# *Pseudomonas* Exotoxin–mediated Selection Yields Cells with Altered Expression of Low-Density Lipoprotein Receptor–related Protein

David J. FitzGerald,* Charlotte M. Fryling,* Alexey Zdanovsky,* Catharine B. Saelinger,‡ Maria Kounnas,§ Jeffrey A. Winkles,§ Dudley Strickland,§ and Stephen Leppla‖

*Laboratory of Molecular Biology, National Cancer Institute, Division of Cancer Biology, Diagnosis and Centers, National Institutes of Health, Bethesda, Maryland 20892; ‡Department of Molecular Genetics, University of Cincinnati, Cincinnati, Ohio 45267; §Holland Laboratory, American Red Cross, Rockville, Maryland 20855; and ‖Dental Institute, National Institutes of Health, Bethesda, Maryland 20892

*Abstract.* The $\alpha_2$-macroglobulin ($\alpha_2$M) receptor/low-density lipoprotein receptor–related protein (LRP) is important for the clearance of proteases, protease–inhibitor complexes, and various ligands associated with lipid metabolism. While the regulation of receptor function is poorly understood, the addition of high concentrations of the 39-kD receptor-associated protein (RAP) to cells inhibits the binding and/or uptake of many of these ligands. Previously, we (Kounnas, M. Z., J. Henkin, W. S. Argraves, D. K. Strickland. 1992. *J. Biol. Chem.* 267:12420–12423) showed that *Pseudomonas* exotoxin (PE) could bind immobilized LRP. Also, the addition of RAP blocked toxin-mediated cell killing. These findings suggested that PE might use LRP to gain entry into toxin-sensitive cells. Here we report on a strategy to select PE-resistant lines of Chinese hamster ovary cells that express altered amounts of LRP. An important part of this strategy is to screen PE-resistant clones for those that retain sensitivity to both diphtheria toxin and to a fusion protein composed of lethal factor (from anthrax toxin) fused to the adenosine diphosphate–ribosylating domain of PE. Two lines, with obvious changes in their expression of LRP, were characterized in detail. The 14-2-1 line had significant amounts of LRP, but in contrast to wild-type cells, little or no receptor was displayed on the cell surface. Instead, receptor protein was found primarily within cells, much of it apparently in an unprocessed state. The 14-2-1 line showed no uptake of chymotrypsin–$\alpha_2$M and was 10-fold resistant to PE compared with wild-type cells. A second line, 13-5-1, had no detectable LRP mRNA or protein, did not internalize $\alpha_2$M–chymotrypsin, and exhibited a 100-fold resistance to PE. Resistance to PE appeared to be due to receptor-specific defects, since these mutant lines showed no resistance to a PE chimeric toxin that was internalized via the transferrin receptor. The results of this investigation confirm that LRP mediates the internalization of PE.

THE $\alpha_2$-macroglobulin ($\alpha_2$M)[1] receptor/low-density lipoprotein receptor–related protein (LRP) is among the largest single-chain proteins yet to be described. It is synthesized as a 600-kD precursor that is converted in the Golgi region to a heavy chain of 515 kD and light chain of 85 kD (Herz et al., 1988, 1990). The heavy chain, which is wholly exposed on the cell exterior, has several low-density lipoprotein receptor– and EGF-like repeats. The light chain, which is noncovalently associated with the heavy chain, contains the transmembrane domain and a cytosolic tail with sequences resembling those known to mediate receptor internalization. LRP plays a major role in the regulation of protease activity and lipid metabolism in mammalian cells. Specifically, the heavy chain is known to bind and internalize several ligands related to protease clearance and lipid metabolism. These include $\alpha_2$M–protease complexes (Jensen et al., 1989; Ashcom et al., 1990; Kristensen et al., 1990), lipoprotein lipase (Chappell et al., 1992), Apolipoprotein E-enriched βVLDL, tissue-type plasminogen activator (tPA) (Kowal et al., 1989; Bu et al., 1992) urokinase-type plasminogen activator (uPA), plasminogen activator inhibitor type 1 complexed with either tPA (Underhill et al., 1992), or uPA (Herz et al., 1992), pregnancy zone protein (Van Leuven et al., 1986), and lactoferrin (Willnow et al., 1992). An

Address all correspondence to David J. FitzGerald, Laboratory of Molecular Biology, National Cancer Institute, Division of Cancer Biology, Diagnosis and Centers, National Institutes of Health, Building 37 4B-03, Bethesda, MD 20892. Tel.: (301) 496-9457. Fax: (301) 402-1969.

1. *Abbreviations used in this paper*: $\alpha_2$M, $\alpha_2$-macroglobulin; DT, diphtheria toxin; LRP, low-density lipoprotein receptor–related protein; PE, *Pseudomonas* exotoxin; RAP, receptor-associated protein; TF, transferrin; WT, wild type.

additional protein, called receptor-associated protein (RAP), binds to the heavy chain and copurifies with it in various protocols for affinity purification of LRP (Strickland et al., 1990). While it is believed that most ligands bind to discrete portions of the heavy chain of LRP, the addition of excess RAP interferes with the binding and/or uptake of all of them. Pseudomonas exotoxin (PE) also binds LRP, and its binding to, and toxicity for, cells is reduced when excess RAP is added to the culture medium (Kounnas et al., 1992).

PE enters cells by receptor-mediated endocytosis (FitzGerald et al., 1980; Saelinger et al., 1985) and ultimately kills the cells it enters by translocating to the cell cytosol ADP-ribosylating elongation factor 2 and shutting down protein synthesis (Pastan and FitzGerald, 1989; Wick et al., 1990). Once inside cells, the toxin is processed by a furinlike protease to generate nonoverlapping fragments of 28 and 37 kD (Ogata et al., 1990, 1992; Moehring et al., 1993; Chiron et al., 1994). The 28-kD fragment is derived from the $NH_2$ terminus of the toxin and contains the toxin's binding domain. The 37-kD fragment is from the COOH terminus of the toxin and contains the translocating domain, the ADP-ribosylating domain, and the sequence REDLK at the COOH terminus, which resembles the endoplasmic reticulum retention sequence KDEL. To be toxic for cells, PE requires the presence of REDLK or KDEL at its COOH terminus (Chaudhary et al., 1990; Seetharam et al., 1991). This has implicated the ER as the organelle most likely to be involved in the translocation of the 37-kD fragment to the cytosol.

The interaction of PE with mammalian cells begins with its binding at the cell surface. Previously, we provided biochemical evidence that PE could bind to the LRP when it was immobilized on nitrocellulose or polystyrene (Kounnas et al., 1992). In ligand blots, PE interacted with the 515-kD heavy chain of the receptor and not with the 85-kD light chain (Thompson et al., 1991; Kounnas et al., 1992). Also, RAP blocked the toxicity of PE but not the toxicity of a chimeric toxin that bound and entered cells via the transferrin receptor. While these initial experiments indicated that PE could bind LRP, there was little evidence that functional LRP was required to mediate toxin entry and delivery to the cell interior. This issue was recently addressed by selection of PE-resistant clones from fibroblasts known to be heterozygous for the LRP gene (Willnow and Herz, 1994). By Southern blot analysis, some PE-resistant clones apparently had lost the wild-type (WT) LRP allele; however, others clearly retained it and were resistant for other reasons.

To investigate the role of LRP in the pathway of PE toxicity, we have selected PE-resistant cells and then screened for cells that retained sensitivity to both diphtheria toxin (DT) and to a fusion protein composed of lethal factor (from anthrax toxin) joined to domain III of PE (Arora et al., 1992). Initially, two lines out of four appeared to have alterations in receptor expression. Using a variety of ligands and antibodies to probe for receptor expression, we determined that the 13-5-1 line lacked detectable LRP protein. Northern blot analysis indicated that, in comparison to WT cells, 13-5-1 cells expressed a significantly reduced level of LRP mRNA. In functional assays, this line failed to bind or internalize chymotrypsin–$\alpha_2$M and exhibited a 100-fold increased resistance to PE. The 14-2-1 line had significant amounts of LRP, but in contrast to WT cells, little or no receptor was displayed on the cell surface. Instead, receptor protein was found primarily within cells, with much of it remaining unprocessed. There was no uptake of chymotrypsin–$\alpha_2$M into 14-2-1 cells and, when incubated with PE, these cells exhibited a 10-fold resistance compared with WT. To determine if the toxin selection and screening procedure readily identifies cells with altered expression of LRP, we examined LRP expression in other lines that were isolated using the same strategy. Characterization of five additional lines revealed that this selection strategy identifies two classes of mutants: those like 14-2-1 cells, which expressed substantial amounts of LRP but failed to process it (as evidenced by the lack of light chain reactivity), and those like the 13-5-1 line, with little or no detectable LRP.

The results presented here show that the strategic use of several bacterial toxins readily permits the identification of cell lines with alterations in receptor expression. They also confirm that PE uses LRP in its "productive" pathway to reach the cytosol and inhibit protein synthesis.

## Materials and Methods

### Reagents

Recombinant PE was expressed in *Escherichia coli* and isolated from the periplasm according to procedures described previously (Ogata et al., 1990). PE from *Pseudomonas aeruginosa* was purchased from List Biological Laboratories, Inc. (Campbell, CA). Construction of the chimeric toxin transferrin (TF)-PE40, which is composed of human TF linked by a thioether bond to a truncated portion of PE lacking the toxin's binding domain, has been described previously (Kounnas et al., 1992). A RAP–protein A fusion protein was expressed in *E. coli* from the plasmid pETA39-5 and was recovered from the soluble fraction of the cytoplasm (see below). The fusion protein was purified on an IgG sepharose column. Rabbit antibodies to the heavy chain of LRP were raised against affinity-purified human receptor as described previously (Ashcom et al., 1990), while antibodies to the light chain were raised against the last 12 amino acids of the LRP light chain (Kounnas et al., 1992).

### Construction of the RAP–Protein A Fusion Protein

Plasmid pRAP1-323 (Williams et al., 1992) was used as the template for PCR amplification of the internal SacI to EcoRI fragment. The amplified fragment was digested with these two enzymes and then ligated into pETAPA125 that had been prepared by digestion with Asp718 and EcoRI and treated with T4 polymerase. pETAPA125 has the T7 promoter at the 5′ end of the structural gene and a terminator at the 3′ end. Induction of expression was by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG).

### Isolation of PE-resistant Cells

CHO K1 cells were from Michael Gottesman (National Institutes of Health) and were grown in α-MEM supplemented with 10 mM Hepes, pH 7.5, 5% Fetal Clone II (Hyclone Laboratories Inc., Logan, UT), and 50 μg/ml gentamicin. T-75 flasks containing $5 \times 10^6$ cells were treated with 5 mM ethyl methane sulfonate in the standard MEM growth medium for 21 h. The cells were detached by trypsin treatment, diluted 1:50, plated in 100-mm dishes, and grown for 4 d to allow expression of mutations. Cells were then treated with PE at 100 ng/ml for 48 h. The medium and the dead cells (>99.9% of the total) were removed and new medium was added. After an additional 4 d, the dishes of mutagenized cells contained ~50 colonies, whereas dishes derived from nonmutagenized cells contained <5 colonies. Well-isolated colonies were picked, expanded, and tested for sensitivity to PE, DT, and the combination of anthrax protective antigen and a fusion protein of anthrax lethal factor and PE domain III (FP33) (Arora et al., 1992). Mutant cell lines that were resistant only to PE were cloned by limiting dilution and their phenotypes confirmed.

### *Immunofluorescence*

To detect total cell-associated LRP, CHO cells grown in 35-mm dishes were fixed with 2.0% formaldehyde and then permeabilized with 0.1% saponin. An affinity-purified rabbit polyclonal antibody to the receptor heavy chain (Ashcom et al., 1990) was added in the presence of saponin and normal goat globulin (4 mg/ml). After 30 min, the primary antibody was removed and cells were washed a minimum of four times each with saponin and normal goat globulin. Then rhodamine-labeled affinity-purified goat anti–rabbit IgG at 25 μg/ml was used to detect the primary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). After extensive washing, the cells were fixed with 3.7% formaldehyde and viewed at a magnification of 640×.

The presence of LRP on the surface of cells was assessed using the same affinity purified rabbit antibody to the heavy chain. Cells were chilled to 4°C and ice-cold antibody (10 μg/ml) in PBS-BSA (BSA at 2 mg/ml) was added for 30 min. At the end of this incubation period, monolayers were washed extensively with cold PBS-BSA. Surface-bound antibody was detected using rhodamine-labeled affinity purified anti–rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) at 25 μg/ml. Cells were washed extensively in PBS, fixed in formaldehyde (3.7%), and viewed at a magnification of 640. Control cells were processed in an identical manner except that the primary antibody step was omitted.

### *Ligand Blots of Membrane Extracts*

Cell lines were grown for 3–4 d in roller bottles or large flasks, washed with PBS, scraped from the plastic surface, and collected by centrifugation. Cell pellets were resuspended in 0.1 M KCl, 20 mM Hepes, pH 7.0, with the following protease inhibitors: 1% aprotonin (Sigma Immunochemicals, St. Louis, MO), 1 mM PMSF (Sigma), 1 mM pepstatin (Boehringer Mannheim Corp., Indianapolis, IN) and 1 μM leupeptin (Boehringer Mannheim Corp.). Gentle sonication was used to disrupt cells. Unbroken cells and nuclei were removed by low-speed centrifugation. Crude membranes were then harvested by high-speed centrifugation (80,000 *g* for 30 min). Detergent extracts were prepared by resuspending membranes at 4°C in 20 mM $NaPO_4$, 1 mM $CaCl_2$, 1% *n*-Octyl-β-D-glucopyranoside (Calbiochem Novabiochem, La Jolla, CA) with the same cocktail of protease inhibitors mentioned above. Residual membranes were removed from the preparation by a second high-speed centrifugation. The supernatant was saved as the membrane extract. Approximately 12 μg of extract from each cell line was dissolved in 2× sample buffer, and individual components were separated by SDS-PAGE under nonreducing conditions, using 8 and 4–20% precast gels (NOVEX, San Diego, CA). After separation by SDS-PAGE, proteins were transferred to Immobilon membranes and probed with 2 μg/ml of either PE or RAP, both of which interact with immobilized receptor.

Since PE interacts with LRP optimally at pH 5.5, PE ligand blots were carried out at this pH (Thompson et al., 1991). The presence of PE was detected using an affinity-purified rabbit anti-PE antibody that had been conjugated with HRP (Jackson ImmunoReseach Laboratories, Inc.). RAP binding to immobilized receptor was detected using RAP–protein A. Binding was carried at neutral pH, and the presence of RAP–protein A was detected using a peroxidase-labeled rabbit IgG.

### *Western Blot Analysis Using Antireceptor Antibodies*

Rabbit antibodies, specific for the heavy and light chains of LRP, were used to detect the presence of the receptor in detergent extracts of membranes prepared from mutant and WT cell. As above, extracts were separated on either 8 or 4–12% polyacrylamide gels, proteins were transferred to Immobilon membranes, and then probed with antibodies. Anti–heavy chain, which had been affinity purified on an LRP column, was added at ~2 μg/ml. The anti–light chain antibody was an IgG preparation and was added to a final concentration of 50 μg/ml. Rabbit antibodies were detected using either a Vecta stain kit (Vector Laboratories, Inc., Burlingame, CA) or with Renaissance Chemiluminescence kit (DuPont-NEN, Boston, MA).

### *Northern Blot Analysis*

Cells were harvested by treatment with trypsin-EDTA, and total RNA was isolated using RNA Stat-60 (Tel-Test, Friendswood, TX) according to the manufacturer's instructions. RNA concentrations were calculated from the absorbance at 260 nm. 10 μg of each sample was denatured and subjected to electrophoresis in a 1.2% agarose gel containing 2.2 M formaldehyde. The gel was stained with ethidium bromide to verify that each lane contained similar amounts of undegraded rRNA. RNA was electroblotted onto Zetabind nylon membranes (Cuno, Inc) and cross-linked by UV irradiation using a Stratalinker (Stratagene Inc., La Jolla, CA). The cDNA probes were full-length LRP (described below), human glyceraldehyde-3-phosphate dehydrogenase, and an 800-bp PstI/XbaI fragment of pHcGAP (American Type Culture Collection, Rockville, MD).

The LRP cDNA clone was constructed by PCR. Primers were designed based on the sequence published by Herz et al. (1988). Fragments of ~1 kb were amplified initially. Thereafter, fragments were joined to form larger pieces until a full-length (13.8-kb) clone had been constructed. To confirm its identity with the known LRP sequence, the clone was sequenced automatically using an ABI 373A DNA sequencer. The full-length clone was produced in plasmid pETα1-14-15, a derivative of pET-3b (Studier and Moffatt, 1986). Miniprep DNA was digested with NotI and EcoRV and the cDNA isolated.

The probes were labeled to high specific activity with [$^{32}$P]-dCTP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) by use of a random primer labeling kit (Boehringer Mannheim Corp.). Hybridization and membrane washing conditions were as previously described (Donohue et al., 1994). Blots were exposed to film (XAR-5; Eastman Kodak Co., Rochester, NY) in cassettes with intensifying screens at −80°C.

### *Inhibition of Protein Synthesis by PE and TF-PE40*

WT, 14-2-1, and 13-5-1 cells were seeded in 24-well plates at ~$10^5$ cells per well. The following day, cells were treated with increasing concentrations of either PE or TF-PE40. After an overnight incubation at 37°C, cytotoxicity was determined by measuring the incorporation of [$^3$H]leucine into new cellular protein. 1-2 h before the addition of TF-PE40, cells were washed in tissue culture medium lacking serum but containing BSA (2 mg/ml). This served to reduce the competition for receptor binding usually seen with the high levels of TF found in serum. Cells remained in serum-free medium for the duration of the experiment. Data are expressed as percent of protein synthesis compared with cells that did not receive toxin.

### *Cell Binding, Internalization, and Degradation Assays*

Cell binding, internalization, and degradation assays were carried out with modifications to the procedures of Isaacs et al. (1988) and Kounnas et al. (1993). CHO cells (WT and mutants) were grown in HAMS F12 medium containing 10% FCS, penicillin/streptomycin, and L-glutamine. The cells were plated at 7 × $10^5$ cells per well in six-well dishes and allowed to grow for 24 h before assay. 1 h before starting the assay, the medium was removed and the cells incubated at 37°C with HAMS F12, 1% Nutridoma serum substitute (Boehringer Mannheim Corp.), 15 mg/ml BSA, and 20 mM Hepes, pH 7.4 (incubation media). For cell uptake and degradation experiments, cells were then washed with incubation medium and $^{125}$I$\alpha_2$M–chymotrypsin (13 μCi/μg) in incubation medium added. At selected time intervals, the media was removed, the cells were washed with cold PBS, and then incubated with trypsin-EDTA for 15 min at 4°C. The cell suspension was then removed from the wells and the cells pelleted by centrifugation. Counts present in the pellet were taken as the amount of ligand that had been internalized. To measure the extent of ligand degradation, TCA (to a final concentration of 10%) was added to the media removed from the cells. After centrifugation, counts in the supernatant were measured. Nonspecific uptake and degradation were measured by including an excess of unlabeled $\alpha_2$M–chymotrypsin.

## *Results*

### *Strategy for Selection of LRP Mutants*

Since PE can bind to LRP and may use this receptor to gain entry into sensitive cells, it was of interest to determine if toxin-resistant lines had alterations in their expression of LRP. CHO cells were mutagenized by exposure to 5 mM ethyl methane sulfate. Surviving cells were grown for 4 d to allow expression of mutations and then challenged with 100 ng/ml of PE. Well-isolated colonies were picked, expanded, and screened for their sensitivity to PE, DT, and a fusion protein composed of lethal factor (from anthrax toxin) fused with the ADP-ribosylating domain of

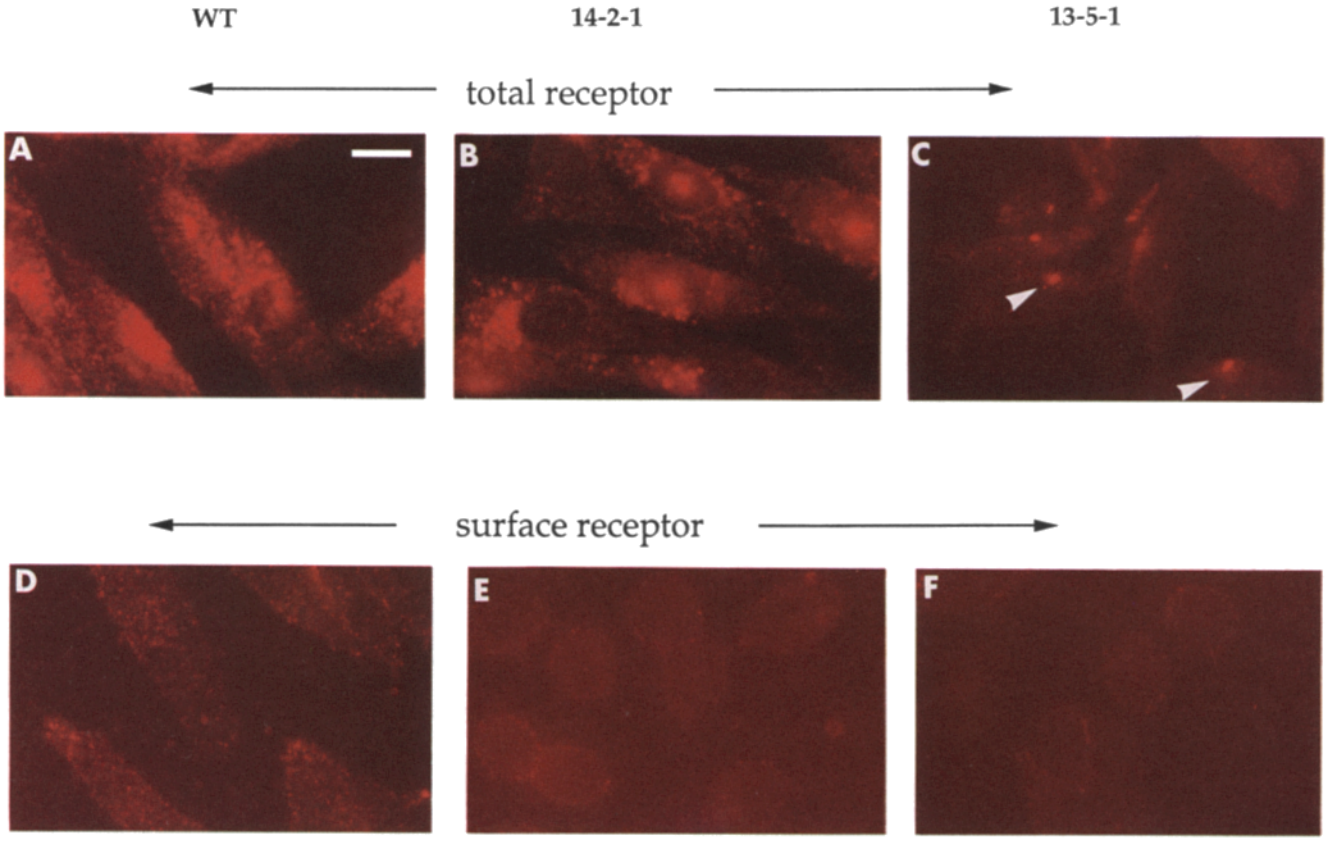


*Figure 1.* Detection of cell-associated LRP by indirect immunofluorescence. Total LRP was detected in WT cells (*A*), 14-2-1 cells (*B*), and 13-5-1 cells (*C*) by the addition of affinity-purified rabbit anti-LRP heavy chain IgG in the presence of 0.1% saponin. Cells were then probed with rhodamine-labeled affinity-purified goat anti–rabbit IgG. Surface-expressed LRP was detected by the addition of rabbit anti-LRP heavy chain IgG to cells at 4°C. As above, the rabbit IgG was detected by the addition of rhodamine-labeled affinity-purified goat anti–rabbit IgG: WT cells (*D*), 14-2-1 (*E*), and 13-5-1 (*F*). Cells were fixed with 3.7% formaldehyde. Bar, 20 μm.

PE (domain III). Mutant cells, specifically resistant to PE, were cloned by limiting dilution, and their phenotypes confirmed (see below). PE-resistant cells that were also resistant to DT or lethal factor–domain III were discarded since they were likely to be elongation factor-2 mutants, acidification mutants, protease mutants, or pleiotropic mutants.

Initially, the status of LRP expression was investigated in two PE-resistant cell lines, 14-2-1 and 13-5-1. Cells were permeabilized with saponin and then probed with a rabbit antibody to the heavy chain of LRP. A rhodamine-labeled goat anti–rabbit IgG was used to visualize the rabbit antibody. In WT cells (Fig. 1 *A*) abundant receptor was seen throughout each cell. Although the strength of labeling was slightly less than in WT cells, the 14-2-1 line exhibited significant amounts of reactivity throughout each cell (Fig. 1 *B*). In contrast to this, the 13-5-1 line exhibited one "spot" of perinuclear reactivity per cell (Fig. 1 *C*, *arrowheads*). The latter reactivity was antibody specific since cells receiving no primary antibody appeared completely dark (data not shown).

To detect surface-expressed LRP, cells were chilled to 4°C and then exposed to the rabbit anti-heavy chain antibodies. Cells were washed extensively, and bound antibody was detected using rhodamine-labeled goat anti–rabbit IgG. WT cells had a punctate pattern of weak fluorescence, possibly corresponding to LRP that had clustered in coated pits. In contrast to this, surface fluorescence for the 14-2-1 and 13-5-1 lines was undetectable on most cells and barely present on a small number of cells (Fig. 1 *D* depicts WT cells, while *E* and *F* show the 14-2-1 and 13-5-1 lines, respectively).

### *Ligand Blot Analysis*

When intact cells were probed with anti-LRP antibodies, it appeared that the two toxin-resistant lines had altered expression of the receptor. To determine if cells possessed functional LRP, ligand blots were performed. Detergent extracts of membranes from WT, 14-2-1, and 13-5-1 cells were analyzed for the presence of PE- and RAP-binding proteins. Solubilized proteins (12 μg total protein/lane) were separated by SDS-PAGE and then transferred to Immobilon P membranes. One set of Immobilon membranes was exposed to PE at pH 5.5 (the optimum pH for toxin binding [Thompson et al., 1991]), while the other set was exposed to RAP at neutral pH.

Results indicated that there was toxin binding to a very high molecular weight component (with the mobility of the heavy chain of LRP; Fig. 2, *single arrow*) in WT and 14-2-1 cells but not in the 13-5-1 line (for comparisons see the first three sample lanes of Fig. 2). In addition, there

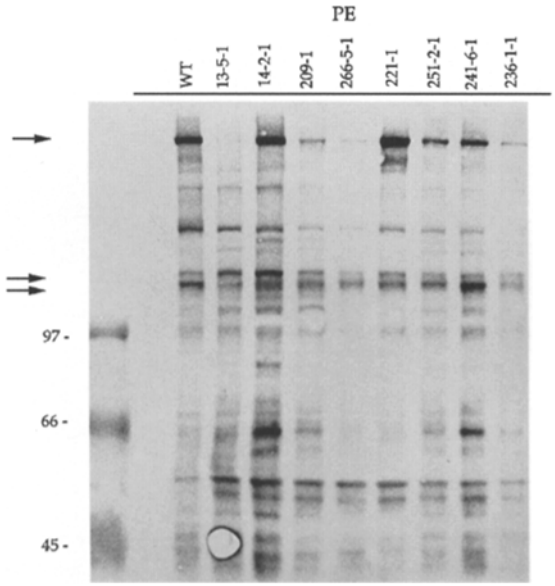


*Figure 2.* Ligand blots to detect membrane-associated PE-binding activity. Detergent-solubilized membrane proteins from nine CHO lines were separated by SDS-PAGE, transferred to Immobilon P membranes, and probed at pH 5.5 for the presence of PE-binding proteins. Toxin binding was detected by the addition of HRP-labeled affinity-purified rabbit anti-PE. Molecular mass markers indicate phosphorylase B (97 kD), albumin (66 kD), and ovalbumin (45 kD).

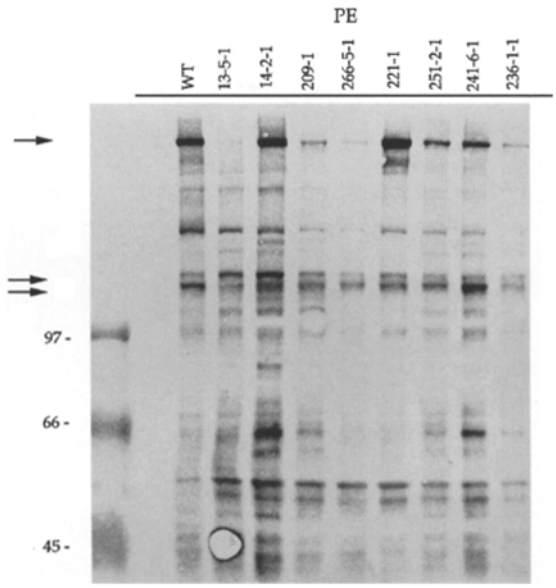


*Figure 3.* Ligand blots to detect membrane-associated RAP binding activity. Detergent-solubilized membrane proteins from nine CHO lines were separated by SDS-PAGE, transferred to Immobilon P membranes, and probed at neutral pH for the presence of RAP-binding proteins. Binding was detected by the addition of HRP-labeled rabbit IgG.

was reactivity with several other unidentified bands. The relevance of this reactivity is not well understood. However, in many different blotting experiments, the only consistent difference between WT and mutant cells was seen at the top of the gel corresponding to the mobility of the heavy chain of LRP. Reactivity with a doublet at ~120 kD (Fig. 2, *double arrow*) was consistently noted in all lines (see below).

To determine reactivity with RAP, Immobilon membranes were exposed to a RAP–protein A fusion protein at neutral pH. Several bands of reactivity were noted. WT and 14-2-1 cells had reactivity near the top of the gel, consistent with binding to the heavy chain of LRP (Fig. 3, *upper arrow*), while the 13-5-1 cell line did not have this band (for comparisons see the first three sample lanes of Fig. 3). In addition, all three cell lines had strong reactivity for a band that migrated at ~120 kD (Fig. 3, *lower arrow*). It is not known if this lower band is related to the two reactive bands seen at approximately the same relative mobility in the PE ligand blots.

Together, the ligand-binding results suggest that functional LRP is present in 14-2-1 cells, possibly at reduced amounts compared with WT cells, but absent in 13-5-1 cells. Also, all three cell lines had approximately similar amounts of a 120-kD component that reacted strongly with RAP.

### *Western Blot Analysis*

To detect the presence of any receptor-related proteins, membrane extracts were probed with antibodies to both the heavy and light chains of LRP. When solubilized proteins from the three lines were probed with antibodies to the heavy chain, the pattern of reactivity was similar to the one seen with the PE ligand blots. The 14-2-1 line had about the same or slightly reduced amounts of heavy chain compared with WT, while the 13-5-1 line had none (see the first three sample lanes of Fig. 4). Reactivity at ~120 kD was seen, but the staining was much weaker than was seen for RAP binding. When extracts were probed with rabbit antibodies to LRP light chain, there was a reactive band of 85 kD in the WT sample, no band at a similar migration in the 13-5-1 cells, and only a faint band in the 14-2-1 cells (Fig. 5, *large arrow*). In the 14-2-1 extracts there was also weak reactivity migrating at the position of the heavy chain of LRP (Fig. 5, *small arrow*). This suggested that in 14-2-1 cells either LRP was not maturing into heavy and light chains or there was some selective loss of the COOH-terminal portion of the light chain. Since the 13-5-1 line was devoid of the LRP heavy chain, it was not surprising that it also lacked the light chain.

### *Northern Blot Analysis*

To determine whether LRP mRNA levels were also reduced in 13-5-1 cells, RNA was isolated from WT and 13-5-1 cells and Northern blot analysis was performed using a full-length cDNA probe for LRP. One major and minor transcript at 13–14 kb were detected (Fig. 6 *A*) in WT but not in 13-5-1 cells. Similar amounts of RNA from each line had been loaded on the gel as determined by reprobing the blot for glyceraldehyde-3-phosphate dehydrogenase mRNA.

In companion experiments, cells were analyzed for RAP expression. Results indicated that while 13-5-1 cells lacked LRP (Fig. 6 *B*), expression of RAP was similar in both

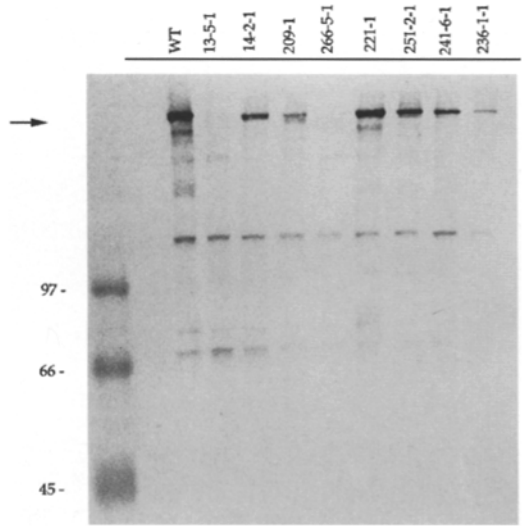


*Figure 4.* Western blot analysis of membrane proteins from nine CHO cell lines. Detergent-solubilized membrane proteins were transferred to Immobilon P membranes and then probed with rabbit anti-LRP heavy chain. Reactive proteins were detected using a Vecta stain kit.

lines (Fig. 6 *C*). Combined, the Northern blot result and the Western blot analysis ruled out the possibility that the absence of LRP protein was due to indirect effects such as poor expression of RAP, which might be needed to stabilize LRP inside cells.

### *Uptake of Radiolabeled $\alpha_2$M–Chymotrypsin*

To assess the ability of mutant cells to bind and internalize a physiologic ligand, the time course of $\alpha_2$M–chymotrypsin uptake and degradation was measured. Fig. 7 demonstrates that $^{125}$I-labeled $\alpha_2$M–chymotrypsin was rapidly taken up (Fig. 7 *A*) and degraded (Fig. 7 *B*) by WT cells, but not by the 14-2-1 or 13-5-1 cell lines. This result is consistent with the immunofluorescence micrographs (Fig. 1, *E* and *F*), which showed that there was little or no LRP exposed on the surface of either the 14-2-1 or 13-5-1 cell lines.

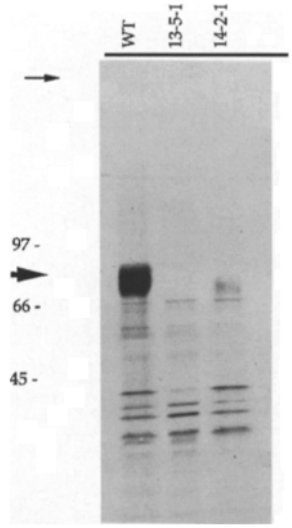


*Figure 5.* Western blot analysis of membrane proteins from WT, 13-5-1, and 14-2-1 cells. Detergent-solubilized membrane proteins were transferred to Immobilon P membranes and then probed with rabbit antibodies prepared against the last 12 amino acids of the LRP light chain. Reactive proteins were detected using a Vecta stain kit.

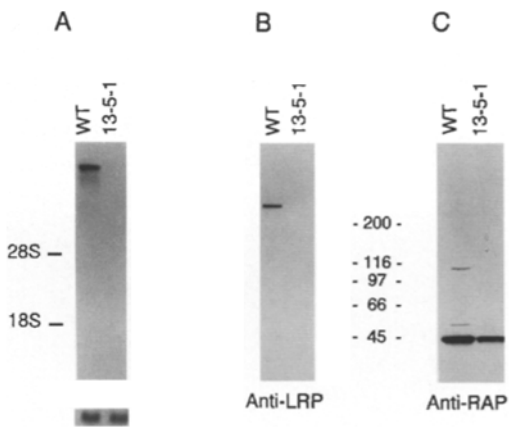


*Figure 6.* Northern and Western blot analysis of 13-5-1 cells for LRP expression. (*A*) RNA from WT or 13-5-1 cells was electroblotted onto Zetabind membranes and probes with a full-length LRP cDNA and a glyceraldehyde-3-phosphate dehydrogenase cDNA. (*B* and *C*) Membrane proteins from WT or 13-5-1 cells were probed with antibodies to the heavy chain of LRP (*B*) or to RAP (*C*).

### *Susceptibility of Cells to PE and TF-PE40*

To investigate the relative toxin sensitivity of cells displaying various configurations of LRP, WT, 14-2-1, and 13-5-1 cells were exposed to increasing concentrations of PE. WT cells had an $IC_{50}$ of 15 ng/ml, 14-2-1 cells of 130 ng/ml, and 13-5-1 cells of >500 ng/ml (Fig. 8 *A*). Clearly, the changes of LRP expression were reflected in the cells' sensitivity to PE. To determine the nature of the lesion causing toxin resistance, the same lines were exposed to TF chemically conjugated to PE40 (PE40 is a truncated form of PE lacking the toxin's cell-binding domain). Cells were washed into serum-free medium and then exposed to increasing concentrations of TF-PE40. TF-PE40 was active on WT cells with an $IC_{50}$ of 270 ng/ml. The 14-2-1 cells exhibited a slight resistance compared with WT, while the 13-5-1 cells were slightly more sensitive (Fig. 8 *B*).

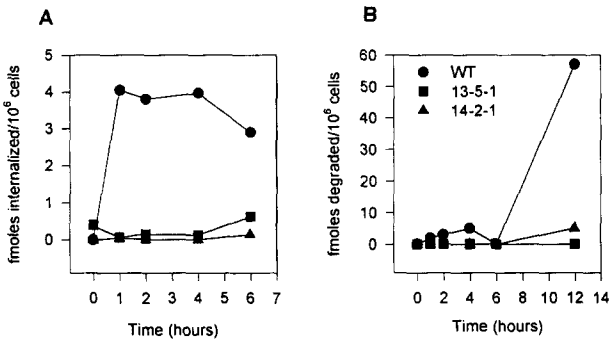


*Figure 7.* Time course of (*A*) uptake and (*B*) degradation of $^{125}$I-$\alpha_2$M–chymotrypsin. WT (●), 14-2-1 (▲), and 13-5-1 (■) cells were plated at a density of $7 \times 10^5$. 1 h before the assay, the medium was removed and replaced with incubation buffer. $^{125}$I-$\alpha_2$M–chymotrypsin (1 nM) was then added. To measure nonspecific uptake, excess cold $\alpha_2$M–chymotrypsin was added; at each time point, the values plotted are corrected for nonspecific uptake or degradation measured by subtracting the amount endocytosed or degraded in the presence of excess unlabeled $\alpha_2$M–chymotrypsin (500 nM).

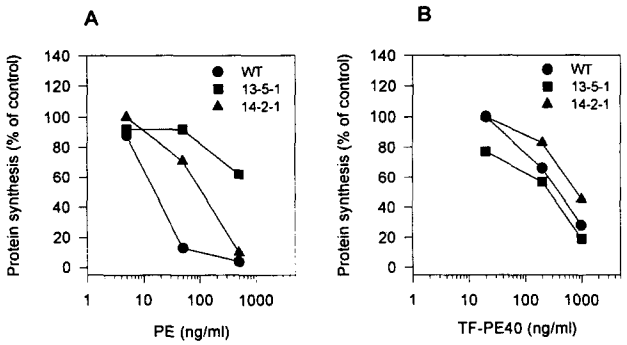


*Figure 8.* Toxicity of (*A*) PE and (*B*) TF-PE40 for cell lines expressing varying amounts of LRP. WT (●), 14-2-1 (▲), or 13-5-1 (■) cells were incubated with increasing concentrations of PE or TF-PE40 for 18 h. To determine the level of toxicity, cells were then pulsed for 1 h with [$^3$H]leucine. Data are expressed as percent of protein synthesis in toxin-treated cells compared with control cells receiving no toxin.

### *Analyses of Other Clones Generated by the Same Strategy*

To determine the incidence of altered LRP expression in other PE-resistant clones that retained sensitivity to DT and lethal factor domain III, additional lines were produced and screened as described above. Membrane extracts were prepared from each line and then, after SDS-PAGE, probed for the presence of receptor heavy chain (see Fig. 4), PE binding (see Fig. 2), and RAP binding (see Fig. 3). One clone, 266-5-1, which had little or no detectable heavy chain, appeared to have the same phenotype as 13-5-1. In cell-killing assays, the 266-5-1 line was 100-fold resistant to PE (data not shown). The other four lines (209-1, 251-2-1, 241-6-1, and 236-1-1) resembled the 14-2-1 cells in so far as they all had detectable LRP heavy chain, albeit at lower levels than in WT cells. In cell-killing assays, these lines exhibited a 30–100-fold resistance to PE. All lines had a band at 120 kD exhibiting strong reactivity with RAP (see Fig. 3, *lower arrow*) and weak reactivity with anti–heavy chain antibodies (see Fig. 4). The identity of the protein corresponding to this band is discussed below.

One cell line, designated 221-1, was obtained using a different kind of selection. This line was selected for resistance to the fusion protein of lethal factor and domain III of PE. It subsequently proved to be approximately threefold more sensitive to PE than WT cells (data not shown). By Western blot analysis, this line had slightly more LRP heavy chain than WT cells. Also, 221-1 was the only mutant line with comparable amounts of LRP light chain to WT cells (data not shown).

## *Discussion*

Bacterial toxins have proved to be useful probes of mammalian cell function. In particular, studies using DT have illuminated the biology of EF-2 by uncovering the post-translational modification of a conserved histidine (residue 715) to a novel amino acid called diphthamide (Van Ness et al., 1980). In unrelated experiments, it was shown ~30 yr ago that ammonium chloride protected cells from the action of DT (Kim and Groman, 1965). This was one of the earliest experiments that led to the unveiling of the process we now know as endosomal acidification (for review see Yamashiro and Maxfield, 1988). In addition, it should be noted that the genetic basis for toxin resistance has been studied extensively in CHO cells (Robbins et al., 1981; Moehring et al., 1984; Kohno et al., 1985; Colbaugh et al., 1988; Yamashiro and Maxfield, 1988).

Here we have used DT to screen against cell lines that express mutations at common steps, such as those mentioned above, in the PE/DT pathway. Our screen also included a fusion protein comprised of lethal factor from anthrax toxin fused with domain III of PE. This hybrid toxin enters and kills cells via cell-bound protective antigen (Arora et al., 1992). Protective antigen requires a furinlike cleavage to render it capable of binding the lethal factor–domain III hybrid. Therefore, by incorporating this second screen, cells expressing mutations in furinlike proteases or "translocation" proteins would also be avoided. In theory, lines expressing PE-specific mutations should emerge. In fact, this strategy produced a very high proportion of cell lines with altered expression of LRP. PE had already been shown to bind LRP in a specific manner (Kounnas et al., 1992). Here we provide further evidence that PE not only binds to this receptor but uses it to enter cells and inhibit protein synthesis. This was shown primarily by documenting that lines exhibiting diminished expression of functional LRP were resistant to native PE but not resistant to a PE-related chimeric toxin that was internalized via another receptor.

Initially, four PE-resistant clones were analyzed. In preliminary studies, using antibodies to the heavy chain of LRP, two of these, 14-2-1 and 13-5-1, appeared to have obvious changes in their expression of LRP. These were studied further using a variety of probes that evaluated both the physical state of the receptor and its functionality.

Immunofluorescence studies of intact cells revealed that the 13-5-1 line had little reactivity for the antisera raised against the heavy chain of LRP. In separate studies (not reported here) we have expressed recombinant fragments corresponding to the entire length of the LRP heavy chain. Many of these fragments reacted with the anti–heavy chain antibodies, indicating that the loss of reactivity in the 13-5-1 cell line was not due to a change in one or two epitopes but a major loss in LRP-related material. In addition, ligand blots of membrane extracts revealed that 13-5-1 cells did not contain a high molecular weight component that was reactive with either PE or RAP. By Northern blot analysis, no LRP-specific transcripts could be detected in 13-5-1 cells. And, functionally, the 13-5-1 line did not bind or internalize $\alpha_2$M–chymotrypsin complexes. The fact that these cells were 100-fold resistant to PE but not resistant to a chimeric toxin that entered cells via the TF receptor confirmed the importance of this receptor in toxin-mediated cytotoxicity.

It is of interest to note that cells lacking detectable LRP were 100-fold resistant to PE. In domain I of PE, lysine 57 is known to mediate binding to LRP (Jinno et al., 1988; Kounnas et al., 1992). When this lysine is changed to glutamic acid, binding is abolished and toxicity for cells is reduced by 100-fold (Jinno et al., 1988; Kounnas et al.,

1992). Thus the reciprocal loss of binding function, by either the receptor or the ligand, resulted in the same 100-fold reduction in cytotoxic activity. The residual toxicity of PE for 13-5-1 cells could be due to lower affinity interactions with another cell surface component.

The situation with the 14-2-1 line was quite different. Probing permeabilized cells revealed substantial amounts of immunoreactive LRP. However, when the surface expression of LRP was assessed, it became apparent why these cells might be resistant to PE. Anti-LRP heavy chain showed little or no surface reactivity. Lack of functional receptor at the cell surface was confirmed by results indicating that these cells also failed to bind and internalize $\alpha_2$M–chymotrypsin complexes. Ligand blots of membrane extracts revealed that the 14-2-1 line expressed LRP in a form that could still bind both PE and RAP. Additionally, Western blots with anti–heavy chain confirmed the presence of substantial amounts of reactive material at apparently the correct size. Probing with an antibody to the LRP light chain revealed that there was reduced reactivity at 85 kD, the expected molecular mass for this chain of the receptor. Anti–light chain antibodies reacted weakly with a species that was close in size to the LRP heavy chain. This result suggested that single-chain LRP was being expressed but not processed efficiently. When assessed for PE sensitivity, these cells proved to be ~10-fold resistant compared with WT.

While it is not proved, data from the analysis of the 14-2-1 line suggest that surface expression of LRP follows the processing of the single-chain precursor and is possibly dependent on it. This is consistent with the pathway proposed by Herz et al. (1990) for maturation of LRP. Single-chain LRP is cleaved after the sequence of RHRR, which is a consensus site for cleavage by a furinlike protease. Future studies will determine whether or not this sequence is retained in the mutant LRP expressed in the 14-2-1 cells or whether there is another defect that results in poor processing.

A reactive band at ~120 kD was detected when membrane extracts were probed with PE, RAP, or antibodies to the LRP heavy chain. This band was seen in all lines, but did not correlate with toxin resistance, and is not a candidate for the toxin's primary receptor. However, the reactivity of this band for RAP was striking. This has prompted a separate study to establish its identity. A preliminary report suggests that the 120-kD protein is the very low density lipoprotein receptor (Battey et al., 1994).

In conclusion, PE uses the heavy chain of LRP as its receptor for toxin internalization. Since PE binds tightly to the heavy chain of LRP at low pH, it is likely that the toxin remains receptor bound after delivery to the endosome. To be active as a toxin, PE must be proteolytically cleaved. In separate experiments, we have shown that PE is cleaved by furin with a pH optimum of 5.0–5.5 (Chiron et al., 1994). Furin-mediated cleavage, which may occur in the endosomal compartment, could be the mechanism by which the COOH-terminal enzymatically active fragment of PE is released from LRP. Once released, this fragment can translocate to the cytosol and ADP-ribosylate elongation factor 2.

We thank K. and S. Argraves for their help and advice with the production of the photomicrographs, D. Hsu and Y. Guo for their help with the Northern blotting, M. Rosenfeld for helpful suggestions with the manuscript and S. Neal for photography.

This work was supported in part by grants HL50787 and GM42581 (D. K. Strickland) and HL39727 (J. A. Winkles).

Received for publication 13 July 1994 and in revised form 24 February 1995.

*References*

Arora, N., K. R. Klimpel, Y, Singh, and S. H. Leppla. 1992. Fusions of anthrax toxin lethal factor to the ADP-ribosylation domain of *Pseudomonas* exotoxin A are potent cytotoxins which are translocated to the cytosol of mammalian cells. *J. Biol. Chem.* 267:15542–15548.

Ashcom, J. D., S. E. Tiller, K. Dickerson, J. L. Cravens, W. S. Argraves, and D. K. Strickland. 1990. The human alpha 2-macroglobulin receptor: identification of a 420-kD cell surface glycoprotein specific for the activated conformation of alpha 2-macroglobulin. *J. Cell Biol.* 110:1041–1048.

Battey, F. D., M. E. Gafvels, D. J. FitzGerald, W. S. Argraves, D. A. Chappell, J. F. I. Strauss, and D. K. Strickland. 1994. The 39-kDa receptor-associated protein regulates ligand binding by the very low density lipoprotein receptor. *J. Biol. Chem.* 269:23268–23273.

Bu, G., S. Williams, D. K. Strickland, and A. L. Schwartz. 1992. Low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor is an hepatic receptor for tissue-type plasminogen activator. *Proc. Natl. Acad. Sci. USA.* 89:7427–7431.

Chappell, D. A., G. L. Fry, M. A. Waknitz, P. H. Iverius, S. E. Williams, and D. K. Strickland. 1992. The low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor binds and mediates catabolism of bovine milk lipoprotein lipase. *J. Biol. Chem.* 267:25764–25767.

Chaudhary, V. K., Y. Jinno, D. FitzGerald, and I. Pastan. 1990. *Pseudomonas* exotoxin contains a specific sequence at the carboxyl terminus that is required for cytotoxicity. *Proc. Natl. Acad. Sci. USA.* 87:308–312.

Chiron, M. F., C. M. Fryling, and D. J. FitzGerald. 1994. Cleavage of *Pseudomonas* exotoxin and diphtheria toxin by a furin-like protease prepared from beef liver. *J. Biol. Chem.* 269:18167–18176.

Colbaugh, P. A., C. Y. Kao, S. P. Shia, M. Stookey, and R. K. Draper. 1988. Three new complementation groups of temperature-sensitive Chinese hamster ovary cell mutants defective in the endocytic pathway. *Somat. Cell Mol. Genet.* 14:499–507.

Donohue, P. J., G. F. Alberts, B. S. Hampton, and J. A. Winkles. 1994. A delayed-early gene activated by fibroblast growth factor-1 encodes a protein related to aldose reduction. *J. Biol. Chem.* 269:8604–8609.

FitzGerald, D. J. P., R. E. Morris, and C. B. Saelinger. 1980. Receptor-mediated internalization of *Pseudomonas* toxin by mouse fibroblasts. *Cell.* 21:867–873.

Herz, J., U. Hamann, S. Rogne, O. Myklebost, H. Gausepohl, and K. K. Stanley. 1988. Surface location and high affinity for calcium of a 500-kd liver membrane protein closely related to the LDL-receptor suggest a physiological role as lipoprotein receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:4119–4127.

Herz, J., R. C. Kowal, J. L. Goldstein, and M. S. Brown. 1990. Proteolytic processing of the 600 kd low density lipoprotein receptor-related protein (LRP) occurs in a trans-Golgi compartment. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:1769–1776.

Herz, J., D. E. Clouthier, and R. E. Hammer. 1992. LDL receptor-related protein internalizes and degrades uPA-PAI-1 complexes and is essential for embryo implantation. *Cell.* 71:411–421.

Isaacs, I. J., J. P. Steiner, P. A. Roche, S. V. Pizzo, and D. K. Strickland. 1988. Use of anti-idiotypic antibodies to establish that monoclonal antibody 7H11D6 binds to the alpha 2-macroglobulin receptor recognition site. *J. Biol. Chem.* 263:6709–6714.

Jensen, P. H., S. K. Moestrup, and J. Gliemann. 1989. Purification of the human placental alpha 2-macroglobulin receptor. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 255:275–280.

Jinno, Y., V. K. Chaudhary, T. Kondo, S. Adhya, D. J. FitzGerald, and I. Pastan. 1988. Mutational analysis of domain 1 of *Pseudomonas* exotoxin. *J. Biol. Chem.* 263:13203–13207.

Kim, K., and N. B. Groman. 1965. In vitro inhibition of diphtheria toxin action by ammonium salts and amines. *J. Bacteriol.* 90:1552–1556.

Kohno, K., T. Uchida, E. Mekada, and Y. Okada. 1985. Characterization of diphtheria-toxin-resistant mutants lacking receptor function or containing nonribosylatable elongation factor 2. *Somat. Cell Mol. Genet.* 11:421–431.

Kounnas, M. Z., R. E. Morris, M. R. Thompson, D. J. FitzGerald, D.K. Strickland, and C. B. Saelinger. 1992. The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes *Pseudomonas* exotoxin A. *J. Biol. Chem.* 267:12420–12423.

Kounnas, M.Z., J. Henkin, W. S. Argraves, and D. K. Strickland. 1993. Low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor mediates cellular uptake of pro-urokinase. *J. Biol. Chem.* 268:21862–21867.

Kowal, R. C., J. Herz, J. L. Goldstein, V. Esser, and M. S. Brown. 1989. Low density lipoprotein receptor-related protein mediates uptake of cholesteryl esters derived from apoprotein E-enriched lipoproteins. *Proc. Natl. Acad. Sci. USA.* 86:5810–5814.

Kristensen, T., S. K. Moestrup, J. Gliemann, L. Bendtsen, O. Sand, and J. L. Sottrup. 1990. Evidence that the newly cloned low-density-lipoprotein related protein (LRP) is the alpha 2-macroglobulin receptor. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 276:151–155.

Moehring, J. M., N. M. Inocencio, B. J. Robertson, and T. J. Moehring. 1993. Expression of mouse furin in a Chinese hamster cell resistant to *Pseudomonas* exotoxin A and viruses complements the genetic lesion. *J. Biol. Chem.* 268:2590–2594.

Moehring, T. J., D. E. Danley, and J. M. Moehring. 1984. In vitro biosynthesis of diphthamide, studied with mutant Chinese hamster ovary cells resistant to diphtheria toxin. *Mol. Cell. Biol.* 4:642–650.

Ogata, M., V. K. Chaudhary, I. Pastan, and D. J. FitzGerald. 1990. Processing of *Pseudomonas* exotoxin by a cellular protease results in the generation of a 37,000-Da toxin fragment that is translocated to the cytosol. *J. Biol. Chem.* 265:20678–20685.

Ogata, M., C. M. Fryling, I. Pastan, and D. J. FitzGerald. 1992. Cell-mediated cleavage of *Pseudomonas* exotoxin between Arg279 and Gly280 generates the enzymatically active fragment which translocates to the cytosol. *J. Biol. Chem.* 267:25396–25401.

Pastan, I., and D. FitzGerald. 1989. *Pseudomonas* exotoxin: chimeric toxins. *J. Biol. Chem.* 264:15157–15160.

Robbins, A. R., R. Myerowitz, R. J. Youle, G. J. Murray, and D. M. J. Neville. 1981. The mannose 6-phosphate receptor of Chinese hamster ovary cells. Isolation of mutants with altered receptors. *J. Biol. Chem.* 256:10618–10622.

Saelinger, C. B., R. E. Morris, and G. Foertsch. 1985. Trafficking of *Pseudomonas* exotoxin A in mammalian cells. *Eur. J. Clin. Microbiol.* 4:170–174.

Seetharam, S., V. K. Chaudhary, D. FitzGerald, and I. Pastan. 1991. Increased cytotoxic activity of *Pseudomonas* exotoxin and two chimeric toxins ending in KDEL. *J. Biol. Chem.* 266:17376–17381.

Strickland, D. K., J. D. Ashcom, S. Williams, W. H. Burgess, M. Migliorini, and W. S. Argraves. 1990. Sequence identity between the alpha 2-macroglobulin receptor and low density lipoprotein receptor-related protein suggests that this molecule is a multifunctional receptor. *J. Biol. Chem.* 265:17401–17404.

Studier, F. W., and B. A. Moffatt. 1986. Use of bacteriophage T7 polymerase to direct selective expression of cloned gene. *J. Mol. Biol.* 189:113–130.

Thompson, M. R., J. Forristal, P. Kauffmann, T. Madden, K. Kozak, R. E. Morris, and C. B. Saelinger. 1991. Isolation and characterization of *Pseudomonas aeruginosa* exotoxin A binding glycoprotein from mouse LM cells. *J. Biol. Chem.* 266:2390–2396.

Underhill, D. M., D. A. Owensby, P. A. Morton, and A. L. Schwartz. 1992. Endocytosis and lysosomal delivery of tissue plasminogen activator-inhibitor 1 complexes in Hep G2 cells. *Blood.* 80:2746–2754.

Van Leuven, F., J. J. Cassiman, and H. Van den Berghe. 1986. Human pregnancy zone protein and alpha 2-macroglobulin. High-affinity binding of complexes to the same receptor on fibroblasts and characterization by monoclonal antibodies. *J. Biol. Chem.* 261:16622–16625.

Van Ness, B. G., J. B. Howard, and J. W. Bodley. 1980. ADP-ribosylation of elongation factor 2 by diphtheria toxin. NMR spectra and proposed structures of ribosyl-diphthamide and its hydrolysis products. *J. Biol. Chem.* 255: 10710–10716.

Wick, M. J., D. W. Frank, D. G. Storey, and B. H. Iglewski. 1990. Structure, function and regulation of *Pseudomonas aeruginosa* exotoxin A. *Annu. Rev. Microbiol.* 44:335–363.

Williams, S. E., J. D. Ashcom, W. S. Argraves, and D. K. Strickland. 1992. A novel mechanism for controlling the activity of alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein. Multiple regulatory sites for 39-kDa receptor-associated protein. *J. Biol. Chem.* 267:9035–9040.

Willnow, T. E., and J. Herz. 1994. Genetic deficiency in low density lipoprotein receptor-related protein confers cellular resistance to *Pseudomonas* exotoxin A. Evidence that this protein is required for uptake and degradation of multiple ligands. *J. Cell Sci.* 107:719–726.

Willnow, T. E., J. L. Goldstein, K. Orth, M. S. Brown, and J. Herz. 1992. Low density lipoprotein receptor-related protein and gp330 similar ligands, including plasminogen activator-inhibitor complexes and lactoferrin, an inhibitor of chylomicron remnant clearance. *J. Biol. Chem.* 267:26172–26180.

Yamashiro, D. J., and F. R. Maxfield. 1988. Regulation of endocytic processes by pH. *Trends Pharmacol. Sci.* 9:190–193.