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Mouse Liver Contains a *Pseudomonas aeruginosa* Exotoxin A-Binding Protein

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The opportunistic pathogen *Pseudomanas aeruginosa* produces several potential virulence factors, including the ADP-ribosylating toxin, exotoxin A (PE). Studies using a burned mouse model have shown that PE consistently inhibits protein synthesis and depletes elongation factor 2 in mouse liver and variably in other organs. One reason for toxin sensitivity could be the presence of a PE receptor on the surface of cells. Therefore we examined detergent extracts of mouse tissues for the presence of toxin-binding proteins. Proteins which specifically bind PE were present in extracts from liver, kidney, lung, spleen, and heart. Because liver appears to be a prominent target for the toxin in a burned animal, we choose to isolate the PE-binding protein from mouse liver and compare this protein to the recently characterized toxin-binding protein from toxin-sensitive mouse LM fibroblasts. The toxin-binding proteins from both sources have a molecular mass of approximately 350 kDa, share similar protease digestion profiles, and are glycosylated. However the glycosylation patterns for the two species are quite different. Both glycoproteins bind toxin with high avidity. The toxin-binding moiety is located, at least in part, on the plasma membrane and thus could represent the receptor involved in internalization of toxin molecules responsible for cell death.

Pseudomonas aeruginosa is an opportunistic pathogen which causes infections in burned patients. It produces several extracellular products which are associated with virulence including a protein toxin, *pseudomonas* exotoxin A (PE) (16). PE is an enzyme which catalyzes the ADP-ribosylation of elongation factor 2, with the end result being inhibition of protein synthesis in target cells (3).

Early studies by Pavlovskis et al. (12, 13) had shown alteration in liver enzymes and reduced protein synthesis in the liver of animals to which purified PE had been administered. Iglewski et al. (4) have shown that parenteral administration of PE to normal mice results in depletion of elongation factor 2 in the heart, kidneys, spleen, lungs, and liver. Elongation factor 2 activity is virtually eliminated from the livers of intoxicated mice (4).

Our studies in a burned mouse model have shown that PE is produced by *P. aeruginosa* multiplying at the burn site (17). Toxin enters the circulation, and by 24 h protein synthesis and elongation factor 2 levels in the liver are significantly reduced (18). The spleen, heart, kidneys, and lungs show a more modest reduction in protein synthesis and in elongation factor 2 activity; these organs are usually affected late in the course of infection (18). Passive treatment of animals with specific antitoxin negates these effects (11, 18). Thus, the liver appears to be the earliest and the most important target for the toxin in burned animals.

Since the substrate for the toxin is in the cell cytoplasm, it is assumed that PE crosses membrane barriers to exert its effect. Using an established cell line, mouse LM fibroblasts, we have shown that toxicity involves the following steps: specific binding to a receptor, entry into endosomes, and subsequent movement to the Golgi region of the cell and to lysosomes (2, 7, 8). At an as yet unidentified point in this journey, some toxin must be activated, perhaps by cleavage of a 37-kDa enzymatically active fragment, enter the cell cytoplasm, and there ADP-ribosylate elongation factor 2 (7, 10).

Recently we have isolated a *Pseudomonas* toxin-binding protein from mouse LM fibroblasts which may be the putative receptor involved in this pathway (20). The purified toxin-binding glycoprotein has an estimated molecular mass of 325 to 350 kDa. To date, this molecule has been identified only in extracts of cultured cells which are sensitive to PE; it has not been found in extracts of human ovarian carcinoma cells (OVCAR-3) which are toxin resistant (14, 20). In this paper we show that PE-binding protein is present in extracts of mouse tissues and of established tissue culture cell lines which are sensitive to PE. In addition, we have purified a toxin-binding protein from mouse liver that is similar to that in LM cells.

MATERIALS AND METHODS

Reagents. PE was obtained from List Biochemicals and was resuspended according to the manufacturer's instructions, aliquoted, and stored at −80°C. The toxin was biotinylated as described; biotinylated PE retained greater than 95% biological activity, as determined by inhibition of protein synthesis, and binding activity, as measured by electron microscopy (8). Goat *Pseudomonas* antitoxin was obtained from List Biochemicals. Affinity-purified swine anti-goat immunoglobulin G labeled with horseradish peroxidase (HRP) and peptide *N*-glycosidase F (PNGase F) were obtained from Boehringer Mannheim Biochemicals.

Cells. Mouse LM fibroblasts (ATCC CCL 1.2, L-M) were obtained from the American Type Culture Collection (ATCC) and were maintained as described previously (8). Chang liver cells (human), HeLa cells (human), and Vero (monkey kidney) cells were obtained from ATCC and main-

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tained in medium containing 10% fetal calf serum, as described by ATCC.

Protein synthesis. Inhibition of protein synthesis was used as an index of toxin biological activity. Monolayers were incubated with PE overnight at 37°C, and incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable material was determined (2).

Preparation of PE affinity column. A PE-Sepharose 4B affinity column was prepared and stored as described previously (20). After each use, the column was washed with 5 M NaSCN in 0.15 M NaCl phosphate-buffered saline (pH 7.2) (PBS) with 0.1% Nonidet P-40 (NP-40) and 0.05% NaN_3. The pH of this buffer was adjusted to 8.5 with NaOH.

Solubilization of PE-binding protein. Solubilized binding protein was prepared from tissue of 20- to 25-g CF-1 mice. Mice were sacrificed by cervical dislocation; tissues were removed, rinsed in cold PBS, weighed, and homogenized in cold buffer A (0.125 M Tris-maleate buffer [pH 6.0] containing 160 mM NaCl, 2.0 mM $CaCl_2$, 1.0 mM EDTA, 0.2 mM pepstatin, 0.2 mM phenylmethylsulfonyl fluoride, and 0.2 mM leupeptin) (1 g of tissue to 6 ml of buffer A), using 30 strokes in a Dounce homogenizer. All further steps were carried out in the cold. The homogenate (approximately 30 mg of protein per ml) was subjected to centrifugation at 500 × *g* for 5 min. The pellet was discarded, and the supernatant was treated in one of two ways. For studies involving detection of PE-binding protein in different tissues, the supernatants containing crude membranes were made 1% with NP-40, stirred for 60 min, and then centrifuged at 100,000 × *g* for 60 min. The resulting detergent extracts were collected and assayed for protein and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For studies involving purification of the binding protein from liver, the supernatant was applied to a discontinuous sucrose gradient consisting of layers of 65, 32.5, and 10% sucrose in PBS (6). Sucrose gradients were centrifuged at 100,000 × *g* for 60 min. Membranes were collected from the 10–32.5% interface, mixed with 4 volumes of PBS, and then centrifuged at 100,000 × *g* for 50 min. The pellets were resuspended in 6 ml of buffer A; NP-40 detergent was added to a final concentration of 1.0%, and the extraction was continued with stirring for 40 min. The material was centrifuged at 100,000 × *g* for 20 min, and the pellet was discarded. The resulting supernatant, called detergent extract, was used for further purification.

Affinity purification of liver PE-binding protein. The affinity purification protocol is a modification of a previously published method (20). All steps were carried out at 4°C. Five milliliters of detergent extract (approximately 0.3 mg of protein per ml) was mixed with 10 ml of 0.5 M NaCl PBS (pH 8.5) containing 0.1% NP-40, 0.05% Na azide, and the same protease inhibitors as in buffer A and was recycled overnight on a PE-Sepharose 4B column (2-ml bead volume) previously equilibrated with this buffer. The column was washed with a minimum of 100 ml of this buffer; then toxin-binding protein was eluted with 20 ml of 1.5 M NaSCN in PBS (pH 8.5) containing 0.1% NP-40 and 0.05% NaN_3. The eluate was collected and concentrated to 2.5 ml by using 30,000-molecular-weight-cutoff Centricell concentrators (Polysciences). This material was desalted on a Pharmacia PD-10 gel filtration column and was then reconcentrated to approximately 1 ml by using 100,000-molecular-weight-cutoff Centricon concentrators (Amicon). The affinity-purified protein was assayed by the Micro BCA method (Pierce) and frozen in aliquots at −80°C. Approximately 50 μg of purified material was obtained from each liver.

Binding protein from kidney was prepared in a similar manner.

Identification of binding protein. Samples containing PE-binding proteins were subjected to SDS-PAGE, transferred to nitrocellulose (5, 21), and assayed for toxin-binding activity in one of two ways. The PE-binding protein blot assay was done as described previously (20) except that PE bound to receptors was detected by sequential incubation with goat anti-PE, swine anti-goat immunoglobulin G labeled with HRP, and 4-chloro-1-naphthol. Control experiments showed that neither the primary nor secondary antibody bound to toxin-binding protein in the absence of PE.

To show specificity of PE binding to the toxin-binding protein, the nitrocellulose sheet was incubated with biotinyl-PE (1.6 μg/ml) in the presence or absence of a 100-fold excess of native toxin. PE was identified with streptavidin-HRP (1 μg/ml) and 4-chloro-1-naphthol. Control experiments showed that streptavidin-HRP did not bind to the 350-kDa protein in the absence of biotinyl-PE.

A competition format of an enzyme-linked immunosorbent assay (ELISA) using biotinyl-PE also was employed to show specificity of PE binding (20).

Glycosylation of binding protein. Commercially available glycan detection and differentiation kits (Boehringer Mannheim) were used to identify carbohydrate constituents of purified binding proteins as previously described (20).

Subcellular distribution. Plasma membrane- and Golgi-enriched fractions were prepared from mouse liver homogenates as described by Croze and Morre (1). The enrichment of isolated plasma membrane and Golgi fractions was assessed by marker enzymes Na^+,K^+-ATPase (9) and galactosyltransferase (15), respectively. Ouabain-sensitive Na^+,K^+-ATPase activity was enriched at least 10-fold in plasma membrane fractions, and galactosyltransferase activity was enriched 80-fold in Golgi fractins over that in the total homogenate. PE-binding proteins were detected either by PE blot assay or by ELISA (20).

RESULTS

Liver represents the major site of PE activity in vivo; therefore, we purified toxin-binding protein from this tissue and compared its characteristics with those of the binding protein previously purified from LM cells. Initial experiments using direct detergent extraction of liver homogenate followed by affinity chromatography on immobilized PE resulted in a high yield of PE-binding protein but significant contamination with other proteins (results not shown). When crude liver membranes were applied to discontinuous sucrose gradients prior to detergent extraction, PE-binding activity was greatly enriched in material collected at the 32.5–10% sucrose interface as estimated by PE blot assay. Most of the PE-binding protein was found in this fraction, as determined by protein staining of SDS gels. This material, when solubilized with NP-40 and subjected to affinity chromatography, yielded homogeneous PE-binding protein when assessed by SDS-PAGE and Coomassie staining (results not shown). Figure 1A shows a high-molecular-weight protein in liver preparations with the same electrophoretic mobility as the binding protein purified from LM cells. Purified liver binding protein showed low levels of contamination with other proteins by silver staining. Virtually all trace contaminating proteins also bound to and eluted from a Sepharose 4B column prepared without PE (results not shown). None of these contaminating proteins could bind PE in the blot assay.

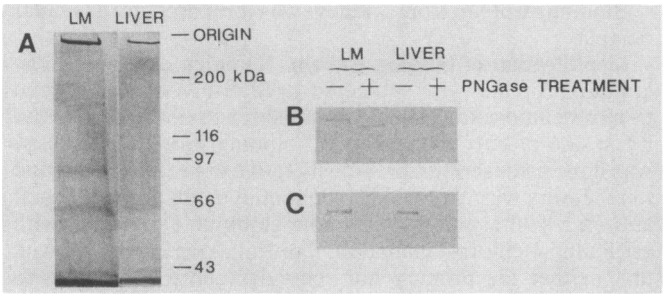

FIG. 1. Comparison of toxin-binding proteins prepared from mouse liver and LM cells. Affinity-purified binding proteins were subjected to SDS-PAGE on a 6% separating gel and then silver stained (A) or transferred to nitrocellulose paper. PE-binding proteins were detected by sequential incubation with PE, goat anti-PE, and swine anti-goat immunoglobulin G labeled with HRP (PE blot assay) (B). Carbohydrate was detected with a commercially available hydrazide-digoxigenin glycan detection kit (C). +, pretreatment of 5 μg of toxin-binding protein with 0.5 U of PNGase F for 1 h at 37°C as described previously (20); −, identical incubation in the absence of enzyme. The molecular mass markers are myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), and ovalbumin (43 kDa).

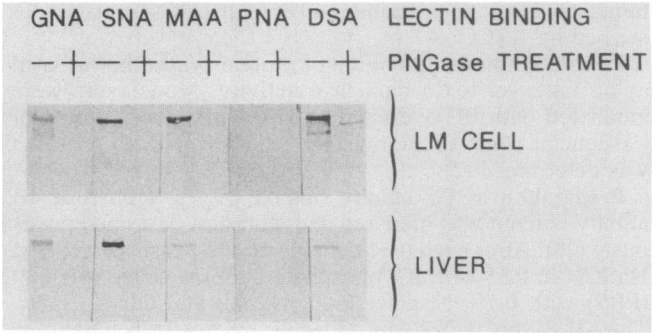

FIG. 3. Identification of carbohydrate in purified PE-binding protein. Affinity-purified binding proteins from LM cells and liver were electrophoresed on a 6% SDS gel and transferred to nitrocellulose paper, and the blot was then probed with the digoxigenin-labeled lectins indicated. Binding protein was incubated with (+) or without (−) PNGase F prior to electrophoresis as described in Materials and Methods.

We have demonstrated two ways that PE binds specifically to toxin-binding protein. First, binding of biotinyl-PE to the 350-kDa protein from liver or LM cells was blocked in the presence of a 100-fold excess of native toxin (Fig. 2). In addition, toxin specifically bound to immobilized liver binding protein in an ELISA format, as shown previously for a purified LM cell preparation (results not shown; 20).

Distribution of toxin-binding protein. The plasma membrane and Golgi apparatus were isolated from mouse liver (1). The plasma membrane and Golgi membranes contain similar levels of toxin-binding activity as measured by the SDS-PAGE PE blot assay (results not shown). Analysis by ELISA of gradient interfaces prepared by the method of Marynen et al. (6) also showed distribution of toxin-binding protein between Golgi-enriched and plasma membrane-enriched fractions.

Glycosylation of PE-binding proteins. The toxin-binding proteins purified from LM cells and from mouse liver are glycosylated, as measured both by hydrazide-digoxigenin and specific lectin-digoxigenin labeling. PNGase F pretreatment of toxin-binding proteins from both liver and LM cells increased their mobility identically (Fig. 1B). Nearly all carbohydrate was removed with this treatment (Fig. 1C). As previously found for LM cell binding protein, removal of carbohydrate did not significantly reduce PE binding (Fig. 1B), suggesting that carbohydrate is not required for toxin binding.

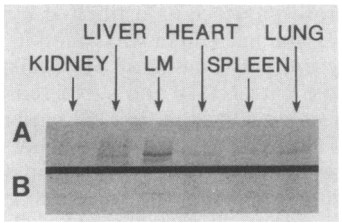

FIG. 2. Specificity of PE binding to toxin-binding proteins. Affinity-purified toxin-binding protein from kidney (0.6 μg), liver (1.4 μg), or LM cells (1.0 μg) and detergent extracts of heart, spleen, and lung (approximately 100 μg each) were subjected to SDS-PAGE and transferred to nitrocellulose paper. The blot was incubated with biotinyl-PE in the absence (A) or presence (B) of a 100-fold excess of native toxin (biotinyl-PE blot assay).

We compared the specific glycosylation patterns of purified binding proteins from LM cells and liver by using a panel of digoxigenin-labeled lectins (Fig. 3). Both glycoproteins reacted with *Galanthus nivalis* lectin (GNA) and extremely strongly with *Sambucus nigra* lectin (SNA); reactivity was removed following treatment with PNGase F. These data suggest that binding proteins from both sources contain high-mannose N-linked oligosaccharides and abundant sialic acid, terminally linked α(2-6) to either galactose or *N*-acetylgalactosamine in complex N-glycan chains. LM cell binding protein reacted strongly with *Maackia amurensis* agglutinin (MAA) and *Datura stramonium* agglutinin (DSA); in contrast, liver PE-binding protein reacted weakly with these two lectins (Fig. 3). These data suggest that the liver PE-binding protein has fewer complex N-glycan structures without terminal sialic acid and less sialic acid terminally linked α(2-3) to galactose than the LM cell binding protein.

When either of the toxin-binding proteins was treated with neuraminidase, sialic acid residues were removed, as determined by loss of reactivity with both MAA and SNA (results not shown). Following this treatment, LM cell toxin-binding protein was strongly recognized by peanut agglutinin (PNA), while recognition of liver toxin-binding protein by PNA was very faint. These data suggest that LM cell binding protein contains O glycans.

We have begun to examine the carbohydrate component of purified kidney binding protein and find that it is significantly different from either LM cell or liver PE-binding protein. Preliminary experiments show that kidney binding protein reacted strongly with PNA (without prior treatment with neuraminidase) and DSA but not with the other lectins. This suggests that the glycosylation pattern of PE-binding proteins may be unique to different tissues.

Proteolytic treatment of toxin-binding proteins. Limited digestion of purified LM cell and liver PE-binding proteins by *Staphylococcus aureus* V-8 protease generated several distinct lower-molecular-weight bands, visible on SDS-PAGE, which retained the ability to bind PE (Fig. 4A). Virtually identical fragmentation patterns for the binding proteins were found, suggesting a similar protein core. Figure 4B shows that complex glycan structures identified

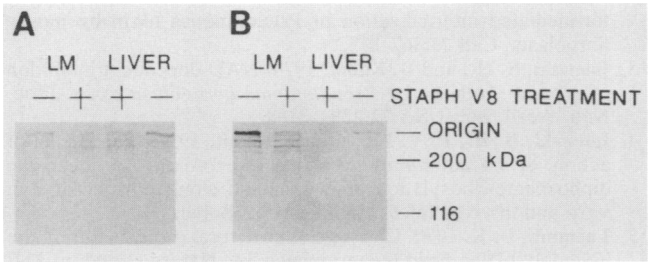

FIG. 4. Effect of limited digestion of LM cell and mouse liver PE-binding proteins by staphylococcal V-8 protease. Affinity-purified binding proteins (3 μg) from liver and LM cells were incubated with 50 ng of staphylococcal V-8 protease for 2 h at 25°C prior to SDS gel electrophoresis. Proteins were electrophoretically transferred to nitrocellulose paper, which was assayed for toxin-binding activity by the PE blot assay (A) or probed with digoxigenin-labeled DSA (B).

by DSA reactivity with LM cell binding proteins also are present on toxin-binding proteolytic fragments.

Tissue distribution of toxin-binding protein. Detergent extracts of several mouse tissues were assayed by the SDS-PAGE toxin blot method for the presence of PE-binding protein (Fig. 5). Detergent extracts from all tissues examined had a toxin-binding species which moved with the same electrophoretic mobility as affinity-purified toxin-binding protein. Unlike affinity-purified binding protein, whole tissue extracts typically had a second toxin-binding species of slightly greater electrophoretic mobility. On occasion, PE binding to an approximately 120-kDa protein in heart extract was detected.

As already indicated, PE binding is specific (Fig. 2). Binding of biotinyl-PE to the 350-kDa protein present in tissue extracts from heart, spleen, and lung or to PE-binding protein purified from liver or kidney was blocked in the presence of a 100-fold excess of native toxin.

Correlation of PE-binding protein with toxicity in established cell lines. The toxin sensitivity of established tissue culture cell lines was compared with the presence of toxin-binding activity in detergent extracts of these cells (Table 1). The biotinyl-PE blot assay is qualitative, and the presence of PE binding was estimated by using a scale from − to 3+ to measure the relative intensity of staining. Ovcar-3 cells were highly resistant to PE and lacked almost all demonstrable toxin-binding activity by PE blot assay. HeLa and Vero cells exhibited moderate toxin sensitivity, and Chang liver cells were nearly as sensitive to PE as were LM cells. Detergent extracts of HeLa and Vero cells had low levels of the 350-kDa toxin-binding protein by the PE blot assay, while Chang cell extracts had activity comparable to extracts from LM cells.

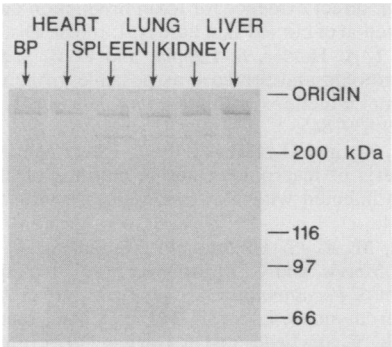

FIG. 5. Distribution of PE-binding moiety in mouse tissue. NP-40 detergent extracts of various tissues were prepared as described in Materials and Methods. Toxin binding was detected after SDS gel electrophoresis by the PE blot assay. Lane BP (purified binding protein from LM cells) contained 1 μg of protein. All other lanes contained between 112 and 120 μg of tissue protein. Samples were electrophoresed in a 6% separating gel.

TABLE 1. Correlation of presence of PE-binding protein with cultured-cell sensitivity to PE

Cell line	TCD_{50}[a] (ng/ml)	PE binding[b]
OVCAR-3	180	+/−
HeLa	32	+
Vero	11	+
Chang liver	0.52	+++
LM	0.11	+++

[a] TCD_{50} is the amount of PE required to inhibit protein synthesis by 50% compared with synthesis in control cells.

[b] Relative intensity estimated visually from SDS-PAGE, biotinyl-PE blot assay.

DISCUSSION

PE is one of several recognized virulence factors produced by *P. aeruginosa* and is especially important in the pathology seen following burn infections in experimental animals. PE is found in sera of burned mice infected with *P. aeruginosa* (17). Production of toxin in burned infected mice results in a rapid and nearly total depletion of liver elongation factor 2 and an inconsistent depletion in other tissues (18). In addition, Pavlovskis and Shackelford (12) reported that intravenous injection of mice with purified PE results in a 50% reduction of protein synthesis in the liver within 4 h and nearly complete inhibition shortly before death. Protein synthesis in the kidneys and spleen is reduced 50% during the terminal stages of intoxication. While the liver appears to show the most profound effect of the toxin, the majority of the toxin is cleared by the kidneys, where it is degraded (12). Similarly, Iglewski et al. (4) have shown that intraperitoneal injection of purified PE reduces the level of elongation factor 2 in all organs examined, except the brain, but that the largest reduction is found in the liver.

We have demonstrated the presence of related high-molecular-weight PE-binding proteins in extracts from mouse heart, spleen, kidney, lung, and liver tissue. The fact that specific PE-binding proteins are associated with each of these tissues could account for their sensitivity to the toxin (4, 12, 18). At least partial reduction in elongation factor 2 content is found in all of these organs in PE-intoxicated mice (4); differences in levels of inhibition could be explained by differences in distribution of the toxin via the circulation, by different processing of the toxin by cells, or by a difference in the number or affinity of PE receptors in the tissues.

Because of the susceptibility of mouse liver to PE intoxication, we focused our attention on possible receptors for PE in this organ. The glycoprotein we have identified has characteristics strikingly similar to the PE-binding protein previously purified from mouse LM cells (20). The toxin-binding proteins purified from LM cells and mouse liver share identical electrophoretic and protease digestion patterns, suggesting a similar if not identical protein core. These proteins also are immunologically related, since both proteins bind antisera raised against the purified mouse LM

binding protein (results not shown). The toxin-binding characteristics of liver and LM cell binding proteins as determined by PE blot assay and by ELISA also are similar. The liver PE-binding protein appears to differ from LM binding protein only in glycosylation. We have two lines of evidence that carbohydrate is not required for PE binding to either protein. Removal of carbohydrate by pretreatment of LM or liver binding protein with PNGase F did not alter subsequent PE binding (Fig. 1; 20). Furthermore, initial experiments in which LM cells were incubated with tunicamycin, an antibiotic which blocks the formation of N-glycosidic-type protein-carbohydrate linkages (19), caused no alteration in the cell's biological response to PE.

The LM cell binding protein has a more complex glycosylation pattern than that purified from either liver or kidney. These differences may reflect alternate forms of protein processing in differentiated versus cultured cells. We have not looked at the role of carbohydrate in receptor compartmentalization or routing, nor have we determined if removal of carbohydrate alters the kinetics of PE binding.

At present we have no definitive proof that the PE-binding protein is the receptor responsible for internalization of toxin molecules which ultimately ADP-ribosylate elongation factor 2. However, several observations suggest that it is an appropriate receptor candidate. To function in entry, the toxin receptor must be located at least in part on the cell surface. We have reported previously that trypsin treatment of LM cell monolayers reduces both the amount of toxin-binding activity in cell detergent extracts (20) and the sensitivity of LM cells to PE by over 2 logs (data not shown). Furthermore, toxin-binding protein was found in plasma membrane-enriched fractions of both liver and LM cells. Interestingly, binding protein also was associated with Golgi- and endoplasmic reticulum-enriched membrane fractions. On the basis of these data, we conclude that the PE-binding protein is located not only on the plasma membrane but also exists in intracellular pools.

We found the toxin sensitivity of established tissue culture cell lines correlated strikingly with the presence of toxin-binding activity in detergent extracts of these cells. The OVCAR-3 cells are at least 3 logs more resistant to PE than are LM cells and lack demonstrable specific toxin-binding activity in the biotinyl-PE blot assay. Detergent extracts of human Chang liver cells, which are nearly as sensitive to PE as are LM cells (22), demonstrate equivalent PE binding by the blot assay. Thus, toxin sensitivity apparently parallels the presence of this 350-kDa toxin-binding moiety in cultured cells. Finally, incubation of PE with increasing concentrations of purified toxin-binding protein reduces its lethality for LM cells in a dose-dependent fashion (20). All of these observations provide indirect evidence that the binding protein is a functional PE receptor. Continued structural and functional analysis of this glycoprotein will be required to prove that it is the receptor which internalizes the toxin molecules involved in inhibition of protein synthesis.

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