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Level of Receptor-Associated Protein Moderates Cellular Susceptibility to Pseudomonas Exotoxin A

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Pseudomonas exotoxin A (PE) enters mammalian cells via a receptor-mediated endocytic pathway. The initial step in this pathway is binding to the multiligand receptor termed the α_2-macroglobulin receptor/low-density lipoprotein receptor-related protein (LRP). Binding of toxin, and of the many other ligands that bind to LRP, is blocked by the addition of a 39-kDa receptor-associated protein (RAP). Here we show that approximately 40% of the cell-associated LRP is on the surface of toxin-sensitive mouse LM fibroblasts and thus accessible for toxin internalization. The remainder is located intracellularly, primarily in the Golgi region. Mammalian cells exhibit a wide range of sensitivity to PE. To investigate possible reasons for this, we examined the expression levels of both LRP and RAP. Results from a variety of cell lines indicated that there was a positive correlation between LRP expression and toxin sensitivity. In the absence of LRP, cells were as much as 200-fold more resistant to PE compared with sensitive cells. A second group of resistant cells expressed LRP but had a high level of RAP. Thus, a toxin-resistant phenotype would be expected when cells expressed either low levels of LRP or high levels of LRP in the presence of high levels of RAP. We hypothesize that RAP has a pivotal role in moderating cellular susceptibility to PE.

Pseudomonas exotoxin A (PE) is one of several potential virulence products produced by *Pseudomonas aeruginosa* and has been shown to be toxic for a variety of mammalian cells. The ultimate action of the toxin results in blockage of protein synthesis and cell death. The toxin enters sensitive cells by receptor-mediated endocytosis. The first step in this process is binding to a specific receptor on the cell surface (34). Toxin then moves to coated pits, is internalized into endosomes, and is delivered at least in part to the Golgi region of the cell. During this journey some of the toxin is cleaved to generate an enzymatically active fragment, which ADP-ribosylates cytoplasmic elongation factor 2 (10, 12, 23, 30, 40, 42).

Genetic and crystallographic studies have shown that the toxin is composed of three domains, each with a unique function (1, 22, 50, 57). The amino-terminal domain contains sequences which mediate receptor binding, the carboxyl-terminal domain contains the catalytic subunit responsible for ADP-ribosylation of elongation factor 2, and the middle domain is involved in processing and translocation into the cytosol (5, 6, 14, 22, 49, 50, 57). Native toxin is a proenzyme and must be activated to exhibit enzyme activity in cell extracts. In vitro pretreatment with urea and dithiothreitol activates the toxin by unfolding the protein (30, 32). In vivo, the toxin appears to be nicked in endosomes, with reduction of the disulfide bond and generation of the active fragment occurring at a later stage (12, 42; unpublished data). While the site of escape into the cytosol has not been fully defined, biochemical data suggest that the endoplasmic reticulum may be involved in toxin translocation (5). However, electron microscopic studies (40) and subcellular fractionation studies (unpublished data) have failed to show measurable amounts of toxin in this compartment.

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The first step in the intoxication process is binding to a cell surface receptor. We have purified and characterized this receptor (11, 54) and have shown that the toxin receptor is immunologically and functionally identical to the α_2-macroglobulin receptor/low-density lipoprotein receptor-related protein (LRP) (25). Willnow and Herz reported that murine embryonic fibroblasts which were selected for resistance to PE lacked LRP, thus confirming the role of receptor in toxicity (62). More recently, FitzGerald et al. have described a line of Chinese hamster ovary cells which have no detectable LRP, cannot internalize α_2-macroglobulin-chymotrypsin, and are highly resistant to PE (10).

LRP is a multifunctional cell surface receptor which mediates the clearance from the circulation of α_2-macroglobulin complexed with proteolytic enzymes and of apoE-enriched β-very low density lipoproteins (2, 17, 27, 28, 52, 60). Several other ligands which bind to LRP have been identified. These include complexes of plasminogen activators and their inhibitors (15, 31, 45), bovine milk lipoprotein lipase (4), and lactoferrin (21, 61). In addition, a minor-group cold virus, HRV2, binds to and is internalized by the LRP on human fibroblasts from a patient with familial hypercholesterolemia (19). Thus, this receptor is truly a scavenger receptor which is capable of clearing a variety of ligands from the circulation. In addition, LRP has been shown to be essential for early development of the mouse embryo (15).

LRP is a large glycoprotein found in most cultured cells and in various animal tissues (27, 60). It is synthesized as a 600-kDa molecule that is cleaved in the Golgi region to yield a light chain of 85 kDa and a heavy chain of 515 kDa. The 515-kDa heavy chain is responsible for ligand binding, while the 85-kDa subunit anchors the receptor complex in the plasma membrane and may mediate receptor internalization (18). A 39-kDa receptor-associated protein (RAP) has been shown to copurify with LRP. RAP interacts with the 515-kDa subunit after synthesis and is believed to remain associated with the receptor as

it is transported to the cell surface (38, 53). RAP has been hypothesized to regulate ligand binding of LRP or to aid in the processing and/or trafficking of the receptor (26, 58, 60). We have shown previously that exogenously added RAP blocks the binding of PE to LM cells and protects these cells from PE-mediated inhibition of protein synthesis (25). RAP also blocks the interaction of other ligands with receptor (7, 15, 16, 61).

Mammalian cells and tissues exhibit a spectrum of susceptibility to pseudomonas exotoxin. This is most evident in an animal model following infection with viable *P. aeruginosa* or injection of purified toxin. In this situation, the liver is the primary target of the toxin, with protein synthesis and functional elongation factor 2 depleted by over 90%. Other organs are variably affected (24, 46, 48, 51). Established tissue culture cell lines also display a spectrum of sensitivity to toxin action (36, 37). Resistance to toxin could be based on lack of susceptibility of elongation factor 2 to ADP-ribosylation, inefficient processing of toxin by resistant cells, the absence of LRP, or endogenous RAP interfering with either the transport of receptor to the cell surface or the binding of toxin to receptor. In this paper, we focus on the role of RAP in determining the susceptibility of mammalian cells to PE.

MATERIALS AND METHODS

Materials. LRP was purified from mouse tissues or tissue culture cells as described previously (11, 54). RAP was prepared as a fusion protein with glutathione *S*-transferase, using the pGEX-2T vector as described before (58). PE and goat anti-PE were purchased from List Biochemicals. Swine anti-goat immunoglobulin G-horseradish peroxidase (HRP) conjugate and goat anti-rabbit immunoglobulin G-HRP conjugate were purchased from Boehringer Mannheim and Kirkegaard and Perry Laboratories Inc., respectively. Peroxidase-free bovine serum albumin was purchased from C-C Biotech Corp., Poway, Calif.

Polyclonal antibodies against the LRP heavy chain were prepared by immunizing rabbits with the 515-kDa human LRP excised from sodium dodecyl sulfate (SDS) gels, as described before (25). Rabbit anti-human placental RAP (Rb 80) and polyclonal antibody to the PE receptor were prepared as described previously (25, 53). A mouse monoclonal antibody which recognizes only human RAP was prepared as described previously (26). A specific anti-endoplasmic reticulum antibody (RIboI) was the kind gift of Günter Blobel and Christopher Nicchitta (41).

Cells. Mouse LM fibroblasts (ATCC CCL 1.2 L-M) were maintained in McCoy's 5A medium containing 10% heat-inactivated fetal calf serum, penicillin, and streptomycin. NIH/OVCAR 3 cells were isolated from the malignant ascites of a patient with ovarian carcinoma and were grown in RPMI medium containing 10% fetal bovine serum, penicillin, and streptomycin. HeLa, Chang liver (human; CCL 13), NIH Swiss 3T3, Vero (CCL 81; monkey kidney), and DU 145 (HTB 81; human prostate carcinoma) cells were maintained in minimal essential medium with 10% fetal calf serum, vitamins, and antibiotics. LoVo (CCL 229; human colon adenocarcinoma) cells were maintained in Hams F12 with 7% fetal calf serum and antibiotics. H441 (ATCC HTB 174, human lung) and Chinese hamster ovary (CHO) cells were maintained in RPMI medium with 10% fetal calf serum and antibiotics.

Protein synthesis. Inhibition of protein synthesis was used as an index of toxin biological activity. Monolayers were incubated with PE in Hanks balanced salt solution-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) either overnight at 37°C or for 1 h at 4°C, followed by a wash and reincubation in fresh medium for 5 h at 37°C. At the end of the incubation period, monolayers were washed, tritiated leucine (2 μCi/ml in leucine-free medium) was added for 60 min at 37°C, and cells were processed as described previously (9).

Ligand blotting. Cell monolayers from various cell lines were collected by scraping, homogenized in 0.25 M sucrose–10 mM Tris-HCl, separated on SDS-polyacrylamide (6 or 12%) gels, and transferred to nitrocellulose membranes (29, 55). The membrane was first blocked with 10% milk (Flavorite non-fat dried) in phosphate-buffered saline (PBS), pH 7.0. Proteins were identified as follows. (i) LRP was identified by immunoblotting, using a 1:1,000 dilution of affinity-purified rabbit polyclonal antibody against LRP, followed by goat anti-rabbit immunoglobulin G-HRP conjugate (diluted 1:2,000 in PBS, pH 7.4). The substrate was 4-chloro-1-naphthol (Bio-Rad Laboratories). (ii) For toxin binding, nitrocellulose paper was incubated sequentially with PE, goat anti-PE (List Biochemicals), and swine anti-goat peroxidase as described before (25). (iii) RAP was identified by immunoblotting, using a 1:200 dilution of affinity-purified rabbit anti-39-kDa protein and then goat anti-rabbit immunoglobulin G conjugated to HRP.

In some experiments, Nonidet P-40 detergent-solubilized LRP was prepared from mouse tissue as described previously (54).

In some experiments, a Bio-Rad scanning densitometer was used to quantitate the immunoblots.

Subcellular fractionation. Monolayers were maintained in culture for 2 days before use. Cells were harvested by scraping in 0.25 M sucrose–10 mM Tris HCl (pH 7.2), collected by centrifugation, and homogenized in 1 ml of 0.25 M sucrose–10 mM Tris HCl by 100 strokes with a Dounce homogenizer with a tight-fitting pestle (on ice). The homogenate was centrifuged at 1,500 × *g* for 20 min, and the resulting supernatant (1 ml) was layered on a 10 to 65% linear sucrose gradient containing 10 mM Tris HCl. The gradient was centrifuged at 4°C for approximately 18 h at 100,000 × *g* in a Beckman SW41 rotor. One-half-milliliter fractions were collected from the bottom of the gradient, and each fraction was assayed for LRP and RAP as described above.

In some experiments, cell monolayers were incubated with 1.0% trypsin–10 mM EDTA in Hanks balanced salt solution for 60 min at 4°C, collected, and washed several times with buffer containing 0.5% soy bean trypsin inhibitor in sucrose-Tris buffer.

The following enzyme activities were assayed by using established protocols: galactosyltransferase (3) and β-glucuronidase (13) to identify Golgi apparatus and lysosomes, respectively. Endoplasmic reticulum was identified by reaction with antibody to ribophorin I (41). Endosomes were identified by allowing cells to internalize HRP (1 mg/ml) for 18 h at 15°C (20). Plasma membrane was identified by the binding of HRP-labeled concanavalin A to cells at 4°C or following surface biotinylation. Cell surfaces were labeled with Sulfo-*N*-succinimide biotin as follows. Monolayers were cooled to 4°C, washed three times with bicarbonate buffer (0.1 M $NaHCO_3$, 0.1 M NaCl, pH 8.2), and incubated with fresh Sulfo-*N*-hydroxysuccinimide biotin (no. 21217; Pierce Chemical Co., Rockford, Ill.) for 4 h at 4°C. Monolayers were then washed with bicarbonate buffer and with 0.01 M PBS (pH 7.2) and incubated with streptavidin-peroxidase (diluted 1:500 in PBS containing 3% bovine serum albumin). Incubation was continued for 1 h at 4°C prior to extensive washing of monolayers. Two peaks enriched in plasma membrane were identified. The lighter peak represented the one in which we detected PE binding at 4°C; since this toxin was susceptible to removal by trypsin, this peak was considered the major site of surface-exposed plasma membrane.

Measurements of surface and intracellular RAP. Measurements of surface and intracellular RAP were performed essentially as described previously (59). Flasks, 75 cm^2, of cells were grown to confluence and washed three times with 10 ml of PBS. The cells were then rinsed with 1 to 2 ml of PBS containing 10 mM EDTA. The wash solution was collected and concentrated with Centricon 10 filter units to a final volume of 1.0 ml, and Nonidet P-40 (final concentration, 0.1%) was added prior to assay. The cells were removed from the dishes by scraping into 5 ml of PBS and centrifuged, and the resulting pellet was brought to 1 ml in PBS containing leupeptin and phenylmethylsulfonyl fluoride. This solution was pipetted several times through a 22-gauge syringe needle, brought to 0.1% Nonidet P-40, and centrifuged for 15 min at 4°C. The resulting supernatant was collected and was considered to represent intracellular RAP. The concentrated EDTA wash of the cells was considered to represent surface-bound RAP. EDTA treatment released neither lysosomal marker enzyme nor cytoplasmic marker enzyme (lactic acid dehydrogenase; Boehringer Mannheim kit 1644793) over that released by treatment with PBS alone.

Quantitation of RAP by ELISA. Quantitation of RAP by enzyme-linked immunosorbent assay (ELISA) was performed as described previously (59). Plastic microtiter plates were coated overnight with 3 μg of monoclonal antibody 7F1 per ml and blocked in Tris-buffered saline–3% bovine serum albumin for 1 h at room temperature. Cell extracts, concentrated cell washes, or gradient fractions to which 0.1% Nonidet P-40 had been added were added to the plate in several dilutions in triplicate. Dilutions of purified RAP in Tris-buffered saline were used as a standard. After overnight incubation at 4°C, the plate was washed three times with Tris-buffered saline–0.05% Tween 20 (wash buffer), and a 1:1,000 dilution of Rb 80 was added for 1 h at room temperature in incubation buffer. The plates were washed three times, and a 1:1,000 dilution of a goat anti-rabbit HRP-conjugated antibody was added for 1 h at room temperature. The plates were washed three times, substrate (*o*-phenylenediamine dihydrochloride; Sigma Fast Tablets) was added, the reaction was stopped with H_2SO_4 buffer, and the color reaction was monitored at 492 nm.

RESULTS

Correlation of toxin sensitivity with LRP expression. Mammalian cells of various origins naturally exhibited as much as a 300-fold differential sensitivity to PE (Table 1). Mouse fibroblast lines such as LM and Swiss 3T3 cells were the most sensitive to PE. Fifty percent tissue culture lethal doses of less than 1 ng/ml were seen routinely for an 18-h incubation with toxin. Chang liver and Vero cells exhibited intermediate sensitivity, while HeLa, DU145, OVCAR 3, lung H441, LoVo, and CHO cells were relatively resistant, requiring at least 200 times more toxin to inhibit protein synthesis than was seen with LM cells. Possible reasons to explain toxin resistance include the

TABLE 1. Differential sensitivity of cells to PE

Cell line	50% tissue culture lethal dose (ng/ml)	
	18 h[a]	5 h[b]
LM	0.13	11
Swiss 3T3	0.90	21
Chang	3.5	1,000
Vero	11	1,250
HeLa	32	ND[c]
DU 145	30	>2,000
OVCAR	50	>1,000
Lung 441	ND	>5,000
LoVo	>50	ND
CHO	45	>5,000

[a] Cell monolayers were incubated for 18 h at 37°C with various concentrations of PE, toxin was removed, and protein synthesis was measured by a 60-min pulse with titrated leucine.

[b] Cell monolayers were cooled to 4°C, and PE was added for 60 min (4°C). Monolayers were washed and reincubated in medium for 5 h at 37°C prior to measurement of protein synthesis.

[c] ND, not determined.

absence of receptor protein or the presence of a nonfunctional receptor. To address this, we determined receptor levels in all cells and attempted to correlate the presence of LRP with toxin susceptibility (Fig. 1). In the experiments shown here, receptor was identified with antibody to LRP. Similar receptor distribution was obtained by probing for the ability to bind PE (not shown). As expected, toxin-sensitive cells, such as LM and Swiss 3T3, had high levels of receptor. Little or no receptor was detectable in HeLa, LoVo, and 441 cells, and thus their resistance could be explained easily. Of more interest were CHO, DU145, and OVCAR 3 cells, which were highly resistant to PE, despite the fact that they had moderate levels of receptor. One possibility was that all receptor was located intracellularly and toxin had no access to it. Another explanation was that the

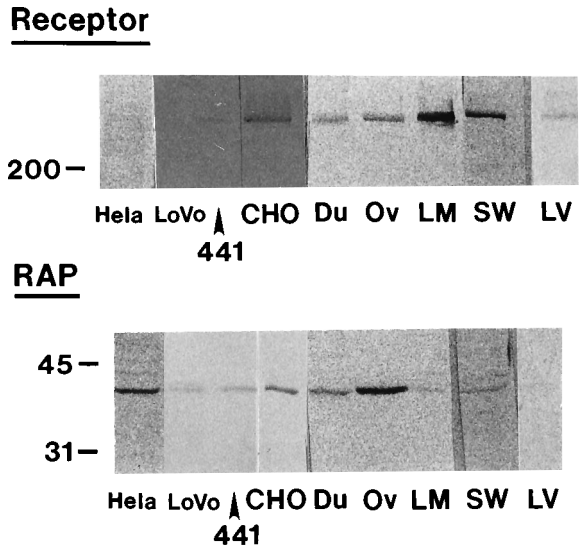

FIG. 1. LRP and RAP concentration in PE-sensitive and -resistant cells. Cell homogenates from several cell lines were subjected to SDS-polyacrylamide gel electrophoresis, and LRP or RAP was identified as indicated. Approximately 25 μg of protein was applied in a lane. (A) 6% gel; receptor identified using rabbit polyclonal antibody to LRP. (B) 12% gel; RAP identified using rabbit polyclonal antibody to RAP. HeLa, human; LoVo, human adenocarcinoma; 441, human lung; Du, Du 145 human prostate carcinoma; OV, OVCAR 3; LM, mouse fibroblast; SW, NIH Swiss 3T3; LV, detergent extract of intact mouse liver.

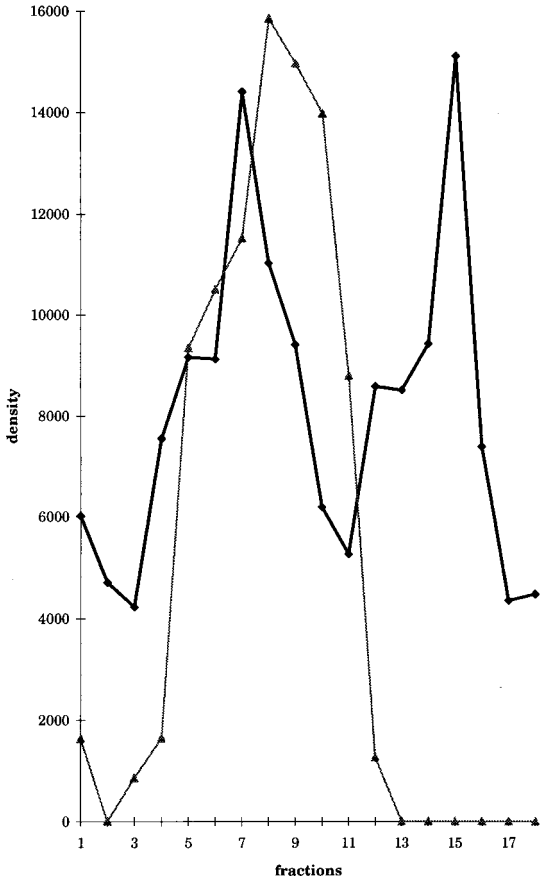

FIG. 2. Subcellular localization of LRP in LM cells. Mouse LM cell monolayers were collected and subjected to subcellular centrifugation, and fractions were assayed for the presence of LRP by polyacrylamide gel electrophoresis and immunoblotting with the appropriate polyclonal antibody. One set of cells was incubated with 1.0% trypsin prior to collection (▲). ◆, no trypsin treatment. The most dense fraction is fraction 1. Fractions 2 to 5, endoplasmic reticulum; fractions 6 to 9, galactosyltransferase; fractions 5 and 6 and 15 and 16, plasma membrane; fractions 17 and 18, endosomes.

LRP was present on the surface but not available for toxin binding, due to the presence of RAP.

Correlation of toxin resistance with RAP expression. Since exogenously added RAP is known to interfere with PE binding to LRP and reduce PE toxicity (25), the levels of cell-associated RAP were investigated. Results from immunoblot analyses indicated that there was a good correlation between the amount of RAP present and the degree of toxin resistance (Fig. 1). Toxin-sensitive cells had minimal levels of RAP, while toxin-resistant cell lines, which had significant levels of receptor, also had significant levels of RAP. While the exact stoichiometric relationship remains to be determined, it is clear that endogenous RAP has a toxin-protective effect for cells.

LRP is present in many tissues but is thought to function as a scavenger receptor primarily in the liver. Also, animals injected with PE apparently die of liver failure, presumably because these cells bind and internalize PE with higher efficiency than other tissues (24, 46, 48, 51). To investigate receptor-RAP expression in the liver, mice were killed and liver tissue was solubilized by detergent extraction. Expression of both LRP and RAP was detected by immunoblot analysis (Fig. 1). Results indicated that liver tissue has receptor but little or no RAP, which correlates with its sensitivity to toxin.

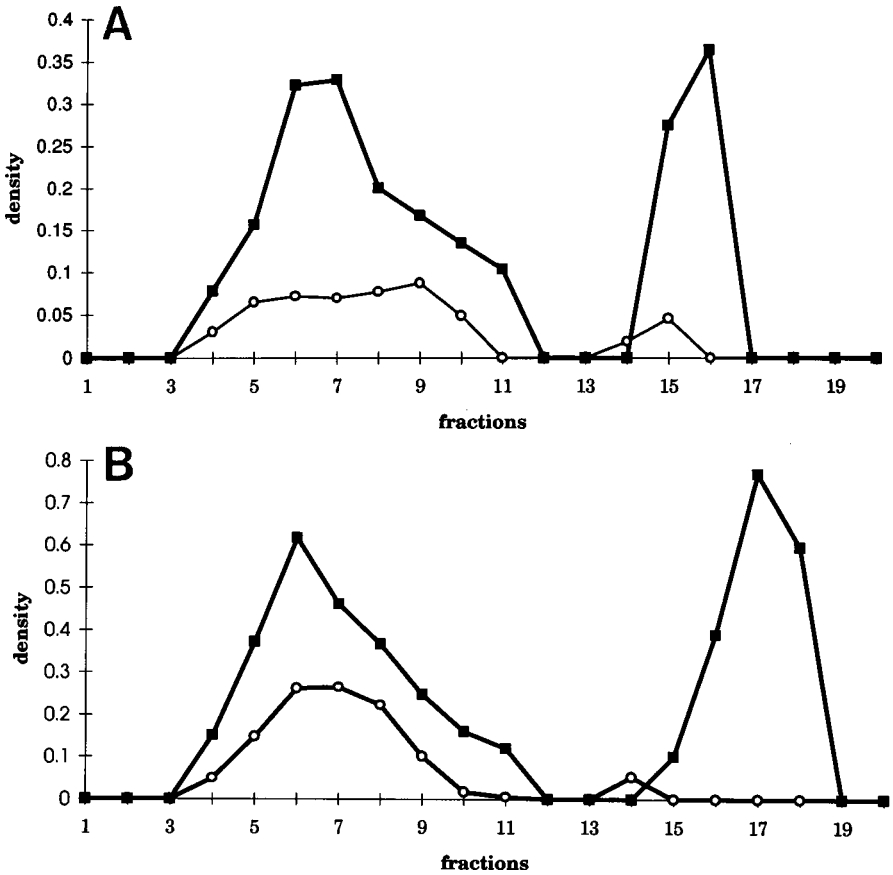

FIG. 3. Colocalization of RAP and LRP in toxin-resistant cells. Chang (A) and DU 145 (B) cells were harvested and subjected to sucrose density gradient centrifugation. Fractions were assayed by SDS-polyacrylamide gel electrophoresis, and RAP (■) or LRP (○) was identified by immunoblotting with the appropriate polyclonal antibody.

Subcellular localization of receptor and RAP. To prevent ligand binding, RAP would need to be associated with the receptor on the cell surface. Initially, receptor and RAP were localized in toxin-sensitive LM cells by using sucrose density gradient centrifugation (Fig. 2). Two peaks of receptor protein were determined, one (fraction 15) comigrating with the major peak of plasma membrane activity and another (fractions 7 and 8) comigrating with galactosyltransferase, the Golgi marker. The lighter LRP peak was obliterated when cells were incubated with trypsin, confirming that this represents surface-associated receptor. While some plasma membrane marker also comigrated with the more dense peak of LRP, this LRP was not susceptible to removal with trypsin and thus is believed to be intracellular, predominantly in the Golgi region. The level of RAP in LM cells was below the level of detection (data not shown).

Treatment of LM cells with trypsin also rendered these cells transiently resistant to PE (unpublished observations). There was no evidence of a cytotoxic effect in cells treated with trypsin followed by immediate addition of toxin. However, if cells were incubated in tissue culture medium for 5 h after trypsin treatment, the addition of toxin caused an 80% reduction in protein synthesis. In addition, surface receptor was rapidly replaced following trypsin treatment and could be detected on the cell surface within 15 min post-trypsin treatment.

Since several naturally toxin-resistant cell lines had high levels of LRP, we determined the subcellular location of receptor and whether RAP was associated with receptor (Fig. 3). The majority of the LRP was detected intracellularly, with only 6 and 12% located on the surface of DU145 and Chang cells, respectively. When DU145 cell monolayers were treated with trypsin, all receptor in the lighter density fractions was removed (data not shown). In contrast to receptor, there was a definite bimodal distribution of RAP. RAP was found associated with endoplasmic reticulum and Golgi markers as well as with endosomal and plasma membrane markers.

Since there are several reports in the literature that the majority of RAP is intracellular, a variety of methods were used to prove that RAP was located on the cell surface and thus available to block binding of toxin to any receptor which is surface associated. Calcium has been shown to be required for the interaction of LRP with RAP (58), and EDTA effectively dissociates the receptor-RAP complex. Therefore, we used an EDTA release assay to measure cell surface RAP (Table 2). Treatment with EDTA released RAP from several toxin-resistant cells, suggesting that at least part of the RAP was on the cell surface. In all cases the amount of RAP released by EDTA was between 0.5 and 5% of the total cell-associated RAP.

To further examine the cellular location of RAP, we focused attention on OVCAR and DU145 cells. Trypsinization of cell monolayers, or treatment with EDTA at an acidic pH, prior to subcellular fractionation consistently reduced the amount of RAP found in the plasma membrane-endosomal fraction (Fig. 4). It should be noted that RAP was quantitated in these experiments by an ELISA, while results in Fig. 3 were obtained

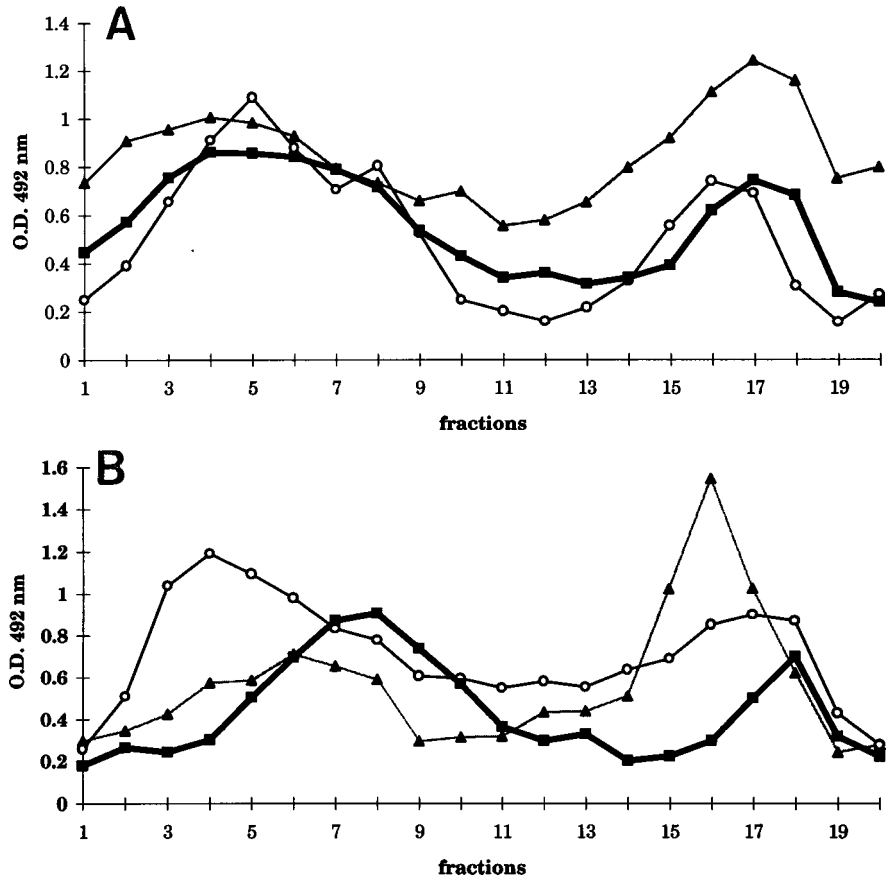

FIG. 4. Location of RAP in toxin-resistant cell lines. OVCAR 3 (A) and DU 145 (B) cell monolayers were assayed for intracellular distribution of RAP. RAP was assayed by ELISA. ▲, untreated cells; ○, cell monolayers treated with 10 mM EDTA (pH 5.5) prior to collection; ■, cell monolayers treated with 1.0% trypsin prior to collection. O.D., optical density.

by immunoblotting. These two methods rely on different antibodies to identify RAP, which could explain the different sensitivities of the assays. Taken together, these data suggest that a small but significant percentage of the total cell-associated RAP is located at the cell surface, where it would be able to block binding of toxin to the small amount of receptor which might cycle there.

Since treatment with EDTA releases surface-associated RAP, we asked if EDTA treatment would increase the amount of PE which bound to resistant cells (Table 3). Treatment of resistant cells with EDTA increased the amount of toxin binding to intact cells four- to fivefold. These data support the hypothesis that some cells are resistant to toxin because toxin binding is blocked by the presence of cell-associated RAP.

TABLE 2. Cellular localization of RAP by EDTA release assay

Cell line	RAP (pM)	
	Cell surface[a]	Intracellular[b]
Chang	9.6	1,001
DU 145	6.4	878
OVCAR	48.4	1,123
HeLa	3.3	674
LoVo	4.0	104

[a] Released by EDTA wash.
[b] Cell associated after EDTA wash.

DISCUSSION

Mammalian cells exhibit differences in their susceptibility to PE (37). While there are many possible explanations for the basis of sensitivity, a susceptible cell would be expected to have a functional receptor, be able to internalize toxin, process the molecule to an active form, and provide an environment in which this form can enter the cell cytoplasm to stop protein synthesis. We have focused on the roles of receptor and RAP in determining cellular susceptibility to toxin.

All toxin-sensitive cells and tissues which we have examined have LRP. While much receptor is located intracellularly, approximately 40% is on the cell surface where it would be able to mediate internalization of PE via clathrin-coated pits. This

TABLE 3. EDTA treatment stimulates PE binding to resistant cells[a]

Cell line	PE bound (pg/μg of cell protein)	
	−EDTA	+EDTA
LM	27.9	30.3
OVCAR	3.8	16.6
Chang	2.2	9.3
DU 145	3.2	14.5

[a] Cell monolayers were treated with 10 mM EDTA (pH 5.5) for 10 min at 4°C. Monolayers were washed and incubated with PE (1 μg/ml) overnight. Cells were harvested, and PE was assayed by ELISA.

biphasic distribution of receptor also was suggested by Moestrup et al. (39) in fluorescent microscopic studies of human tissue samples. In LM cells, surface-associated receptor is removed by trypsin treatment but is rapidly replaced when cells are incubated in tissue culture fluid. It is assumed that this is due to trafficking of receptor from the large pool in the Golgi apparatus to the cell surface.

Since RAP blocks the binding of ligand to receptor (25), it would be predicted that the amount of RAP would be low in toxin-sensitive cells. This is true in LM and Swiss 3T3 cells, which are highly susceptible to the action of the toxin. It is also true in the liver, which is the primary target of toxin in vivo. The liver also is involved in clearing complexes of α_2-macroglobulin–proteolytic enzymes and tPA PAI-1 from the circulation (56, 63). Thus, it is not surprising that the levels of RAP in liver tissue are low, allowing binding of relevant ligands to receptor.

A different picture is seen in resistant cells. In the case of HeLa, lung adenocarcinoma, and LoVo cells, there is virtually no detectable LRP. It is presumed that these cell lines are resistant to PE, because they are not able to bind toxin and internalize it via the receptor-mediated endocytic pathway. The minimal toxicity observed following long-term incubation with high levels of toxin could be due to the presence of very small amounts of surface LRP or to that toxin which is internalized nonspecifically (nonreceptor mediated) and escapes into the cell cytosol. These results complement those of Willnow and Herz (62) and FitzGerald et al. (10), who showed that cells lacking LRP were resistant to PE.

Of more interest is that several naturally toxin-resistant cells do have LRP. In the cell lines examined, most of the receptor is located intracellularly and thus presumably would not be available to bind PE. However, all of these cells also have RAP, which would be able to moderate binding of toxin. RAP is located both intracellularly and on the cell surface. Because of the high amount of RAP relative to receptor, it is assumed that receptor in resistant cells is not able to function in toxin internalization. Removal of surface-associated RAP facilitates an increase in the binding of toxin to resistant cells. Unfortunately, it has not been possible to measure the effect of EDTA treatment on toxicity. The furinlike protease involved in toxin processing is calcium dependent, and processing of PE is inhibited by EDTA treatment (12). These data support the hypothesis that one explanation for PE resistance is the presence of RAP in cells, which prevents binding to LRP.

There is some discrepancy in the literature as to the subcellular localization of RAP. The distribution appears to depend on the cell type and the method of study used. Immunohistochemical studies of rat kidney tissue and rat L2 yolk sac cells suggest that RAP is most abundant in the lumen of the endoplasmic reticulum, where it would be able to modify receptor folding and/or trafficking (33). RAP has a C-terminal endoplasmic reticulum retrieval signal which might facilitate its location in this organelle. By using cell surface radioiodination and/or immunocytochemistry, RAP has been detected on the surface of gingival fibroblasts (53), renal proximal tubule cells (47), mouse F9 tetracarcinoma cells (8), and a rat yolk sac carcinoma cell line (33, 43). Immunocytochemical localization studies by Farquhar and colleagues (44, 47) suggest that cell surface RAP can be detected only in unfixed tissue, while RAP is detectable in the rough endoplasmic reticulum in fixed tissue. However, all work suggests that the great majority of RAP is intracellular. The data presented here suggest that the majority of RAP is intracellular, associated with the Golgi apparatus as well as the endoplasmic reticulum. However, some RAP is on the cell surface, where it can modify ligand binding.

It is not known what signals are involved in directing the intracellular movement of receptor, nor is it known whether different signals are operative in resistant cells compared with sensitive cells. It could be hypothesized that in the absence of RAP, receptor is able to cycle to the cell surface, but the complexes of RAP and receptor are only inefficiently routed further than the Golgi area of the cell. Recently, Melby et al. (35) reported that several polarized cell lines are more sensitive to PE when the toxin is added to the basolateral side than when it is added apically. This would suggest, at least for these cells, that the LRP is located on the basolateral surface and that a signal for trafficking in polar cells may exist.

Mammalian cells exhibit a spectrum of sensitivity to PE. Sensitivity requires surface LRP to facilitate toxin binding and internalization by receptor-mediated endocytosis and thus entrance into the appropriate pathway for expression of toxicity. As expected, cells which lack receptor are resistant to PE because they are not able to internalize toxin by an efficient mechanism. Of more interest are resistant cells which have LRP but in which binding of toxin to receptor is blocked by the presence of excess RAP.

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