analysis (13, 25) suggests only a single class of CPE receptor.

Elucidation of the role of the 70-kDa protein in CPE action could also prove critical to a molecular understanding of CPE action. However, the membrane events in CPE action may prove complicated and difficult to study. For example, some evidence (13; this study) suggests that specifically bound CPE exists in two forms in membranes, as either noncomplexed CPE or complexed CPE. This might indicate that once inserted into membranes, some dissociation of CPE from the CPE complex occurs. The relationship between these two forms of CPE and the relative importance of each form for biologic activity remains to be determined. The relationship between the 70- and 50-kDa proteins and CPE insertion into membranes also must be determined. The results shown in Fig. 3 indicating that complex formation increases with time and that complex remains intact at times after CPE insertion occurs (13) suggest that the eucaryotic proteins associated with the complex are also inserted, at least transiently. It remains to be determined whether the 50- and 70-kDa proteins are integral or surface membrane proteins.

The simplest explanation for the CPE complex data is that one CPE molecule (35,000 M_r) is present together with one 50- and one 70-kDa eucaryotic protein to form a complex of approximately 160 kDa. However, other possible explanations of the complex data cannot be eliminated by present information. For example, the 50-kDa protein may be a degradation product of the 70-kDa protein, which suggests multivalent CPE binding to a 70-kDa receptor (approximately two CPE molecules would bind to a 70-kDa protein).

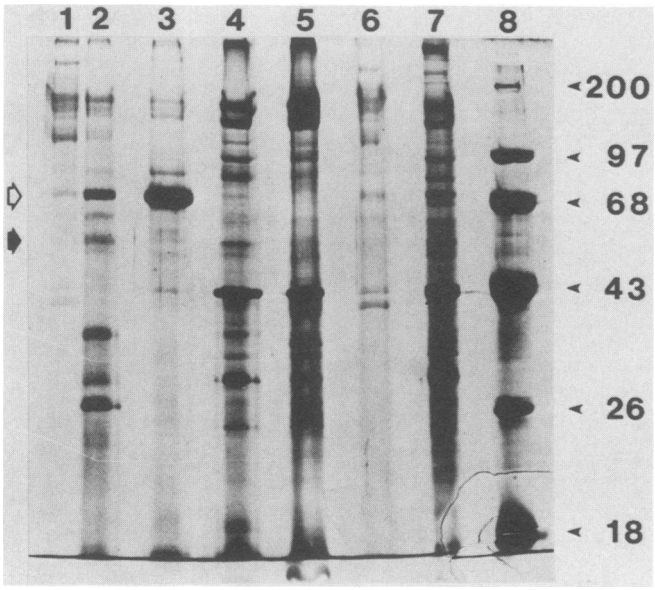

FIG. 5. CPE complex proteins from BBMs partially purified by SDS-PAGE without sample boiling compared with CPE-binding proteins from BBMs partially purified by affinity chromatography. Silver-stained gel shows the following: lane 1, electroeluted control (no CPE) gel (8 μg); lane 2, CPE complex (12 μg); lane 3, affinity chromatography partially purified CPE-binding protein (note 70-kDa protein) from CHAPS extractions (11 μg); lane 4, affinity chromatography partially purified CPE-binding protein (note 50-kDa protein) from Tergitol NP-40 extractions (18 μg); lane 5, control affinity column preparation from Tergitol NP-40 extractions (15 μg); lane 6, control affinity column preparation from CHAPS extractions (8 μg); and lane 7, total BBM proteins (14 μg). Positions of 70-kDa protein (open arrow) and 50-kDa protein (closed arrow) are shown on left side of figure. Positions of molecular size markers (in kilodaltons) are indicated on right side of figure and in lane 8.

The affinity chromatography data obtained by rapid analytic procedures at 4°C argue against, but do not disprove, this possibility. Alternatively, the CPE complex may represent oligomerization of CPE after membrane exposure. In this view, the 50- and 70-kDa eucaryotic proteins copurifying with complex would be simply contaminants. This possibility seems less likely, however, because (i) the hypothetical contaminants are virtually identical from both Vero cells and BBMs even though the starting cell or membrane lysates are very dissimilar (Fig. 4) and (ii) by affinity chromatography, specific interactions can be shown between CPE and proteins of identical size to complex-associated proteins. However, the relationship between the 50- and 70-kDa eucaryotic proteins and CPE complex must be proved in further studies.

In summary, our research indicates that a substantial percentage of CPE bound to membranes is associated with a complex of 160,000 M_r. Understanding of the significance of this complex is currently incomplete but may prove important to understanding CPE-membrane interactions.

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