Identification of Residues in α-Macroglobulins Important for Binding to the α2-Macroglobulin Receptor/Low Density Lipoprotein Receptor-related Protein*

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The α2-Macroglobulin receptor/lipid protein receptor-related protein (α2MR/LRP) is a 600-kDa endocytic membrane-bound receptor belonging to the low density lipoprotein receptor family (1–3), which includes the low density lipoprotein (LDL) receptor (4), epithelial glycoprotein 330 (5), and the very low density lipoprotein (VLDL) receptor (6).

α2MR/LRP is expressed in a broad spectrum of cell types as a 4525-amino acid residue single chain precursor (3). It is processed into a 85-kDa transmembrane β-chain and an approximately 515-kDa α-chain noncovalently associated with the extracellular part of the β-chain (8, 9).

The β-chain contains one transmembrane segment and a short cytoplasmic tail with two copies of the NPXY sequence that function as signals for endocytosis in coated pits (10). The α-chain contains four clusters of 2, 8, 10, and 11 cysteine-rich LDL receptor ligand-binding repeats flanked by epidermal growth factor type repeats (EGF).

α2MR/LRP is multifunctional in the sense that it is capable of binding ligands of different classes. These include α-macroglobulin-proteinase complexes (α2M-proteinase) (1, 2), plasminogen activator inhibitor-plasminogen activator complexes (PAI-1-PA) (11), lipoprotein lipase (LPL) (12), apolipoprotein E (apoE) (13, 14), bovine pancreatic trypsin inhibitor (BPTI) (15), lactotransferrin (16), α2-macroglobulin-associated protein (α2M-RAP) (9, 17, 18), and Pseudomonas exotoxin A (19). Several of these ligands do not compete for binding to the α2MR/LRP receptor.

Each domain of the α-Macroglobulin family of proteinase binding proteins is recognized by the α2MR/LRP except one, hen ovomacroglobulin (ovoM) (21). The αα2M-RAP has been mapped to the region comprising residues 776–1399, which includes EGF repeat number 4, LDL receptor ligand binding repeat number 3–10, and EGF repeat number 5–6 (domain numbering from NH2-terminal) (20). It should be noted that α2M-proteinase and PAI-1-PA do not compete for binding to α2MR/LRP (11).

In this study mutant RBDs of both α2M and ovoM have been produced in order to study the receptor interaction further. Two lys residues spaced by three amino acid residues are found to be critical for receptor binding. On the basis of this observation, the mutational evidence provided by other investigations on α2M-RAP (45), LPL (43, 44), and BPTI (15) and comparison of the sequences of the ligands versus the sequence of ovoM, a common sequence motif of importance for receptor binding is tentatively deduced and presented.

MATERIALS AND METHODS

Preparations of Mutant RBDs—In vitro mutagenesis of α2M-RBD (residues 1299–1451) of human α2M was performed either on single-stranded template using the T7-GEN in vitro mutagenesis kit as described by the manufacturer (U. S. Biochemical Corp.) or on double-stranded template using polymerase chain reaction essentially as described (28).

Before expression the mutant insert was ligated into pT-HF Xa as described (29) and modified by insertion of an oligonucleotide encoding the sequence GSPQPQNSSTSTTLPR (27). The nucleotide sequence of the construct was verified by DNA sequencing using Sequenase version 2.0 (U. S. Biochemical Corp.).

Recombinant protein was produced by expressing the plasmid
pT,H,F, with the proper mutant insert in E. coli DH1 cells. Refolding, processing, and purification was carried out according to Ref. 27.

Characterization of the Expressed Variants—The variants of RBDv were analyzed by SDS-polyacrylamide gel electrophoresis in order to determine whether its single intrachain disulfide bridge had been formed indicating correct folding (23, 24). Gel filtration on a TSK3000SW column in 0.1M sodium phosphate, pH 7.0, was used to investigate whether the products were monomeric or noncovalently as-sociated. The amino acid composition of mutant a2M-RBDv was determined after acid hydrolysis with 6M HCl, 0.1% phenol, and 5% thioglycolic acid for 20 h at 110°C in vacuo using the procedure described (30) modified to permit determination of half-cystine as cysteine (31).

Binding Assay—In vitro binding of 125I-a2M-MA to a2M/LRP immobilized in microtiter wells and competition for a2M binding sites by mutant RBDvs were performed as described (9, 27). The concentration of mutant a2M-RBDv stock solutions was determined after acid hydrolysis (mean of three analysis). The dissociation constant of each mutant was determined on the basis of at least two independent experiments.

RESULTS

Selection of Target Sites for Mutagenesis—The selection of residues for mutagenesis studies was based on comparison of the primary structures of the receptor binding domains of nine known receptor binding macros globulins with the recently determined sequence of the nonbinding hen ovoM (32). As is evident from Fig. 1, the sequences of the RBD part of nine receptor binding a2-macroglobulins are very conserved. Because previous experiments revealed that blocking Lys residues of human a2M-RBD abolished receptor binding (24), conserved Lys residues were considered. The residues Lys-1306, Lys-1333, Lys-1361, Lys-1370, Lys-1374, Lys-1425 as well as Arg-1384, Glu-1377, and Asp-1428 are all highly conserved among the nine receptor binding a2-Ms and were hence chosen as targets for mutagenesis studies. Intriguingly, in ovoM no lysine residues are present in positions 1333, 1374,
and 1425. Some of the residues selected for study were mutated into glutamic acid, glutamine, or asparagine residues to preserve hydrophilic character of residues, others were mutated into alanine. A list of the mutations performed is shown in Fig. 2.

In an attempt to induce binding capability in the corresponding ovomacroglobulin domain, the mutant constructs ovoM-Q1358K, ovoM-D1359K, and the chimeric mutant ovoM-(1355–1373), where residues 1355–1373 of ovoM were replaced with residues 1371–1389 of α-M, were produced.

Expression and Characterization of Variant Receptor Binding Domains—In a previous paper (27) we described the refolding of wild type human α-M-RBDv (residues 1299–1451) and demonstrated that we were able to produce and refold the domain and purify it in milligram quantity. All variant domains except the chimeric ovoM-M-RBD yielded a product with the intramolecular disulfide bridge formed correctly as judged by SDS-polyacrylamide gel electrophoresis analysis. The products were soluble and monomeric under nondeaturing conditions as determined by gel filtration on TSK3000SW, from which the product eluted as an approximately 20-kDa protein.

These results showed that none of the introduced mutations radically changed the folding properties of RBDv, indicating that none of the residues mutated affected interactions critical for the folding or the stability of the final structure. Furthermore, for each variant the amino acid composition was in agreement with the introduced mutation. Final yields of pure α-M-RBDv variants were approximately 0.1–0.5 mg/liter culture. The chimeric product resulting from replacement of 20 residues in ovoM with 19 residues from human RBD seemed to depend on one or more Lys residues (24). In this work, guided by analysis of the pattern of Lys residues in the RBD part of receptor binding α-M-acoglobulins and the non-binding ovoM, we have identified, by site-directed mutagenesis, two Lys residues located at positions 1370 and 1374, which are of importance for receptor binding. As seen from Fig. 1, Lys-1374 is replaced by an Asp residue in α-M, but, curiously, Lys-1370 is conserved in that protein. From sequence comparisons several other sites of potential importance for receptor binding were also identified, but introduction of mutations had no effect on receptor binding.

α-MR/LRP recognizes a variety of apparently unrelated ligands and itself consists of repeating sets of similar structural modules. It can be speculated whether these ligands essentially bind in similar ways but with minor variations reflecting individual specificity in recognizing different sites on the receptor. If indeed a common structural framework is prerequisite for receptor recognition, it might be reflected in the primary structure of the ligands. It is, therefore, relevant to collate information from mutagenesis and deletion experiments for all known α-M/LRP ligands and inspect sequence information for elements of unity or similarity. Fig. 3 shows our interpretation of such collated information, and the proposed alignment of sequence.
quence segments that are known or suggested to be binding motifs of LPLs, α-macroglobulins, αM-RAPs, PAI-1s, tissue plasminogen activators, PA, and BPTI does indeed exhibit some common primary structure features suggestive of an overall similarity in binding motifs.

The most important similarities are as follows. Position 1 preferentially contains an aromatic residue. At position 4 a hydrophobic residue is preferred, most commonly a Leu residue. Position 5 most frequently contains a basic residue. At position 8 a hydrophobic residue is present, most often a Val residue. Position 10 harbors the common Lys residue. Position 14 contains an acidic residue. RAP, however, contains a Glu residue instead. At position 22 a hydrophobic residue is present. Position 24 is an Arg residue, and position 32 is a hydrophobic residue.

Compared with the main group of binding motifs, BPTI contains a deletion at position 15-21. This deletion might well be compensated by other polypeptide segments brought into vicinity by disulphide bridging to cysteine 22. Explicitly, the residues at positions 5, 10, 14, and 24 (Arg-42, Lys-46, Asp-50, and Arg-53) are all exposed on the surface and located less than 10 Å apart in the crystal structure determined for BPTI (42).

Although speculative, this alignment is consistent with the two regions shown in our alignment and, finally, residues that proved important for the binding of BPTI to the αM/MLrp include residues Arg-42 (position 5) and Lys-46 (position 10) of BPTI (15).

In conclusion, we have provided evidence for the participation of Lys-1370 and Lys-1374 in the molecular interaction of Lys-13 and Lys-1374 in the molecular interaction between αM and its receptor. Several other mutations were performed, but none affected binding significantly. These results proved consistent with data obtained with other ligands, suggesting a common sequence motif for receptor recognition. This motif seems to originate from convergent evolution since the results proved consistent with data obtained with other ligands, suggesting a common sequence motif for receptor recognition.
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