INFECTION AND IMMUNITY, Oct. 1991, p. 3381–3386
0019-9567/91/103381-06$02.00/0
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Vol. 59, No. 10

Anthrax Protective Antigen Interacts with a Specific Receptor on the Surface of CHO-K1 Cells

VINCENT ESCUYER AND R. JOHN COLLIER*

Department of Microbiology and Molecular Genetics and The Shipley Institute of Medicine, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115

Received 29 April 1991/Accepted 27 June 1991

The interaction of protective antigen (PA), a component of the anthrax toxin, with receptors on the Chinese hamster ovary cell line CHO-K1 was characterized. Protective antigen binding at 4°C is highly specific, concentration dependent, saturable (K_d = 0.9 nM), and reversible. Scatchard analysis indicates the presence of a single class of PA binding sites at a concentration of 10,000 ± 2,000 per cell. Pretreatment of cells with a number of different proteases strongly inhibits PA binding, suggesting that the receptor may be at least partially proteinaceous. Direct chemical cross-linking of radioiodinated PA to the cell surface results in the appearance of a major band exhibiting an apparent molecular mass of 170 kDa, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The appearance of this band is completely inhibited by a 200-fold molar excess of unlabeled PA, indicating a high specificity for this interaction. Our results suggest that a cell surface protein(s) of 85 to 90 kDa is, or constitutes a portion of, a specific receptor for the PA.

Many bacterial toxins affect eukaryotic cells by enzymatically modifying an intracellular target and must therefore cross the plasma membrane to reach the cytoplasm (24). The molecular mechanisms by which these toxins enter the cell remain poorly understood. Generally, the first step of the intoxication process is the binding of the toxin molecule to a specific receptor on the cell surface (7). This step is crucial in that it determines the specificity of host cell sensitivity as well as the efficiency of the intoxication process. Few cell surface toxin receptors have been formally identified. Receptors for cholera toxin, botulinum toxin, and tetanus toxin are thought to be gangliosides, whereas receptors for diphtheria toxin, *Shigella* toxin, and probably *Pseudomonas* exotoxin A are believed to be proteins or glycoproteins (7). For many other toxins, however, the identity of the receptor remains to be determined, and it is of ongoing interest to obtain more information on such molecules.

Anthrax toxin, which is produced by *Bacillus anthracis*, the causative agent of the disease anthrax, is unique in that it is composed of three distinct proteins: protective antigen (PA; 82,684 Da), edema factor (EF; 89,840 Da), and lethal factor (LF; 90,237 Da). The genes for all three proteins have been cloned and sequenced (3, 8, 25, 29, 30, 38, 39). None of the anthrax toxin components is active alone, but these components interact in binary combinations to give two distinct physiological responses in experimental animals. Coinjection of PA and LF (lethal toxin) causes death in susceptible animals, while intradermal injection of PA and EF (edema toxin) produces an edema in guinea pigs or rabbits (1, 9, 34). EF has been shown to be an adenylate cyclase which is dependent on the eukaryotic activator calmodulin for activity (18, 19). Many types of cultured cells are sensitive to edema toxin and show a dramatic increase in the intracellular concentration of cyclic AMP upon intoxication with the edema toxin (18). No enzymatic activity for LF has been found, and its mode of action remains unknown.

Recently, Leppla et al. (21) proposed a model for the entry of anthrax toxin into sensitive cells. PA first binds to a receptor on the cell surface and is then proteolytically cleaved by a membrane protease. Proteolytic cleavage removes a 20-kDa N-terminal polypeptide (PA_{20}), leaving a 63-kDa piece (PA_{63}) bound to the receptor. PA_{63} acts as a ligand for either LF or EF, and the complexes PA_{63}-LF and PA_{63}-EF are thought to be internalized by receptor-mediated endocytosis. Neither LF nor EF can bind to cells if PA is not already present on the cell surface. Therefore, it is clear that PA plays a central role in cellular intoxication and that its binding to the cell surface is a critical step in this process. In this work, we demonstrate that the Chinese hamster ovary cell line CHO-K1 possesses a specific receptor for PA and that this receptor, or a portion of it, is protein in nature.

MATERIALS AND METHODS

Cells, media, and culture conditions. CHO-K1 cells were obtained from the American Type Culture Collection (Rockville, Md.). Cells were grown in nutrient mixture Hams F12 (Irvine Scientific, Santa Ana, Calif.) containing 10% fetal bovine serum, 500 U of penicillin G per ml, and 500 μg of streptomycin sulfate per ml at 37°C in an atmosphere of 5% CO_2.

Enzymes and chemicals. Phospholipases A2 (from *Naja naja* venom) and D (from peanut), neuraminidase (from *Clostridium perfringens*), trypsin, pronase, mannose, glucose, galactose, inositol, and ethanolamine were purchased from Sigma Chemical Co. (Saint Louis, Mo.). Lysozyme, glucosamine, galactosamine, *N*-acetylglucosamine, *N*-acetylgalactosamine, methyl-α-D-mannopyranoside, concanavalin A, wheat germ agglutinin, phytohemagglutinin, fetuin, and bovine brain ganglioside mix were obtained from Calbiochem (San Diego, Calif.). Phospholipase C (from *Bacillus cereus*) was from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Diphtheria toxin was from Connaught Laboratories (Willowdale, Ontario, Canada). Exotoxin A was a gift from Kevin Killeen.

Radiolabeling of PA. PA was purified from the culture supernatants of the noncapsulogenic Sterne strain of *B. anthracis* by the method of Leppla (20) and modified as previously described (2). Radiolabeled PA was prepared by

* Corresponding author.

incubating 50 to 60 μg of PA with 250 μCi of iodinated Bolton-Hunter reagent (New England Nuclear, Boston, Mass.) according to the manufacturer's recommendations. Radiolabeled PA was separated from unreacted reagent on an exocellulose GF-15 column (Pierce, Rockford, Ill.). The elution buffer was 0.1 M Tris-HCl, pH 7.5, containing 10 mM KI and 0.01% gelatin. We consistently obtained a specific activity between 2×10^6 and 6×10^6 cpm/μg of PA. Radiolabeled PA migrated as a single band on a sodium dodecyl sulfate (SDS)-polyacrylamide gel, with an apparent molecular mass of 82 kDa.

PA binding assay. CHO-K1 cells were seeded in 24-well culture plates (Costar, Cambridge, Mass.) at a density of 2×10^5 cells per well and were incubated for 18 h at 37°C prior to use. For binding studies, monolayers were washed three times with cold phosphate-buffered saline, pH 7.4 (PBS), and radiolabeled PA at the indicated concentrations in Eagle minimum essential medium (Irvine Scientific) buffered to pH 7.4 with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and containing 0.1% bovine serum albumin was added to the mixture. Cell monolayers were incubated at 4°C for the appropriate time, washed four times with PBS, solubilized with 0.1 M NaOH, and counted in an LKB 1272 gamma counter. Nonspecific binding was determined by incubating cells with radiolabeled PA in the presence of a 200-fold molar excess of unlabeled PA.

Chemical cross-linking of PA to the cell surface. CHO-K1 cells were grown to confluency in 100-mm (diameter) tissue culture dishes and washed three times with cold PBS. Cells were incubated with 0.2 to 0.3 μg of radioiodinated PA per ml in Hanks' balanced salt solution buffered to pH 7.4 with 25 mM HEPES and containing 0.01% bovine serum albumin for 6 h at 4°C. After incubation with PA, cells were washed five times with cold PBS. PA cross-linking to cells was achieved by the addition of disuccinimidyl suberate (DSS) (Pierce) at a final concentration of 0.5 mM for 30 min at 4°C. Cells were washed three times with cold PBS, removed from the plates with a rubber policeman, suspended in cold PBS, and centrifuged for 10 min at $1,500 \times g$. Nonspecific cross-linking was determined by adding a 200-fold molar excess of unlabeled PA. The cell pellet was resuspended in 100 to 150 μl of electrophoresis sample buffer and heated at 100°C. Samples were analyzed by electrophoresis on a 6% SDS–polyacrylamide gel. Autoradiography was performed at −70°C with Kodak XAR film and intensifying screens.

RESULTS

Demonstration of a PA receptor on CHO-K1 cells. CHO-K1 cells were chosen for these experiments because of their sensitivity to edema toxin (18). Therefore, in accordance with the model proposed by Leppla et al. (21), these cells should bind PA with a high specificity. The classical technique used to prevent the internalization of the ligand in ligand-receptor interaction studies is to incubate the cells with the ligand at 4°C (6). Figure 1 shows the kinetics of the association of radiolabeled PA with CHO-K1 cells at 4°C. The amount of PA associated with the cells reaches its maximum after 5 to 6 h. The nonspecific association of radiolabeled PA at 4°C, determined by adding a 200-fold molar excess of unlabeled PA, was low (10% or less of the total association). In subsequent studies, PA was incubated with cells for 6 h at 4°C to allow maximal binding.

The dose-response curve of PA binding to CHO-K1 cells is shown in Fig. 2. Maximal binding under these conditions occurred at a PA concentration of 0.5 μg/ml. Analysis of the

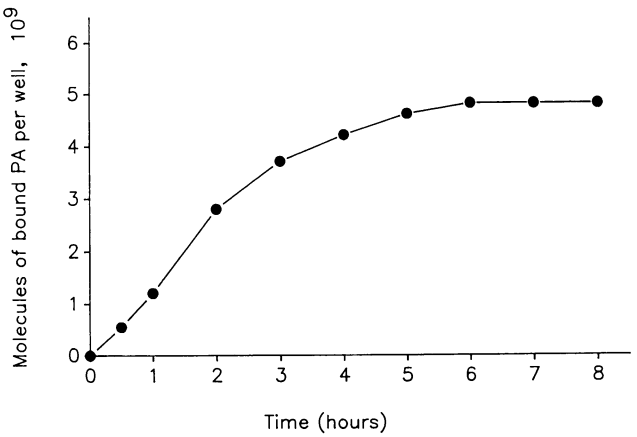

FIG. 1. Kinetics of specific binding of ^{125}I-labeled PA to CHO-K1 cells at 4°C. Cells were incubated with iodinated PA (0.05 μg/ml), and, at the times indicated, triplicate samples were processed as described in Materials and Methods. Specifically bound PA was determined by subtracting values obtained in the presence of a 200-fold molar excess of unlabeled PA. Results are the means of two independent experiments. Standard errors of the means were consistently less than 5%.

binding data by the method of Scatchard (31) indicates a single class of binding sites (10,000 ± 2,000), with an apparent dissociation constant (K_d) of 0.9 nM (Fig. 2, inset). Competition experiments between iodinated and unlabeled PA gave an experimental K_d of 0.6 nM, in good agreement with the calculated value obtained from Scatchard analysis (Fig. 3).

The reversibility of PA binding is shown in Fig. 4. Cells were incubated with radiolabeled PA for 6 h at 4°C, a 500-fold molar excess of unlabeled PA was added, and the fraction of radiolabeled PA remaining bound was determined as a function of time. The addition of unlabeled PA clearly results in a decrease in the association of radiolabeled PA with CHO-K1 cells, indicating that the association is reversible. Dissociation of bound PA in the absence of unlabeled PA was less than 5%. Diphtheria toxin and exotoxin A from *Pseudomonas aeruginosa* were not able to compete with PA for binding even at a 500-fold molar excess, supporting the notion that PA binds to its own specific receptor.

Similar binding experiments were performed at 37°C with cells that had been prefixed with paraformaldehyde, as described by Manhart et al. (22). Results were identical except that the kinetics of PA binding were faster, with maximal binding being reached after 2 h of incubation (data not shown). We also tried to extend this work to another mammalian cell line, Vero (monkey kidney cells). This cell line exhibited only a very low level of specific PA binding, which made it unsuitable as a model for the identification of a PA receptor (data not shown).

Characterization of the receptor. To elucidate the nature of the interaction between PA and its receptor, three basic approaches were used: (i) competitive inhibition of PA binding to cells with sugars, gangliosides, and glycoproteins; (ii) receptor blockage with lectins; and (iii) enzymatic alteration of the receptor.

Table 1 shows the effect of 13 monosaccharides, gangliosides, and glycoproteins on PA binding to CHO-K1 cells. Some of these compounds have been shown to interfere with the binding of toxins, such as cholera toxin or shigella toxin, to their receptors (5, 16), but none of the compounds tested

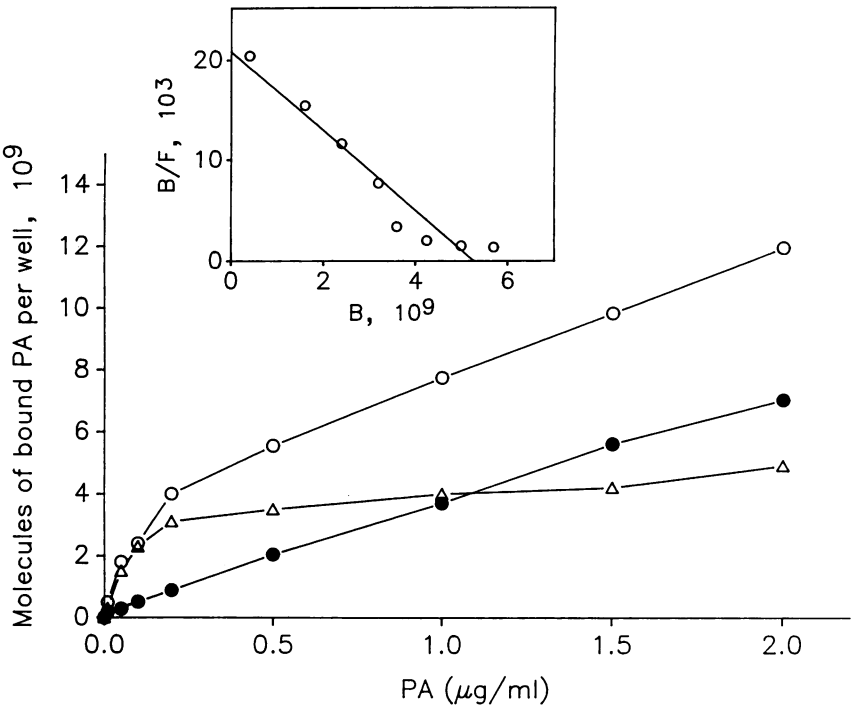

FIG. 2. Concentration dependence of PA binding to CHO-K1 cells at 4°C. Cells were incubated for 6 h at 4°C with radiolabeled PA (0.01 to 2 μg/ml) in the absence (○) or presence (●) of a 200-fold excess of unlabeled PA. Binding was determined as described in Materials and Methods. Specific binding (△) represents the difference between ○ and ● values. Values represent the means of triplicates from two independent experiments. Standard errors of the means were less than 10%. Inset, Scatchard analysis of PA binding (bound molecules of PA [B] were plotted versus bound PA/free PA [B/F].

was found to inhibit PA binding significantly. The effect of three lectins, with well-characterized structural binding specificities as receptor blockers, was also studied. Preincubation of CHO-K1 cells with these lectins did not affect PA binding.

Table 2 summarizes the results obtained after pretreatment of CHO-K1 cells with enzymes designed to alter various cell surface structures. Lysozyme, neuraminidase, and three different phospholipases had no effect on the ability of PA to bind CHO-K1 cells. The results obtained with phospholipases indicate that phospholipids are probably not involved in PA binding. However, pretreatment of cells with 50 μg of trypsin per ml or 20 μg of pronase per ml (concentrations just below that required to detach the cells from the plastic surface or alter cell morphology) strongly inhibited PA binding to CHO-K1 cells (90 and 70%, respec-

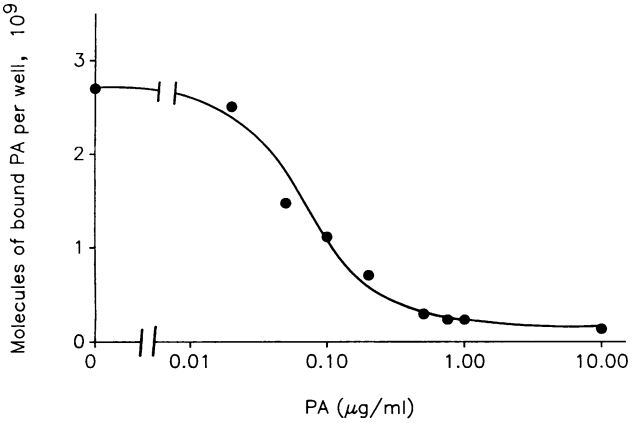

FIG. 3. Inhibition of ^{125}I-PA binding to CHO-K1 cells with unlabeled PA. Cells were incubated with radiolabeled PA (0.05 μg/ml) and with the indicated concentrations of unlabeled PA. Binding was determined as described in the text. The concentration of unlabeled PA that inhibited the binding by 50% of the maximum binding is an approximation of the K_d.

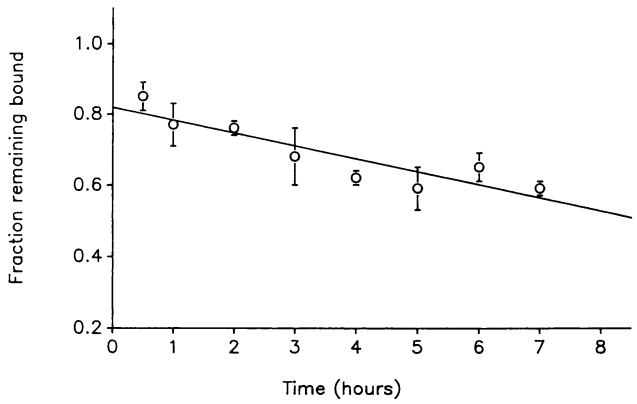

FIG. 4. Dissociation of ^{125}I-PA from CHO-K1 cells at 4°C. Cells were incubated with radiolabeled PA (0.05 μg/ml) for 6 h at 4°C. Unlabeled PA (50 μg/ml) was then added, and the fraction of PA remaining bound to cells was determined at different times as described in Materials and Methods. Values represent the means of two experiments. The line was fitted by regression analysis.

TABLE 1. Effect of sugars, gangliosides, glycoproteins, and lectins on PA binding to CHO-K1 cells[a]

Inhibitor (mM or mg/ml)	Inhibition of binding (%)
ATP (10 mM)	10
Glucose (50 mM)	6
Galactose (50 mM)	6
Mannose (50 mM)	0
Glucosamine (50 mM)	0
Galactosamine (50 mM)	0
N-Acetylglucosamine (50 mM)	0
N-Acetylgalactosamine (50 mM)	0
Inositol (50 mM)	0
Ethanolamine (50 mM)	8
Methyl-α-D-mannopyranoside (50 mM)	0
Fetuin (0.05 mg/ml)	6
Gangliosides (mixed bovine brain) (1.00 mg/ml)	12
Wheat germ agglutinin (0.01 mg/ml)	3
Concanavalin A (0.01 mg/ml)	0
Phytohemagglutinin (0.01 mg/ml)	0

[a] Cells were incubated for 6 h at 4°C with iodinated PA (0.1 μg/ml) in the presence of each inhibitor at the indicated concentration. Nonspecific binding was determined by addition of a 200-fold molar excess of unlabeled PA. Triplicate samples were processed as described in the text.

tively). To eliminate the possibility that this inhibition was due to a direct effect of the proteases on PA itself, cells were washed extensively before further incubation with PA and the last wash was tested for its ability to degrade PA. No degradation of PA was detected after analysis by SDS-polyacrylamide gel electrophoresis (data not shown). These results suggested that the PA receptor may be at least partially proteinaceous.

Chemical cross-linking of PA to the CHO-K1 cell surface. In an attempt to identify the PA-specific receptor, bound PA was chemically cross-linked to the CHO-K1 cell surface. Chemical cross-linkers have been used successfully in identifying several cell surface receptors including the diphtheria toxin receptor (4, 26, 27, 40). In the current work, the homobifunctional cross-linker DSS was used. By using cross-linkers of varying lengths (one example is shown in Fig. 5, lane 3), we found the length of the cross-linker to be particularly important in this experiment. DSS gave the most consistent results. CHO-K1 cells were incubated with ^{125}I-PA in the absence or presence of excess unlabeled PA, followed by cross-linking with DSS. Cells were lysed, and the soluble fraction was subjected to electrophoresis on a 6% denaturing polyacrylamide gel as described in Materials and Methods. Upon exposure of the gel to X-ray film, we

TABLE 2. Effect of various enzymes on PA binding to CHO-K1 cells[a]

Enzyme (U/ml or μg/ml)	Inhibition of binding (%)
Lysozyme (5,000 U/ml)	0
Phospholipase A2 (25 U/ml)	5
Phospholipase C (20 U/ml)	9
Phospholipase D (200 U/ml)	0
Neuraminidase (10 U/ml)	0
Trypsin (50 μg/ml)	90
Pronase (20 μg/ml)	70

[a] Cells were incubated with the enzyme in Eagle minimal essential medium at the indicated concentration for 30 min at 37°C. Cells were then washed with cold PBS and incubated with 0.1 μg of iodinated PA per ml at 4°C. Binding was determined as described in the text.

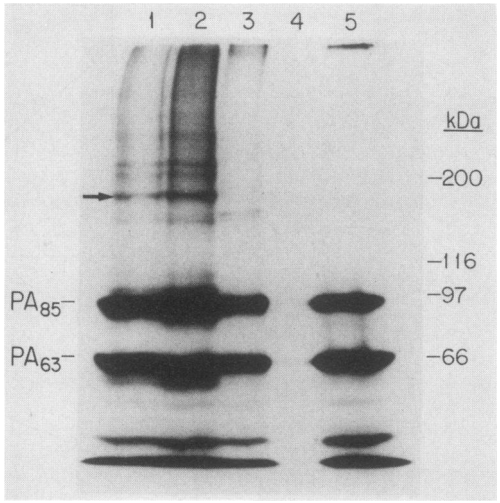

FIG. 5. Chemical cross-linking of PA to the surface of CHO-K1 cells. Radiolabeled PA was cross-linked to the surface of CHO-K1 cells as described in Materials and Methods. Positions of the marker proteins are indicated at the right. Lanes 1 to 5: CHO-K1 cells plus 0.1 mM DSS (lane 1), 0.5 mM DSS (lane 2), 0.5 mM DSS (lane 3), 0.5 mM DSS–200-fold molar excess of unlabeled PA (lane 4), or no DSS. The position of the major 170-kDa band is indicated by an arrow.

observed one prominent band with an apparent molecular mass of 170 kDa, in addition to the bands corresponding to PA_{85} (85 kDa) and PA_{63} (63 kDa) (Fig. 5, lanes 1 and 2). The 170-kDa band was absent when cross-linking was carried out with a 200-fold molar excess of unlabeled PA (Fig. 5, lane 4), when the cross-linker was omitted (lane 5), or when PA was cross-linked to itself (data not shown). Assuming a 1:1 ratio of radiolabeled PA and its receptor, we calculated the molecular mass of the cell surface receptor to be 85 to 90 kDa. Minor bands are also evident on the autoradiograph. A lower molecular mass band(s) (150 kDa) may result from degradation of the receptor or may represent a subunit of the receptor. Interestingly, the difference in size between this band(s) and the major band is almost the same as the difference in size between PA_{85} and PA_{63}. The alternate explanation is that this band could represent the complex between PA_{63} and the receptor. The significance of the high molecular mass bands (200 kDa or greater) is unclear. These bands could represent lower-affinity binding sites present at the cell surface or proteins functionally associated with the receptor and able to interact with PA after it binds to its receptor.

DISCUSSION

In this work, we have shown that the association of PA with CHO-K1 cells at 4°C satisfies the criteria defined by Cuatrecasas (6) for the characterization of a true receptor: specificity, high affinity, saturability, and reversibility. Recently, Singh et al., working on the macrophage cell line J774-A.1, reported that they found approximately 8,000 PA binding sites per cell, with a K_d of 1×10^{-9} to 5×10^{-9} M (32, 33). However, these authors did not present a detailed study of PA binding to this cell line, and whether the PA receptor is the same for the two cell lines remains to be determined.

The results obtained with the proteases strongly suggest

that the PA receptor is at least partially proteinaceous, and cross-linking experiments allowed the identification of a cell surface PA binding component(s) which is a putative candidate(s) for the PA-specific receptor. However, further experiments are required to demonstrate that this protein(s) is the true PA receptor. We are presently trying to purify the 85- to 90-kDa protein(s) from CHO-K1 cell membranes and show that it is able to specifically bind PA in vitro. Such an approach has been used successfully by Mekada et al. in studies of the diphtheria toxin receptor (23).

It is now well established that several bacterial toxins such as diphtheria toxin, *Shigella* toxin, and probably *Pseudomonas* exotoxin A are internalized by receptor-mediated endocytosis (10, 14, 15). In each case, the receptors involved are thought to be cell surface glycoproteins (16, 28, 37). There is evidence that the internalization of lethal toxin and edema toxin is also receptor mediated (21). However, it is not clear at this time whether the PA receptor is a glycoprotein. Preliminary experiments in which CHO-K1 cells were treated with tunicamycin, an antibiotic which blocks the formation of protein-carbohydrate linkages of the N-glycosidic type (36), indicate that PA binding is not affected by this treatment. This could mean that either the PA receptor is not a glycoprotein or, if it is a glycoprotein, that the carbohydrate portion is not involved in the interaction with PA (13). Other remaining questions include the physiological role of the receptor, its possible role in PA processing at the cell surface, and its participation in the translocation of the toxin across the cell membrane. Recent studies of LF and EF indicate that toxicity requires a passage through an acidified endocytic vesicle (11, 12). Koehler and Collier have shown recently that when PA is added in vitro to artificial lipid membranes at low pH, it becomes inserted and forms ion-conducting channels (17). It has been proposed that this channel provides a hydrophilic pore enabling EF and LF to pass through the endosomal membrane. Whether the PA-specific receptor is involved in vivo in the interaction of PA with cell membranes, as seems to be the case for diphtheria toxin (35), is under investigation. This work is the basis for further structural and functional analyses of the PA receptor which may yield answers to these questions.

ACKNOWLEDGMENTS

We thank Sims Kochi and Philip Hanna for helpful discussions.

This work was supported by National Institutes of Health grants AI22021 and AI22848. V.E. is the recipient of a European Molecular Biology Organization fellowship.

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