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Evidence that a Region(s) of the *Clostridium perfringens* Enterotoxin Molecule Remains Exposed on the External Surface of the Mammalian Plasma Membrane When the Toxin Is Sequestered in Small or Large Complexes

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In studies performed to investigate the topology of *Clostridium perfringens* enterotoxin (CPE) when this toxin is associated with intestinal brush border membranes (BBMs), it was shown that radiolabeled CPE antibodies react more strongly against intact CPE-treated BBMs than against control BBMs. Immunoprecipitation studies then demonstrated that CPE antibodies are able to react with both small and large CPE-containing complexes while these complexes are still present in intact BBMs. Therefore, at least a portion of the CPE molecule appears to remain surface exposed in BBMs throughout the action of this toxin.

Clostridium perfringens enterotoxin (CPE), a single 35-kDa polypeptide, produces symptoms associated with *C. perfringens* type A food poisoning and, possibly, other diseases of humans and domesticated animals (12, 13, 18). The cytotoxic action of CPE appears to involve a unique series of four early plasma-membrane-associated events, including the following: (i) the binding of CPE to a 50-kDa mammalian membrane protein receptor, resulting in formation of a small 90-kDa complex (23); (ii) the development of a postbinding physical change to this small complex (see discussion below); (iii) the formation of a large complex (160 kDa), resulting from an interaction between the physically changed small complex and a 70-kDa membrane protein (16, 23, 24); and (iv) the development of extensive plasma membrane permeability alterations for small (<200-kDa) molecules (9, 11, 13, 14), which leads to secondary effects such as morphologic damage and shutdown of macromolecular synthesis (7, 13, 15).

Although the precise molecular mechanism by which CPE affects membrane permeability remains unclear, it has been hypothesized (13, 19, 23) that CPE resembles the pore-forming bacterial toxins (1–3) and inserts itself into plasma membranes as part of the postbinding physical change that takes place in CPE small complex. Circumstantial evidence supporting this possibility includes the following: (i) with time, specifically bound CPE becomes progressively more difficult to release from membranes by use of externally applied proteases (17, 19); (ii) specifically bound CPE does not dissociate, even when CPE-containing membranes are treated with agents known to release peripherally bound proteins (17, 19); (iii) CPE association with membranes shows a biphasic pattern consistent with CPE binding being rapidly followed by a physical event such as insertion (19); and (iv) specifically bound CPE acquires the amphiphilic characteristics expected of an insertion-capable toxin (23).

Although CPE clearly interacts with plasma membranes during its action, surprisingly little information regarding the topology of membrane-associated CPE is available. Since antibodies have proven to be useful probes to study the membrane topology of other insertion-capable toxins (4, 6, 26), the current study has used similar antibody probe techniques to explore whether "inserted" CPE, i.e., CPE that has undergone the postbinding physical change occurring after small-complex formation, remains exposed on brush border membrane (BBM) surfaces at 4°C (where only a small complex forms in BBMs) and 22°C (where both small and large complexes form in BBMs).

Before performing antibody probe experiments, it was important to confirm that CPE insertion could be demonstrated with our current reagents. For this purpose, a previously described phenomenon (17, 19), i.e., the ability of specifically bound CPE to develop resistance to protease-induced release from BBMs, was measured as an indicator of CPE insertion. For these experiments, electrophoretically pure CPE was prepared and radioiodinated as described previously (17, 21). BBMs (100 μg) were incubated, in the presence or absence of a 50-fold excess of unlabeled CPE (for calculating ^{125}I-CPE specific binding, see below), with this ^{125}I-CPE (either 0.75 μg for 4°C experiments or 0.5 μg for 22°C experiments) for 30 min at 4°C or for 20 min at 22°C. (Higher ^{125}I-CPE concentrations and longer incubation times were used for 4°C samples throughout the current study to partially compensate for reduced CPE binding activity at 4°C [16] and thereby increase the probability that BBMs treated with CPE at 4°C would contain sufficient amounts of bound CPE to allow detection with antibody probes.) ^{125}I-CPE specific binding levels were then calculated for each experimental parameter by subtracting radioactive counts bound to BBMs in the presence of 50-fold excess unlabeled CPE (i.e., counts which represent nonspecific ^{125}I-CPE binding) from radioactive counts bound to BBMs under the same incubation conditions except for the absence of unlabeled CPE (i.e., counts which represent total ^{125}I-CPE binding), as described previously (23). The percentage of this specifically bound CPE that was inserted, i.e., protected from protease-induced release from BBMs, was then determined for both 4°C and 22°C samples, as described previously (17, 19). The following should be noted: (i) after completion of the binding incubation and several washings with

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phosphate-buffered saline (PBS) (23), CPE-containing BBMs were further incubated for 5 min at either 4°C or 22°C (as used for binding) before the addition of proteases, in order to ensure the completion of CPE insertion (17, 19); (ii) for each sample, all further manipulations (e.g., washings and protease treatments) were conducted at the same temperature used initially for ^{125}I-CPE binding; and (iii) higher concentrations of proteases were used for 4°C samples than for 22°C samples, as described previously (16, 17, 19), to obtain approximately equivalent protease activities at both incubation temperatures.

Consistent with previous studies (16), ^{125}I-CPE insertion was detectable at both 4 and 22°C with our current reagents (see Fig. 1, parameters 2 and 3) since (i) 80 and 87% of specifically bound ^{125}I-CPE remained membrane associated after CPE-containing BBMs were treated at 4°C with Pronase or trypsin, respectively, and (ii) 68 and 83% of specifically bound ^{125}I-CPE remained membrane associated when CPE-containing BBMs were treated at 22°C with Pronase or trypsin, respectively. This protection of inserted CPE is not explainable by the use of inactive proteases since pretreating BBMs with the same trypsin or Pronase preparations used for the insertion experiments resulted in a sharp reduction in the ability of BBMs to bind ^{125}I-CPE subsequently; i.e., compared with control BBMs, trypsin- or Pronase-pretreated BBMs specifically bound 50 and 80% less ^{125}I-CPE, respectively, at 4°C and 59 and 88% less ^{125}I-CPE, respectively, at 22°C (see Fig. 1, parameters 4 and 5).

Radiolabeled antibodies react with BBMs containing CPE. Having confirmed that our current reagents would reproduce CPE insertion, it was appropriate to determine whether CPE antibodies react more strongly with CPE-treated BBMs than with control BBMs, as would be expected if some membrane-associated CPE remains surface exposed. For these studies, immunoglobulin G (IgG) was purified from either rabbit polyclonal anti-CPE serum (RPC-IgG), prepared as described previously (10), or normal rabbit serum (NRS-IgG), purchased from Cappel Inc. (Durham, N.C.), with an Immuno Pure IgG purification kit (Pierce, Rockford, Ill.). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis confirmed the purity of the resultant IgG preparations (data not shown). Purified RPC-IgG or NRS-IgG was radioiodinated with Enzymobeads (Bio-Rad, Hercules, Calif.) as described previously (25). Radioimmunoassays (RIA) (25) confirmed (data not shown) that ^{125}I-RPC-IgG retained specific reactivity for CPE on the basis of the following. (i) ^{125}I-RPC-IgG binding levels correlated with the amount of CPE present per RIA well, while no significant ^{125}I-NRS-IgG binding occurred with RIA wells containing even large amounts (2 μg) of CPE. (ii) ^{125}I-RPC-IgG binding to CPE-containing RIA wells could be abrogated by the addition of a 50-fold excess of unlabeled RPC-IgG but not by adding a 50-fold excess of NRS-IgG. (iii) Neither ^{125}I-RPC-IgG nor ^{125}I-NRS-IgG reacted with RIA wells containing bovine serum albumin (6 μg).

When these radiolabeled antibodies became available, BBMs containing inserted CPE were prepared at either 4°C (where only a small complex forms in BBMs [23]) or 22°C (where both small and large complexes form in BBMs [23]), as described for Fig. 1 experiments. After several PBS washings, these BBMs were probed with 2 μg of either ^{125}I-RPC-IgG or ^{125}I-NRS-IgG, in the presence or absence of a 50-fold excess of unlabeled homologous or heterologous antibody, for either 30 min at 4°C or 20 min at 22°C (both washings and antibody treatments were performed at the same temperature initially used for CPE binding). Following several more PBS washes at 4 or 22°C (as used for CPE binding), radioactivity associated with BBM pellets was determined with a gamma counter. Results from these experiments indicate that ^{125}I-RPC-IgG was twofold or greater than fivefold more reactive against CPE-containing BBMs (parameter 2) than against control BBMs (parameter 1) at 4°C (Fig. 2A) or 22°C (Fig. 2B), respectively.

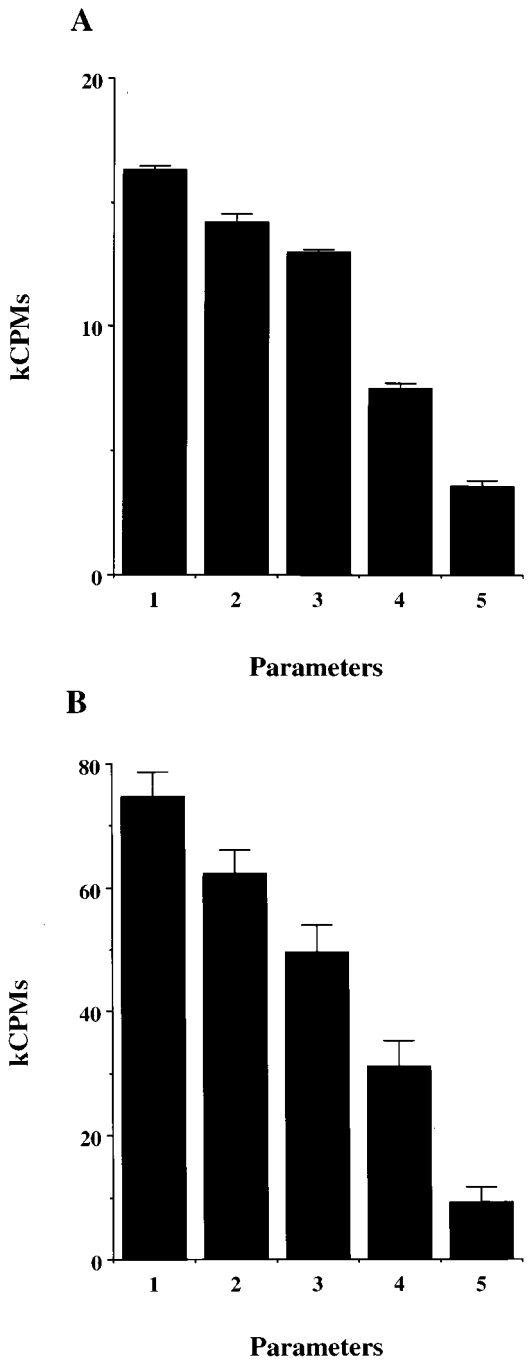

FIG. 1. Demonstration of ^{125}I-CPE specific binding and insertion at 4°C (A) and 22°C (B). Numerical parameters 1 to 5 represent ^{125}I-CPE specific binding to BBMs (parameter 1), specifically bound radioactivity that remains BBM-associated after ^{125}I-CPE-containing BBMs, prepared as described for parameter 1, were either trypsin treated (parameter 2) or Pronase treated (parameter 3), and ^{125}I-CPE specific binding to BBMs that had been pretreated with an aliquot of the same preparation of trypsin used with parameter 2 samples (parameter 4) or with the same preparation of Pronase used with parameter 3 samples (parameter 5), before the addition of ^{125}I-CPE. Results show means obtained from triplicate samples in three independent experiments, while error bars represent standard deviations.

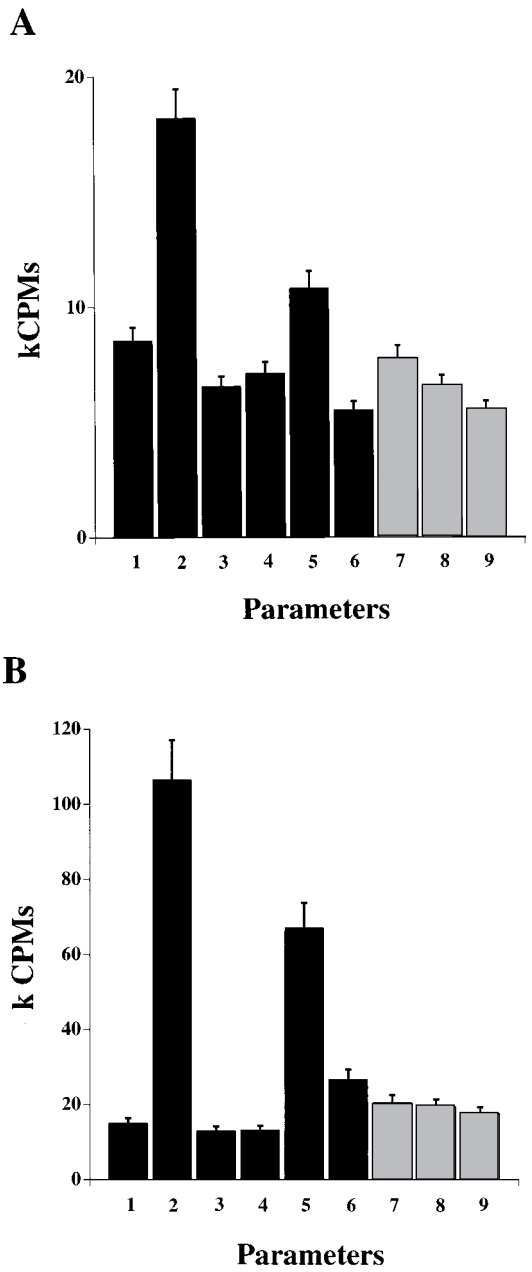

FIG. 2. Binding of ^{125}I-RPC-IgG (solid bars) or ^{125}I-NRS-IgG (shaded bars) to BBMs containing CPE bound and inserted at either 4°C (A) or 22°C (B). Numerical parameters 1 to 9 represent binding of 2 μg of the specified radiolabeled antibody to (i) control BBMs alone (parameters 1 and 7), (ii) BBMs containing inserted CPE (parameters 2 and 8), (iii) same as ii except CPE-containing BBMs were treated with the specified radiolabeled antibody in the presence of a 50-fold excess of either unlabeled RPC-IgG (parameter 3) or unlabeled NRS-IgG (parameter 9), (iv) same as ii except ^{125}I-RPC-IgG was preincubated with a 50-fold excess of CPE (parameter 4), and (v) BBMs pretreated with either trypsin (parameter 5) or Pronase (parameter 6) prior to being CPE treated and then incubated with ^{125}I-RPC-IgG. Results shown represent mean values obtained from triplicate samples in three independent experiments, while error bars represent standard deviations.

This increased ^{125}I-RPC-IgG reactivity for CPE-containing BBMs appears to involve a specific interaction between the radioiodinated antibodies and CPE present in these BBMs on the basis of the following. (i) The addition of a 50-fold excess of unlabeled RPC-IgG (Fig. 2, parameter 3) but not a 50-fold excess of unlabeled NRS-IgG (data not shown) reduced ^{125}I-RPC-IgG reactivity for CPE-containing BBMs to the same level as that observed between ^{125}I-RPC-IgG and control BBMs. (ii) No differences were detected between ^{125}I-NRS-IgG reactivity for CPE-containing BBMs and that for control BBMs (Fig. 2, parameters 7 and 8). (iii) Preincubating ^{125}I-RPC-IgG for 20 min with a 50-fold excess of native CPE reduced the reactivity of this ^{125}I-RPC-IgG for CPE-containing BBMs (Fig. 2, parameter 4) to the same level as that observed between ^{125}I-RPC-IgG and control BBMs.

Quantitative analysis of the enhanced ^{125}I-RPC-IgG reactivity for CPE-containing BBMs compared with that for control BBMs was performed to evaluate whether this enhanced reactivity involves recognition of at least some specifically bound CPE present in the CPE-treated BBMs (it is important to note that only specifically bound CPE produces cytotoxic effects [5, 20, 25]). Under the binding conditions used, it can be calculated (from Fig. 1A and CPE radioiodination data not shown) that our BBM samples contained ~45 ng of specifically bound and ~8 ng nonspecifically bound CPE at 4°C. Since RIA results (data not shown) indicate that 8 ng of free CPE binds 6.5 kcpm of ^{125}I-RPC-IgG at 4°C, yet >10 kcpm more ^{125}I-RPC-IgG was shown to bind to CPE-containing BBMs (Fig. 2A, parameter 2) compared with that of control BBMs (Fig. 2A, parameter 1) at 4°C, it can be concluded that at least some ^{125}I-RPC-IgG must be reacting with specifically bound CPE in the 4°C samples. Similarly, while the BBMs treated with CPE at 22°C were calculated to contain ~210 ng of specifically bound and ~50 ng of nonspecifically bound CPE on the basis of Fig. 1B results and radioiodination data (not shown) and RIA analysis indicated that 50 ng of free CPE binds ~30 kcpm of ^{125}I-RPC-IgG at 22°C (data not shown), Fig. 2B results show that our CPE-containing BBMs (parameter 2) actually bound >90 kcpm more ^{125}I-RPC-IgG than control BBMs (parameter 1) at 22°C. That is, more antibody reactivity was detected at 22°C than would be expected if CPE antibodies were recognizing only the nonspecifically bound CPE present in these BBMs.

Further support for the increase in ^{125}I-RPC-IgG reactivity for CPE-containing BBMs compared with that of control BBMs involving, at least in part, recognition of specifically bound CPE was obtained when it was demonstrated that pretreating BBMs with trypsin or Pronase before the addition of CPE reduced subsequent ^{125}I-RPC-IgG binding to these BBMs by 40 and 70% (Fig. 2A, parameters 5 and 6), respectively, at 4°C compared with that of CPE-treated BBMs that had not been pretreated with proteases (Fig. 2A, parameter 2). This reduction correlates well with the 55 and 78% reductions in ^{125}I-CPE specific binding observed (Fig. 1A, parameters 4 and 5) at 4°C for trypsin- or Pronase-pretreated BBMs, respectively (note that protease pretreatment of BBMs has little effect on nonspecific CPE binding at either 4 or 22°C [data not shown and reference 17]). Similarly, pretreating BBMs with trypsin or Pronase at 22°C, before the addition of CPE, reduced ^{125}I-RPC-IgG binding to these BBMs by 37 and 75% (Fig. 2B, parameters 5 and 6), respectively, compared with that of CPE-treated BBMs that had not been protease pretreated (Fig. 2B, parameter 2), a result that again correlates with the 59 and 88% reductions in ^{125}I-CPE specific binding observed (Fig. 1B, parameters 4 and 5) at 22°C when BBMs were pretreated with trypsin or Pronase, respectively. The inhibition of either ^{125}I-CPE specific binding to protease-pretreated BBMs (Fig. 1) or ^{125}I-RPC-IgG reactivity for CPE-treated, protease-pretreated BBMs (Fig. 2) is not explainable by residual protease activity remaining in BBM samples after the proteases had been removed and the pretreated BBMs had been washed

on the basis of the following findings. (i) Azocoll protease assays (16) detected no extracellular protease activity remaining in washed, protease-pretreated BBMs. (ii) Incubating either ^{125}I-CPE or ^{125}I-RPC-IgG with the washed, protease-pretreated BBMs did not affect the ability of these radiolabeled molecules to bind subsequently to control BBMs or to CPE-containing BBMs, respectively (data not shown).

RPC-IgG interacts directly with membrane-bound CPE sequestered in either a small or large complex. These antibody reactivity studies suggest that some specifically bound and inserted CPE remains surface localized when this toxin is sequestered in a small complex since enhanced ^{125}I-RPC-IgG reactivity was detected for BBMs treated with CPE at both 4°C (where only a small complex forms [23]) and 22°C (where both small and large complexes form [23]). However, the Fig. 2 experiments have not resolved whether CPE also remains surface exposed when sequestered in CPE large complex. Therefore, to determine more definitively when in its action the CPE molecule remains surface exposed, immunoprecipitation studies were also performed. For these experiments, ^{125}I-CPE, in the presence or absence of a 50-fold excess of unlabeled CPE (to identify membrane species containing specifically bound CPE), was added to BBMs at either 4 or 22°C, as described for Fig. 1 experiments. These intact BBMs containing inserted ^{125}I-CPE were treated with 10 μg of either RPC-IgG or NRS-IgG (as described for Fig. 2 experiments). After several washings, the BBMs were solubilized with PBS containing 0.1% Triton X-100 for 30 min (for 22°C samples) or with 1% Triton X-100 in PBS for 20 min (for 4°C samples). A 1:1 slurry of Sepharose-protein A beads (Sigma Chemical Co., St. Louis, Mo.) in PBS was then added to BBM extracts for 1 h (it is important to note that, for each sample, antibody incubations, washings, solubilizations, and protein A bead incubations were performed at the same temperature used for ^{125}I-CPE binding). Samples were then microcentrifuged to remove the protein A beads, and 40 μl of the resultant immunoprecipitation supernatant was mixed with an equal volume of either native sample buffer (23) (for 4°C samples) or SDS-PAGE sample buffer (24) (for 22°C samples). Samples in native sample buffer were then electrophoresed to visualized the small complex (23), while samples in SDS-PAGE buffer were electrophoresed (without sample boiling) to visualize the large complex (24). After electrophoresis, the gels were dried and autoradiographed on X-Omat X-Ray film (Kodak, Rochester, N.Y.) for 16 to 20 h at −70°C.

Given this immunoprecipitation procedure, radioactive species disappearing from supernatants (i.e., disappearing from the gel lanes) correspond to immunoprecipitated ^{125}I-CPE-containing species that had been surface exposed when still present in intact BBMs. By this criterion, immunoprecipitation analysis independently confirms the Fig. 2 results suggesting that the ^{125}I-CPE-containing small complex appears to be surface exposed, since all of the ^{125}I-CPE-containing small complex disappeared from supernatants of ^{125}I-CPE-containing BBM samples that were treated with RPC-IgG (Fig. 3, lane +RPC-IgG, −) prior to being extracted with Triton X-100 and incubated with protein A beads. This immunoprecipitation apparently involves a specific interaction between the small complex and RPC-IgG since no similar decrease in radioactivity was detected in supernatants when (i) ^{125}I-CPE-containing BBM samples were treated with NRS-IgG prior to Triton X-100 extraction and incubation with protein A beads (Fig. 3, lanes +NRS-IgG) or (ii) non-antibody-treated, ^{125}I-CPE-containing BBM samples were Triton X-100 extracted and directly incubated with protein A beads (Fig. 3, lane ProA Beads Only).

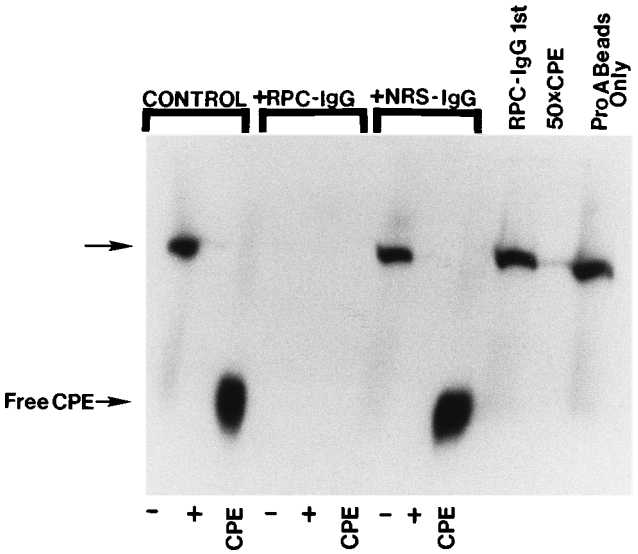

FIG. 3. Triton X-100 native gel electrophoretic analysis of immunoprecipitation supernatants from BBMs treated with ^{125}I-CPE at 4°C. ^{125}I-CPE, in the presence (+) or absence (−) of a 50-fold excess of unlabeled CPE, was bound and inserted into BBMs at 4°C. These BBMs were treated with RPC-IgG or NRS-IgG (see text) and then extracted with 1% Triton X-100. After incubation of extracts with protein A beads, the samples were microcentrifuged to remove immunoprecipitated materials, and 40 μl of each remaining supernatant was analyzed by Triton X-100 electrophoresis to identify the CPE small complex (23). Free ^{125}I-CPE (i.e., no BBMs) in PBS containing 1% Triton X-100 was included as a control in all experimental sets described below (lanes labeled CPE). Lanes: Control, a set of microcentrifugation supernatants from ^{125}I-CPE-treated BBM samples that was extracted with 1% Triton X-100 and then directly microcentrifuged; +RPC-IgG and +NRS-IgG, microcentrifugation supernatants from two sets of ^{125}I-CPE-treated BBM samples that were incubated with RPC-IgG or NRS-IgG, respectively, prior to Triton X-100 extraction, incubation with protein A beads, and microcentrifugation; RPC-IgG 1st, microcentrifugation supernatant from a BBM sample that was preincubated with RPC-IgG, washed, and then treated with ^{125}I-CPE before being extracted with 1% Triton X-100, incubated with protein A beads, and microcentrifuged; 50× CPE, microcentrifugation supernatant from an ^{125}I-CPE-treated BBM sample that was incubated with RPC-IgG and then Triton X-100 extracted in the presence of a 50-fold excess of unlabeled CPE, prior to being incubated with protein A beads and microcentrifuged; and ProA Beads Only, microcentrifugation supernatant from a ^{125}I-CPE-treated BBM sample that was extracted with Triton X-100, incubated directly with protein A beads, and then microcentrifuged (i.e., no antibodies were ever added to this sample). The top arrow depicts the migration of the small complex in the gel system, while the bottom arrow indicates the location of free ^{125}I-CPE. It is important to note that if large complex had been present in these samples, it would have migrated near the very top of these gels (see reference 23 and data not shown).

Two additional control experiments further support the immunoprecipitation of radiolabeled CPE small complex in these +RPC-IgG samples involving a direct interaction between RPC-IgG and ^{125}I-CPE sequestered in the small complex while this small complex was still present in intact BBMs. First, since no significant radioactivity disappeared from supernatants (Fig. 3, lane RPC-IgG 1st) if BBMs were pretreated with 10 μg of RPC-IgG, before being washed and treated with ^{125}I-CPE (followed by extraction with Triton X-100 and incubation with protein A beads), immunoprecipitation of radiolabeled CPE small complex from +RPC-IgG samples apparently does not result from a reaction between the extracted small complex and residual unbound antibodies remaining in samples as a result of insufficient washing. Second, since virtually all of the radiolabeled small complex still disappeared from supernatants of RPC-IgG-treated, small-complex-containing BBM samples even if a 50-fold excess of free CPE was present during the Triton X-100 extraction and protein A incubation steps of

the immunoprecipitation (Fig. 3, lane 50× CPE), it appears unlikely that immunoprecipitation of the radiolabeled small complex in +RPC-IgG samples can be explained by RPC-IgG initially reacting with the nonspecifically bound ^{125}I-CPE in ^{125}I-CPE-treated BBMs and then, during extraction or incubation with protein A beads, dissociating from this nonspecifically bound ^{125}I-CPE and reacting with the extracted small complex. That is, if RPC-IgG were dissociating from extracted nonspecifically bound CPE, the presence of excess free unlabeled CPE in the 50× CPE samples during the extraction and protein A treatment steps should have competed for dissociated RPC-IgG and thereby prevented a significant decrease in the amount of the radiolabeled small complex remaining in supernatants of the 50× CPE samples after immunoprecipitation, yet this effect was not observed. The 50× CPE result also provides additional evidence that the disappearance of specifically bound supernatant radioactivity from +RPC-IgG immunoprecipitation samples is not due to unbound antibodies remaining in samples after washing, since, in this case, the presence of excess free CPE during extraction of the 50× CPE samples should also have competed against the extracted small complex for reactivity with any unbound antibodies and thereby prevented a significant decrease in the amount of radiolabeled small complex present in the 50× CPE supernatants after immunoprecipitation; however, this effect was not observed.

A similar immunoprecipitation analysis was also conducted at 22°C, a temperature at which both small and large complex formation occurs (23) (Fig. 4, lane control, −) (see Fig. 4 legend), to determine whether CPE in CPE large complex also remains surface exposed. When BBMs treated with ^{125}I-CPE at 22°C were reacted with RPC-IgG, extracted with Triton X-100, and then incubated with protein A beads, all specifically bound ^{125}I-CPE, i.e., ^{125}I-CPE sequestered in both large and small complexes, disappeared from the supernatants of these samples (Fig. 4, lanes +RPC-IgG, −). This immunoprecipitation appears to involve a specific interaction between RPC-IgG and the ^{125}I-CPE molecule present in both complex types since there was no similar decrease in supernatant radioactivity if BBMs treated with ^{125}I-CPE at 22°C were (i) reacted with NRS-IgG prior to extraction and incubation with protein A beads (Fig. 4, lanes +NRS-IgG) or (ii) extracted and treated with protein A beads alone (Fig. 4, lane ProA Beads Only). This immunoprecipitation of radiolabeled small and large complexes by RPC-IgG at 22°C does not appear to result from insufficient washing of unbound antibodies or from postextraction dissociation of RPC-IgG from nonspecifically bound ^{125}I-CPE, on the basis of the behavior (see discussion of Fig. 3 results) of the RPC-IgG 1st and 50× CPE samples in Fig. 4.

When these immunoprecipitation results along with observations (8) indicating that virtually all BBMs prepared by the method (22) used in this study are sealed vesicles with a right-side-out orientation are considered, it can be concluded from our current studies that some region(s) of the CPE molecule remains exposed on the external (rather than cytoplasmic) surface of the plasma membrane, whether CPE is sequestered in a small or large complex. This finding is compatible with the second step in CPE action, i.e., the postbinding physical change to the small complex, involving a conformational change to the small complex that leaves some of the CPE region(s) surface exposed in the small (and large) complex but renders the entire CPE molecule more resistant to protease-induced release from BBMs. It is important to note that our current results do not necessarily preclude the possibility that some of the CPE region(s) may be inserted into the lipid bilayer of BBMs, since streptolysin O remains accessible to antibodies after it has inserted into membranes (6). The current results must eventually be integrated with other ongoing studies to fully resolve the topology of CPE in membranes and, in particular, to address whether CPE action involves an insertion step.

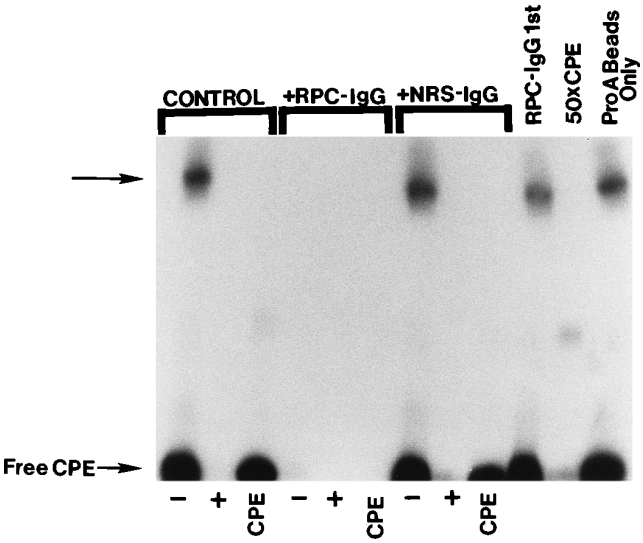

FIG. 4. SDS-PAGE analysis of immunoprecipitation supernatants from BBMs treated with ^{125}I-CPE at 22°C. ^{125}I-CPE, in the presence (+) or absence (−) of a 50-fold excess of unlabeled CPE, was bound and inserted into BBMs at 22°C. These BBMs were treated with RPC-IgG or NRS-IgG (see text) and then extracted with 0.1% Triton X-100. After incubation of extracts with protein A beads, the samples were microcentrifuged to remove immunoprecipitated materials and 40 μl of each remaining supernatant was analyzed by SDS-PAGE (no sample boiling) electrophoresis to identify the CPE large complex (24). Free ^{125}I-CPE (i.e., no BBMs) in PBS containing 0.1% Triton X-100 was included as a control in all experimental sets (lanes labeled CPE). Lanes: Control, a set of ^{125}I-CPE-treated BBMs samples that were extracted with 0.1% Triton X-100 and then directly microcentrifuged; +RPC-IgG and +NRS-IgG, microcentrifugation supernatants from two sets of ^{125}I-CPE-treated BBM samples that were incubated with RPC-IgG or NRS-IgG, respectively, prior to Triton X-100 extraction, incubation with protein A beads, and microcentrifugation; RPC-IgG 1st, microcentrifugation supernatant from a BBM sample that was preincubated with RPC-IgG, washed, and then treated with ^{125}I-CPE before being extracted with 0.1% Triton X-100, incubated with protein A beads, and microcentrifuged; 50× CPE, microcentrifugation supernatant from a ^{125}I-CPE-treated BBM sample that was incubated with RPC-IgG and then Triton X-100 extracted in the presence of a 50-fold excess of unlabeled CPE, prior to being incubated with protein A beads and microcentrifuged; and ProA Beads Only, microcentrifugation supernatant from a ^{125}I-CPE-treated BBM sample that was extracted with 0.1% Triton X-100, directly incubated with protein A beads, and then microcentrifuged (i.e., no antibodies were ever added to this sample). The top arrow depicts the location of the large complex on these gels, while the bottom arrow indicates the migration of free ^{125}I-CPE. It is important to note the following. (i) The small CPE complex itself is not visible on this SDS-acrylamide gel because the small complex is labile to the effects of SDS (23); ^{125}I-CPE that had been localized in the small complex prior to exposure to SDS now runs as "free specifically bound ^{125}I-CPE" (23), visible at the bottom of the − lanes on the gel (this small complex-associated radioactivity disappears from the − lane of the +RPC-IgG sample, indicating that the small complex is also surface exposed in 22°C samples). (ii) The small amount of higher-M_r radioactivity visible in the 50× CPE lane is aggregated ^{125}I-CPE (this aggregation is induced by the presence of excess unlabeled CPE in this sample [17]).

Finally, additional immunoprecipitation studies demonstrated (data not shown) that CPE small or large complexes can react with RPC-IgG after they have been extracted from BBMs. This finding opens the possibility of isolating extracted small or large complexes by use of affinity columns containing immobilized CPE antibodies. This could represent a significant advance, since the unavailability of workable quantities of isolated small and large complexes has been hindering further investigations into the role of these species in CPE action.

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