Molecular Dissection of Ligand Binding Sites on the Low Density Lipoprotein Receptor-related Protein**

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The low density lipoprotein receptor-related protein (LRP) is a large multifunctional receptor that is involved in the cellular uptake of a number of functionally diverse ligands including apoE-rich remnant lipoproteins, lipoprotein lipase, \( \alpha \)-macroglobulin-protease complexes, plasminogen activator-inhibitor complexes, and the active protease tissue-type plasminogen activator. Ligand binding and competition experiments suggest that most LRP ligands bind to specific, independent sites on the large 515-kDa subunit of the receptor. In a previous study (Moestrup, S. K., Holtet, T. L., Etzerodt, M., Thøgersen, H. C., Nykjaer, A., Andreasen, P. A., Rasmussen, H. H., Sottrup-Jensen, L., and Gliemann, J. (1993) J. Biol. Chem. 268, 13601–13606), ligand blotting was used to localize the binding sites for urokinase-type plasminogen activator-plasminogen activator inhibitor-1 (PAI-1) complexes and for \( \alpha \)-macroglobulin to a proteolytic fragment of LRP containing the second cluster of complement-type cysteine-rich repeats. Here, we have used a recombinant DNA approach to express functionally restricted chimeric “LRP-minireceptors” containing two different regions of the extracellular domain of the receptor in cultured cells. Receptor-associated protein, a negative modulator of LRP activity, is bound and internalized by cells transfected with either construct. A minireceptor containing the cluster of eight complement-type cysteine-rich repeats followed by four epidermal growth factor precursor homologous domains binds and internalizes \(^{125}\)I-labeled plasminogen activator-PAI-1 (PAI-1) complexes. It also mediates the cellular uptake of the uncomplexed protease tissue-type plasminogen activator (tPA), suggesting that the tPA and PAI-1 binding sites on LRP are in close vicinity and might promote cooperative binding of tPA-PAI-1 complexes. However, \( \alpha \)-macroglobulin is not internalized by this minireceptor suggesting that this ligand requires the presence of a single epidermal growth factor repeat which is contained in the previously studied proteolytic fragment but is absent from the minireceptor.

LRP is a member of the low density lipoprotein (LDL) receptor gene family which at present comprises four distinct cell

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RAP, receptor-associated protein; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; VLDL, very low density lipoprotein; YPACK, tyrosylprolylarginyl chloromethyl ketone; DMEM, Dulbecco’s modified Eagle’s medium.

folded regions of the extracellular region of LRP fused to the membrane spanning and cytoplasmic domains of the receptor. Like wild type LRP, these minireceptors actively endocytose ligands from the extracellular environment but their ligand recognition profile is restricted.

MATERIALS AND METHODS

LDL receptor-deficient Chinese hamster ovary cells (IdlA7 cells) were kindly provided by Monty Krieger (34). Human α2-macroglobulin activated with methylamine (α2M*) was a gift from Dudley Strickland kindly provided by Monty Krieger (34). Human α2-macroglobulin activated with methylamine (α2M*) was a gift from Dudley Strickland. Human α2-macroglobulin activated with methylamine (α2M*) was a gift from Dudley Strickland. Recombinant PAI-1 was provided by Edwin Madison, Scripps Research Institute, tyrosylprolylarginyl membrane spanning and cytoplasmic domains of the receptor.

Expression of Recombinant Forms of LRP in IdlA7 Cells—A PCR fragment encoding the LRP leader peptide and the first 5 amino acids of the mature amino terminus (nucleotide 467–538; GenBank®/EMBL accession no. for LRP is X13916) was cloned into expression plasmid pT113. Conservative nucleotide changes (nucleotide 535C→T, nucleotide 538T→C) were introduced to generate a SmaI restriction site at the 3’ end of the fragment. To construct the region II of LRP two additional cDNA fragments were derived by PCR from the human LDL cDNA encoding region II (nucleotides 3026–8017) and the COOH-terminal membrane anchor and cytoplasmic tail of the receptor (nucleotides 13012–14099). These fragments were blunt-end cloned into the SmaI digested plasmid pT113. The resulting plasmid vectors were used for PCR amplification: 5′-TGCTGGTGACTCATGGGACGCTGACCGGCGG-3′ and 5′-GGAGGGTGACTGTTAAGAACCGGATTGCGGCGG-3′ to amplify the leader peptide; 5′-AAGACTTGGACGGCTGACGAGCATGTCGTTTG-3′ and 5′-CGAGGTGGACGGCTGACGAGCATGTCGTTTG-3′ to amplify region II; 5′-GTGCCCTGTGCCCTCCTCACCACGCCTCTGGGACTTGGGATCCCTATGCCA-3′ and 5′-GTGCCTGTGCCCTCCTCACCACGCCTCTGGGACTTGGGATCCCTATGCCA-3′ for the membrane anchor and cytoplasmic tail. For cloning of region IV, a PCR fragment encompassing the extracellular region IV (nucleotides 10466–13018) was cloned blunt end into SmaI digested plasmid pT113. Amplification was performed with the following PCR primers: 5′-AAGACTTGGACGGCTGACGAGCATGTCGTTTG-3′ and 5′-CTGCAGTAAAGGCGACGGCTGACGAGCATGTCGTTTG-3′.

The resulting plasmid was cut with HindIII and NotI and ligated with a HindIII/NotI restriction fragment containing the membrane anchor and the cytoplasmic tail of LRP (starting at nucleotide 12659). Region II and IV constructs were cotransfected into IdlA7 cells together with pCMV8 to generate plasmid pT113. Conservative nucleotide changes were introduced by PCR using primers 5′-CTCGAGTTAAGGCACGCATGTGCCGTTGTCCAGC-3′ and 5′-CTCGAGTTAAGGCACGCATGTGCCGTTGTCCAGC-3′ to amplify region I; 5′-CTCGAGTTAAGGCACGCATGTGCCGTTGTCCAGC-3′ and 5′-CTCGAGTTAAGGCACGCATGTGCCGTTGTCCAGC-3′ to amplify region II; 5′-CTCGAGTTAAGGCACGCATGTGCCGTTGTCCAGC-3′ and 5′-CTCGAGTTAAGGCACGCATGTGCCGTTGTCCAGC-3′ to amplify region III; 5′-CTCGAGTTAAGGCACGCATGTGCCGTTGTCCAGC-3′ and 5′-CTCGAGTTAAGGCACGCATGTGCCGTTGTCCAGC-3′ to amplify region IV.

Cellular Degradation of 125I-Labeled Ligands—6 × 10⁶ IdlA7 cells/well were seeded into 12-well plates and grown for 24 h. The medium was replaced by DMEM (without glutamine) containing 0.2% (w/v) bovine serum albumin and the indicated iodinated ligands. Cellular degradation of 125I-labeled proteins was measured as previously described (35) and is expressed as nanograms of 125I-labeled trichloroacetic acid-soluble (non-iodide) material released into the culture medium per mg of total cell protein.

RESULTS

The extracellular domain of LRP consists of four separate regions, each of which is characterized by LDL receptor ligand binding (complement)-type repeats which are followed by one or more epidermal growth factor (EGF) precursor homologous domains. The organization of this domain structure is shown in Fig. 1 and Roman numerals have been assigned to the individual regions. Using PCR we have constructed LRP minireceptors in which either region II (eight complement-type cysteine-rich repeats) or region IV (11 complement-type repeats) was fused to one EGF precursor homologous domain (Figs. 1 and 5). Region II comprises amino acids 836–2501 of the extracellular portion of wild type LRP and largely overlaps with the fragment that has been shown earlier to be the only region within the receptor that binds uPA-PAI-1 and α2-macroglobulin light chain (residues 776–1399 (33). Region IV construct starts at amino acid residue 3316 of wild type LRP. No ligand binding sites have as yet been mapped to this extracellular segment of the receptor.

Chinese hamster ovary cells that do not express functional LDL receptors (IdlA7 cells) (34) were transfected with cDNA constructs expressing either region II or IV (Fig. 1) and cell lines which stably expressed the chimeric proteins were cloned. Using a polyclonal anti-LRP antibody (Fig. 2a, lanes 1 and 2) directed against the cytoplasmic tail of LRP (4), strong expression of the minireceptors was detected by Western blotting of membrane extracts prepared from the cell lines. The expression levels of both region constructs are comparable to each other and greatly exceed the expression of endogenous LRP present in the Chinese hamster ovary cells. Region IV which contains the authentic proteolytic processing site (39) of the
wild type LRP is detected as the unprocessed precursor (region IV) or as the processed ~80 kDa amino-terminal (region IV*) and 85-kDa carboxy-terminal fragment (lanes 2 and 4). Region IV is not completely processed presumably because the intracellular protease which specifically cleaves LRP at a tetrabasic site in the eighth EGF precursor homologous domain (39) becomes limiting due to the overexpression of the “minireceptor.” In control experiments not shown, we have transfected a soluble form of region IV which lacks the transmembrane segment. Both processed and unprocessed forms appear in the medium demonstrating that the unprocessed minireceptor progresses normally through the secretory pathway. Region II does not contain the processing site.

Ligand blot analysis of regions II and IV LRP constructs reveals binding of 125I-labeled GST-RAP fusion protein to region II and to unprocessed and processed forms of region IV (Fig. 2A, lanes 3 and 4). The binding to region II is stronger than that to region IV. Region II also binds 125I-labeled tPA-PAI-1 complex (lane 7), while no binding of tPA-PAI-1 complex to either the processed or unprocessed form of region IV could be detected (lane 8). 125I-Labeled tPA that is not complexed by PAI-1 does not show detectable binding to either region or to wild type LRP present in the same membrane extracts (lanes 5 and 6).

The binding properties of region II and of wild type LRP for tPA-PAI-1 complexes on ligand blots are identical (Fig. 2B). tPA-PAI-1 complex binds strongly to region II in membrane preparations prepared from stably transfected cell lines (lane 1). Bacterially produced GST protein has no effect on the binding of the complex (lane 3). GST-RAP, on the other hand, completely abolishes binding of the 125I-tPA-PAI-1 complex to region II and to endogenous wild type LRP (lane 4). As has previously been observed for the binding of 125I-tPA-PAI-1 complexes to wild type LRP binding of the complex to region II is also dependent upon the presence of Ca2+ and is sensitive to EDTA (lane 2).

To determine if regions II and IV minireceptors are transported and internalized normally in the transfected cells we determined the rate of degradation of 125I-labeled ligands by the cultured cells. Fig. 3 shows a comparison of the ability of non-transfected IDA7 cells (open circles) and of cells lines expressing similar amounts of region II (R-II, closed triangles) and region IV (R-IV, closed circles) to degrade either 125I-labeled GST-RAP (Fig. 3A) or 125I-labeled αM* (Fig. 3B). Parental IDA7 cells are least efficient in degrading GST-RAP. Regions IV and II degrade this ligand with a 3–5-fold higher rate, respectively. The degradation rate of 125I-GST-RAP by the transfected cell lines therefore parallels the relative binding to the same regions in ligand blots (Fig. 2). In a total of seven independent experiments we were not able to demonstrate a reproducible difference in the ability of the individual transfected and non-transfected cell lines to degrade 125I-αM*. The observed rate of degradation reflects the basal activity of wild type LRP in these cells.

In contrast to 125I-αM* which was not internalized by regions II and IV minireceptors 125I-labeled tPA-PAI-1 degradation was increased on average 8-fold in the region II cells (Fig. 4A). The degradation rate of this LRP-ligand by region IV cells was indistinguishable from that of the untransfected parental cells. Region II not only harbors the PAI-1 binding site but contains also the site to which uncomplexed tPA binds, presumably through its EGF domain (40, 41). 125I-Labeled tPA that had been inactivated with the peptide inhibitor YPACK and is therefore unable to form a complex with PAI-1 was degraded at an on average 5-fold higher rate by region II cells than by region IV cells or by the untransfected parental cells (Fig. 4B).

As has been observed previously (19) and as is also shown in Fig. 2, uncomplexed tPA neither binds to LRP nor to the expressed protein containing region II on ligand blots. To distinguish between the possibilities whether the binding of tPA-PAI-1 complexes to LRP is mediated by the PAI-1 moiety or by a conformational change in tPA which might be induced by complex formation of the protease with the inhibitor we performed the ligand blot experiment shown in Fig. 5. tPA-PAI-1 complex (lane 2) but not free tPA (lane 1) binds to LRP in rat liver membrane extracts. Addition of excess PAI-1 (lane 3) blocks this binding as efficiently as GST-RAP fusion protein (lane 5). This suggests that at least on ligand blots the binding of the complex to LRP is mediated by the PAI-1 moiety.
FIG. 3. Degradation of 125I-GST-RAP and 125I-a,M* by transfected and non-transfected cell lines. Replicate monolayers of parental non-transfected idlA7 cells (idlA7) or transfected idlA7 cell lines expressing LRP regions II (R-II) or IV (R-IV) received 1 ml of DMEM (without glutamine) containing 0.2% (w/v) bovine serum albumin, and either 1 mg/ml 125I-GST-RAP (740 cpdng/mg, Panel A) or 5 ug/ml 125I-a,M* (566–800 cpdng/mg, Panel B). After incubation at 37 °C for the indicated periods of time, the amount of 125I-GST-RAP (Panel A) or 125I-a,M* (Panel B) degradation products secreted into the medium was determined. Each value represents the mean of duplicate incubations of 1 (Panel A) or 4 (Panel B) individual experiments.

FIG. 4. Degradation of 125I-tPA-PAT-1 and 125I-tPA-YPACK by transfected and non-transfected cell lines. Replicate monolayers of parental non-transfected idlA7 cells (idlA7) or transfected idlA7 cell lines expressing LRP regions II (R-II) or IV (R-IV) received 1 ml of DMEM (without glutamine) containing 0.2% (w/v) bovine serum albumin, and either 100 ng/ml tPA, 2662 cpdng/mg tPA, Panel A) or 125I-tPA-YPACK (100 ng/ml tPA, 2662 cpdng/mg tPA, Panel B). After incubation at 37 °C for the indicated time, the amount of 125I-tPA-PAT-1 (Panel A) or 125I-tPA-YPACK (Panel B) degradation products secreted into the medium was determined. Each value represents the mean of duplicate incubations.

**DISCUSSION**

Using a PCR-based cut-and-paste approach we have constructed LRP minireceptors and have shown them to be functionally expressed on the surface of transfected cells. On ligand blots of membrane proteins from stable cell lines transfected with constructs containing either region II or IV of LRP (Fig. 1), GST-RAP fusion protein binds strongly to regions II and IV whereas tPA-PAT-1 complexes bind only to region II. The intensity of the respective signals on ligand blots agrees well with the rate by which transfected cells degrade 125I-GST-RAP and 125I-tPA-PAT-1 complexes, respectively. As with wild type receptor, binding of 125I-tPA-PAT-1 to the region II minireceptor requires Cu2+ and can be abolished by coincubation with GST-RAP. Therefore, region II contains all the structural elements required for tPA-PAT-1 recognition and dissociation.

In experiments not shown here we have also observed binding of 125I-tPA-PAT-1 complexes to region II on ligand blots. This binding was comparable to the binding of 125I-tPA-PAT-1 complexes and was also paralleled by an increased degradation of tPA-PAT-1 complexes by region II cells. Furthermore, on ligand blots (Fig. 5) and in degradation experiments (21) PAI-1 has been shown to compete for the binding of plasminogen activator-PAT-1 complexes to LRP indicating that the binding of plasminogen activator-inhibitor complexes can be mediated through PAI-1. Uncomplexed tPA does not bind to LRP on ligand blots (see Ref. 19 and Figs. 2 and 5). However, cells transfected with the region II construct efficiently endocytose 125I-tPA/YPACK suggesting that binding sites for both tPA and PAI-1 may be present on the receptor and that both these sites are located within region II, thus possibly promoting cooperative binding of tPA-PAT-1 complexes. This result is in agreement with previous findings showing that uncomplexed tPA can compete for the cellular uptake of tPA-PAT-1 complexes through LRP (29). In contrast, in the study by Camani et al. (39) neither uPA-PAT-1 complexes nor free PAI-1 were able to compete for the degradation of tPA-PAT-1 complexes. The mechanism of this non-reciprocal cross-competition is unclear at present.

Moestrup et al. (33) have previously used ligand blotting techniques to show that uPA-PAT-1 complex and a,M-macroglobulin light chain both bind to a proteolytic fragment of LRP containing amino acid residues 776–1399. Because a,M-macroglobulin light chain is homologous to the receptor binding site in a,M-macroglobulin and because these two proteins compete with each other for receptor binding, it has been proposed that they bind to the same site on LRP. As depicted in Fig. 1 the proteolytic fragment contains the fourth EGF repeat of LRP, the second cluster of ligand binding type cysteine-rich repeats, and approximately half of the following EGF precursor homologous domain. In a previous study (14) a,M* did not compete with tPA-PAT-1 in cellular degradation experiments indicating that the two ligands bind to different sites.

In the present study, we were able to dissociate these sites. Our experiments show that region II transfected cells endocy-
tose and degrade tPA-PAI-1 complexes efficiently, while neither region II nor IV transfected cells show an increased uptake of myosin (200 kDa), and phosphorylase a.

Alternatively, RAP might exert its inhibitory function by an alternative mechanism. It might envisage a model in which RAP binds to multiple independent sites on LRP, which might be useful to identify the individual amino acids that participate in the ligand interaction and to define the relevant function underlying the early embryonic lethality caused by the absence of this multifunctional receptor (20, 22).

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REFERENCES

Ligand Recognition by LRP