Functional Expression of the Chicken Low Density Lipoprotein Receptor-related Protein in a Mutant Chinese Hamster Ovary Cell Line Restores Toxicity of *Pseudomonas* Exotoxin A and Degradation of α₂-Macroglobulin*

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The low density lipoprotein receptor-related protein (LRP) is responsible for the clearance of several physiological ligands including a complex of proteinase and α₂-macroglobulin (α₂M) and for the entrance of *Pseudomonas* exotoxin A (PEA) into cells. We have prepared expression plasmids for the full-length chicken LRP (designated LRP100) and two intermediates encoding 25 and 67% of the receptor (designated LRP25 and LRP67, respectively) using overlapping cDNA fragments. LRP25 and LRP67 encode the N-terminal 22 and 64%, respectively, of LRP100 plus the transmembrane and intracellular domains. Transient transfection of these plasmids into COS-7 cells yielded recombinant proteins of extracellular domains. Transient transfection of these plasmids into COS-7 cells yielded recombinant proteins of expected molecular mass and immunoreactivity. However, LRP100 was incompletely processed into α₂ (515-kDa) and β (85-kDa) chains and was poorly transported from the endoplasmic reticulum to the Golgi compartment. Stable transformants of LRP100, LRP67, and LRP25 were generated in a mutant Chinese hamster ovary cell line that lacked expression of endogenous LRP and was resistant to PEA. All forms of recombinant LRP proteins were transported from the endoplasmic reticulum to the Golgi apparatus in Chinese hamster ovary cells as shown by their sensitivity to endoglycosidase H and resistance to neuraminidase. Cell surface iodination and subcellular fractionation studies indicated that all three LRP variants were expressed on the plasma membrane. Furthermore, expression of the three LRP variants restored, to various degrees, sensitivity to PEA and the ability to degrade methylamine-activated α₂M (α₂M*). These data suggest that deletion of large internal portions of LRP, including the processing site, does not prevent transport of LRP to the plasma membrane, nor does it abolish the interaction of LRP with α₂M* or PEA. This LRP expression system may allow for the characterization of domains within LRP responsible for its multifunctionality.

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1 The abbreviations used are: LRP, low density lipoprotein receptor-related protein; ER, endoplasmic reticulum; RAP, receptor-associated protein; apo, apolipoprotein; α₂M, α₂-macroglobulin; CHO, Chinese hamster ovary; PEA, *Pseudomonas* exotoxins A; α₂M*, methylamine-activated α₂M; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; Endo H, endoglycosidase H; GST, glutathione S-transferase.
to LRP. With the recognition of the possible link between apoE metabolism and the development of Alzheimer’s disease, the potential role of LRP in the pathophysiology of the central nervous system has also been suggested (18).

LRP has also been suggested to serve as a receptor for Pseudomonas exotoxin A (PEA) (19, 21). PEA consists of three functional domains and exerts its toxicity by irreversibly inhibiting protein translation. Following internalization, PEA protein is cleaved by a cellular protease and translocated to the cytosol. The N-terminal domain of PEA mediates cell binding, while the central domain contains the translocating activity and acts as the substrate for proteolytic cleavage. The C-terminal domain of PEA possesses the enzymatic activity and catalyzes ADP-ribosylation of elongation factor 2, resulting in inhibition of protein synthesis and cell death (22, 23). The primary target of PEA is the liver (25, 26). A mutant Chinese hamster ovary (CHO) cell line has been isolated that lacks LRP and is resistant to the toxic effects of PEA (20).

In this study, we prepared expression plasmids for the full-length chicken LRP and two deletion variants using LRP cDNA fragments and expressed them in an LRP-deficient CHO cell line (LRP-null). We used the cytotoxicity of PEA to monitor the function of the recombinant receptor and found that expression of the full-length receptor in the LRP-null cell line restored its sensitivity to the toxin. Furthermore, the expressed chicken LRP also mediated the uptake and degradation of $\alpha_{2}$-M*. This expression system will facilitate investigations of the pathophysiology and cell biology of this enormous cell surface receptor.

**EXPERIMENTAL PROCEDURES**

**Materials**—DNA restriction enzymes and endoglycosidase H (Endo H) were purchased from New England Biolabs. Neuraminidase was purchased from Boehringer Mannheim or New England Biolabs. All reagents for cell culture were purchased from Life Technologies, Inc. ProMix™ (a mix of [35]S)methionine and [35]S)cysteine (1000 Ci/mmol), carrier-free Na[125]I, horseradish peroxidase-conjugated goat anti-rabbit IgG antibody, and the enhanced chemiluminescence (ECL) reagents for sensitivity to the toxin. Furthermore, the expressed chicken LRP also mediated the uptake and degradation of $\alpha_{2}$-M*. This expression system will facilitate investigations of the pathophysiology and cell biology of this enormous cell surface receptor.
washing the cells three times with PBS. The cell pellet was resuspended in 160 μl of buffer I (200 mM Tris-maleate, pH 6.0, 2 mM CaCl₂, 0.5 mM PMSF, 2.5 μM leupeptin) and were lysed with 40 μl of 7% Triton X-100 at 4 °C overnight. The lysate was then diluted to 0.5 ml with buffer I containing 1.4% Triton X-100 and precleared with protein A-agarose beads (40 μl of 50% suspension) for 2 h. After removing the protein A beads, an aliquot of the supernatant was incubated with anti-α-chain or anti-β-chain antibody (overnight at 4 °C) to precipitate LRP. The immune complex was recovered with protein A-agarose beads, washed extensively with buffer I containing 1.4% Triton X-100, and eluted into buffer R. Following separation by SDS-PAGE, the radiolabeled LRP was visualized by autoradiography.

Preparation of RAP-GST Fusion Protein—The Salmonella japonicum glutathione S-transferase (GST)/39-kDa expression plasmid containing human 39-kDa protein (RAP) cDNA was obtained from D. Strickland. The purification of GST/39-kDa protein from E. coli (DH5α) and of the 39-kDa protein after thrombin cleavage were carried out essentially as described by Herz et al. (31).

Purification, Iodination, and Uptake/Degradation of α2M*—Human α2M was purified from fresh plasma (obtained from the Blood Bank of the Ottawa Civic Hospital) by Zn²⁺-chelate affinity chromatography as described previously (32). Preparation of α₂M* was performed according to previously described procedures (12). The purified α₂M* (100 μg in 250 μl of PBS, pH 7.3) was iodinated with one IODO-BEAD and 1 mCi of Na¹²⁵I for 15 min. Unincorporated ¹²⁵I was removed by passing the reaction mixture over a D-Salt Excellulose column equilibrated with PBS containing 0.1% bovine serum albumin. ¹²⁵I-α₂M* (8.9 × 10⁶ cpm/ng) was collected in the void volume of the column effluent. LRP-null and LRP-transfected cells (confluent in 12-well dishes) were incubated with ¹²⁵I-α₂M* (2.5 nM, 0.4 ml/well) in the presence or absence of RAP in F-12 medium containing 5 mM CaCl₂ and 6 mg/ml bovine serum albumin for up to 4 h at 4 °C for α₂M* binding or 37 °C for α₂M* degradation. For degradation, the cells were then placed on ice, and the medium was collected into tubes containing ice-cold trichloroacetic acid (final concentration, 20%). After 30 min, trichloroacetic acid-insoluble material was pelleted by centrifugation, and the supernatant was removed for determination of trichloroacetic acid-soluble, non-iodide radioactivity as described previously (33). For α₂M* binding, the cells were washed twice with cold PBS containing 6 mg/ml bovine serum albumin and twice with cold PBS. The cells were then solubilized with 0.1 N NaOH, and radioactivity was quantified.

Protein Assays—Proteins were determined by the bicinchoninic acid method (Pierce) according to the manufacturer’s instructions.

RESULTS

Transient Expression of LRP in COS-7 Cells—The expression plasmid encoding the full-length chicken LRP (LRP100) was prepared by combining 12 LRP cDNA fragments as indicated in Fig. 1A. Two intermediate plasmids, LRP25 and LRP67, representing −25 and −67%, respectively, of the full-length receptor, were also generated during the preparation of LRP100 (Fig. 1B). Fig. 2 shows immunoblot analysis of the LRP’s treated with and without Endo H (Fig. 2A) or neuraminidase (Fig. 2B), and Fig. 2C represents the domain structures of...
LRP. LRP25 encodes the N-terminal 984 amino acids (including the 21-residue signal peptide, cluster I, and the first three class A repeats of cluster II) plus the C-terminal 125 amino acids (including the transmembrane domain and the intracellular domain) of LRP100. LRP67 contains the N-terminal 2865 amino acids (including clusters I and II and nine class A repeats of cluster III) plus the transmembrane and intracellular domains.

All three LRP variants exhibited the expected molecular mass in transfected COS-7 cells and reacted with a specific antibody raised against the C-terminal 17 amino acids of the chicken LRP β-chain (Fig. 2C). However, the native LRP in the chicken liver membrane (cLM lanes in Fig. 2, A and B) and endogenous LRP in COS-7 cells (probed with an antibody against human LRP; data not shown) were fully processed into α- and β-chains. Incomplete proteolytic cleavage of the recombinant LRP100 is most likely attributable to the impaired intracellular transport from the ER to the trans-Golgi network (where furin resides) based upon analysis of the carbohydrate moiety. Thus, while the native LRP in the chicken liver membrane was fully resistant to Endo H and sensitive to neuraminidase (left two lanes in Fig. 2, A and B), all three recombinant LRPs were sensitive to Endo H and resistant to neuraminidase. The high molecular mass species (~200 kDa) found in the LRP25-transfected cells (Fig. 2A) were probably self-associated dimer. Dimerization of LRP25 and LRP67 was also observed when PAGE was performed under nonreducing conditions (data not shown). We attempted to improve LRP processing by cotransfection of
LRP100 with RAP (to enhance ER-to-Golgi transport) or furin. Although RAP and furin expression was increased, processing of LRP100 was not significantly improved (data not shown). In these experiments (data not shown). As expected, the LRP protein was processed into the plasma membrane (Fig. 3B) and plasma membrane (middle two lanes in Fig. 3A, and B), indicating that the majority of the surface-presented LRP is proteolytically processed. Surface iodination experiments with intact LRP100-transfected cells also demonstrated that the processed α-chain was the predominant form presented on the cell surface, although a small amount of unprocessed LRP was iodinated in the absence of PEA. B, degradation of α2M*. Top, cells (~230 μg of protein/well) were incubated with 125I-α2M* (5.8 × 10^4 cpm/ng, 20 μg/ml) at 37 °C, and the trichloroacetic acid-soluble, non-iodide radioactivity in the medium was determined at the indicated times. Bottom, the inhibitory effect of RAP on α2M* degradation was determined by co-incubation of 125I-α2M* with increasing concentrations of RAP for 4 h at 37 °C.

Expression of Chicken LRP

We tested several different cell lines and found that CHO-K1 cells were a suitable host for functional expression of recombinant LRP. Stably transfected cells expressing LRP100 were generated in a mutant CHO-K1 cell line that lacked expression of endogenous LRP (Fig. 3, A and B, LRP-null). This LRP-null cell line was resistant to PEA toxicity and was unable to internalize activated α2M (20). In LRP100-transfected cells, the majority of LRP protein was processed into α- and β-chains that could be detected in both the microsomes (Fig. 3A) and plasma membrane (Fig. 3B). The unprocessed LRP100 was found in the microsome fraction but was not detectable in the plasma membrane (middle two lanes in Fig. 3A, and B), indicating that the majority of the surface-presented LRP is proteolytically processed. Surface iodination experiments with intact LRP100-transfected cells also demonstrated that the processed α-chain was the predominant form presented on the cell surface, although a small amount of unprocessed LRP was iodinated in these experiments (data not shown). As expected, the LRP α-chain was sensitive to neuraminidase (right two lanes in Fig. 3, A and B). The LRP β-chain, like the native β-chain (left two

### Table I

**Degradation of 125I-α2M* and PEA toxicity index in CHO cells transfected with wild-type or truncated mutant chicken LRP cDNAs**

| Cell lines | 125I-α2M* binding | 125I-α2M* degradation | Corrected 125I-α2M* degradation | PEA toxicity | Corrected PEA toxicity |
|------------|-------------------|------------------------|---------------------------------|-------------|------------------------|
| CHO-K1     | 2.15              | 42.6                   | 19.8 (100%)                     | 97 ± 1 (n = 3) | 45                     |
| LRP-null   | 0                 | 6.37                   | NA                              | -2 ± 5 (n = 3) | NA                     |
| (13–5–1)   |                   |                        |                                 |             |                        |
| LRP100     |                   |                        |                                 |             |                        |
| P2B3       | 2.86              | 49.2                   | 17.2 (87%)                      | 79 ± 3 (n = 3) | 27 (66%)               |
| LR67       | 3.13              | 39.5                   | 12.6 (64%)                      | 45          | 14 (31%)               |
| P2A2       | 23.9              | 120                    | 5.02 (25%)                      | 92          | 3.8 (9%)               |
| P5A3       | 1.28              | 31.4                   | 24.5 (124%)                     | 32          | 25 (56%)               |
| LRP25      | 16.1              | 106                    | 6.58 (33%)                      | 47          | 2.9 (6%)               |
| P7B3       | 35.7              | 156                    | 4.37 (22%)                      | 42          | 1.2 (3%)               |
| P6B4       | 2.58              | 18                     | 6.98 (35%)                      | 10          | 3.9 (9%)               |

- Corrected 125I-α2M* degradation values were calculated by dividing the amount of 125I-α2M* (ng/mg protein) degraded at 37 °C by the amount (ng/mg protein) of 125I-α2M* bound at 4 °C. Values in parentheses represent the percentage of the normal α2M degradation activity of LRP.
- Values for PEA toxicity represent the percentage of decrease in protein synthesis.
- Corrected PEA toxicity values were calculated by dividing the decrease in protein synthesis (percentage of no PEA) by the amount of 125I-α2M* (ng/mg protein) bound at 4 °C. Values in parentheses represent the percentage of the normal LRP-related PEA toxicity.
- NA, not applicable.
lanes in Fig. 2B), was also sensitive to neuraminidase (middle two lanes in Fig. 3, A and B), indicating that the LRP β-chain is sialylated.

**Functional Analysis of Recombinant LRP in Transfected Mutant CHO Cells**—We tested if expression of LRP100 in LRP-null cells would restore the toxicity of PEA. Preliminary time course experiments indicated that at 200 ng/ml, PEA exerted maximal inhibitory effect on protein synthesis after a 12-h incubation (data not shown). The effect of PEA dose was assessed in PEA toxicity assays using cells that had been treated with PEA for 18 h (Fig. 4A). In wild-type CHO-K1 cells, incorporation of \(^{35}\text{S}\)methionine/cysteine into cell protein decreased with increasing PEA dose, while LRP-null cells were insensitive to the toxin. In LRP100-transfected cells, expression of the full-length LRP restored the toxicity of PEA to LRP-null cells. The PEA dose required to reduce protein synthesis to 50% of untreated cells (IC\(_{50}\)) decreased from >500 ng/ml in LRP-null cells (19) to 50 ng/ml in the LRP100-transfected cells (Fig. 4A). The IC\(_{50}\) for wild-type CHO-K1 cells was approximately 25 ng/ml, similar to an earlier observation (19). When the apparent PEA toxicity observed in wild-type CHO-K1 cells and in LRP100-transfected cells was corrected for the number of receptor molecules on the surface (as determined by \(^{125}\text{I}-\alpha_2\text{M}\) binding at 4 °C), the chicken LRP gave a value that was two-thirds of the endogenous LRP (Table I, fourth column).

Expression of LRP100 also restored the ability to bind, internalize, and degrade \(\alpha_2\text{M}\). While LRP-null cells were unable to degrade \(^{125}\text{I}-\alpha_2\text{M}\), LRP100-transfected cells released trichloroacetic acid-soluble, non-iodide radioactivity at a rate similar to the wild-type CHO-K1 cells (Fig. 4B, top). Measurement of the cell-associated radioactivity (at 37 °C) revealed that the failure of LRP-null cells to degrade \(^{125}\text{I}-\alpha_2\text{M}\) was attributable to their inability to bind or internalize the ligand (data not shown). Degradation of \(^{125}\text{I}-\alpha_2\text{M}\) by CHO-K1 or LRP100-transfected cells could be effectively prevented, in a dose-dependent manner, by RAP (Fig. 4B, bottom) or unlabeled \(\alpha_2\text{M}\) (data not shown). Analysis of the \(\alpha_2\text{M}\) binding and degradation data indicated that the ability of the chicken LRP to degrade \(\alpha_2\text{M}\) was equivalent (87%) to that of the endogenous receptor (Table I, fourth column).

**Functional Analysis of Two Deletion LRP Variants**—We next tested whether LRP25 and LRP67 could function as receptors for PEA and \(\alpha_2\text{M}\). In three LRP67-transfected cell lines, two bands representing the sialylated LRP67 (mature form) and the asialyl form of LRP67 were observed (Fig. 5C). The major species (in comparison with the asialyl form) in the plasma membrane fraction (Fig. 5, B and C, bottom) was sensitive to neuraminidase digestion (Fig. 5C) and was the major species (in comparison with the asialyl form) in the plasma membrane fraction (Fig. 5, B and C, bottom). Cell surface presentation of the mature form of LRP67 was also demonstrated by surface iodination experiment (data not shown). In contrast, the asialyl form of LRP67 was found predominantly in the microsomal membrane fraction (Fig. 5, B and C, top) and was sensitive to Endo H digestion (Fig. 5B).

Among the three LRP67-transfected cell lines, clone P2B4 (a high expressor) was as sensitive to PEA as CHO-K1 cells, whereas in clones P2B4 and PS2A3 (low expressors) the toxicity of PEA was only partially restored when compared with LRP-null cells (Fig. 6A). The difference in toxicity of PEA between the cell lines is attributable to the differing levels of expression and cell surface presentation of LRP67 among the clones (Fig. 5, A–C). When the high expressor P2A2 and low expressor...
P2B4 were tested for their ability to degrade $\alpha_2M^*$, we found that both clones degraded 125I-$\alpha_2M^*$ and that the ability to degrade 125I-$\alpha_2M^*$ was correlated closely to the level of LRP67 expression (Fig. 6B, left). The LRP67-mediated 125I-$\alpha_2M^*$ degradation could be prevented by RAP (Fig. 6B, right) and unlabeled $\alpha_2M^*$ (data not shown). When the $\alpha_2M^*$ degradation data were corrected for the differing level of expression, the LRP67-transfected cells gave results (64 and 124% in two clones) that were comparable with that of LRP100 (87%) (Table I, fourth column). Similarly, the sensitivity to PEA observed in LRP67-transfected cells gave results (64 and 124% in two clones) that were comparable with that of LRP100 (87%) (Table I, fourth column). In addition, LRP67-mediated binding of 125I-$\alpha_2M^*$ could be abolished by RAP (Fig. 8C). The efficiency of $\alpha_2M^*$ degradation (<30% of normal) and the cytotoxicity of PEA (<10% of normal) were much lower in cells expressing LRP25 than in cells expressing LRP100 or LRP67 (Table I).

**DISCUSSION**

In this study, the full-length chicken LRP was stably expressed in a mutant CHO-K1 cell line that lacks endogenous LRP. Biochemical experiments, together with functional analyses, indicated that the recombinant LRP protein was glycosylated, proteolytically processed into $\alpha$- and $\beta$-chains, and presented on the cell surface as a functional receptor. Thus, we have provided conclusive experimental evidence using expressed recombinant protein that LRP indeed serves as a receptor for $\alpha_2M$ (34) as well as confirmed that it serves as a gate for receptor-mediated entrance of PEA into cells (18). In addition, analysis of two receptors with internal deletions has revealed that at least part of the sequence elements responsible for the binding of $\alpha_2M$, RAP, and PEA may be located within the amino terminus of the receptor.

It has been proposed that LRP contains multiple binding sites for RAP. Existence of RAP-binding sites in clusters II and IV of LRP has been shown by studies using anchored minireceptors (11), soluble LRP fragments (35), or proteinase and CNBr digests of LRP (10). Using anchor-free, soluble LRP fragments that contained each of the four clusters of the class A ligand binding repeats, Bu and co-workers (7) have shown that RAP binds avidly to clusters II and IV and less avidly to

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**FIG. 7.** Endoglycosidase H or neuraminidase digestion of LRP25 expressed in 13-5-1 cells. The experiments (A, B, and C) were performed in essentially the same manner as in Fig. 5, except that the LRP25-transfected cells were used.

**FIG. 8.** Functional analysis of LRP25 in transfected 13-5-1 cells. The experiments (A and B) were performed in essentially the same manner as in Fig. 6, except that LRP25-transfected cells were used. PEA toxicity assay (A) for clone P7B3 was repeated with similar results. C, effect of RAP on the cell surface binding of $\alpha_2M^*$. Cells were incubated with 125I-labeled $\alpha_2M^*$ at 4°C for 2 h in the presence of the indicated concentration of RAP, and the cell-associated radioactivity was determined. TCA, trichloroacetic acid.
cluster III but does not bind to cluster I. They have also shown that there are at least five independent RAP-binding sites within LRP (two in cluster II, one in cluster III, and two in cluster IV) and that RAP binding activity seems to be conferred primarily by the class A motifs (8). The current studies with the anchored minireceptor LRP25 (Fig. 5B) have provided new evidence that a RAP-binding site may reside within the first three class A repeats of cluster II.

The αM binding site has been assigned to cluster II of LRP, but the binding activity may not be conferred solely by the class A repeats. Studies with proteolytic fragments of LRP have suggested that some epidermal growth factor type repeats flanking cluster II of class A motifs may also contribute to binding of αM-proteinase complexes (10). A membrane-anchored minireceptor that contained all eight class A repeats of cluster II but not the neighboring fourth epidermal growth factor repeat did not show binding to αM (11). Our observations of binding and degradation of αM* by LRP25-transfected cells indicate that structural determinants essential for binding of αM* are encoded by the amino-terminal 22% of the LRP molecule, a region that also contains sequence determinants for binding of RAP.

This study is the first attempt to define sequence elements within LRP that are required for the entrance of PEA. Evidence that at low concentrations PEA might enter cells via LRP-mediated endocytosis includes (i) binding of PEA to LRP α-chain and inhibition of binding by RAP (19) and (ii) resistance to PEA toxicity of cells lacking LRP expression (20, 21). The LRP-null cell line (13-5-1) used in the present study was initially selected for its increased (100-fold) PEA resistance and its toxicity. It is noted that even in cells that express an exogenous LRP molecule, a region that also contains sequence determinants for binding of RAP.

In summary, we have established an expression system for LRP, a multifunctional cell surface receptor involved in the catabolism of proteinases and lipid-associated proteins. The availability of an in vitro LRP expression system will assist in the identification of structural determinants that are responsible for the multiligand binding activity of LRP and will also facilitate investigations of the involvement of LRP in the development of premature atherosclerosis and Alzheimer’s disease.

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