

Expression and refolding of a high-affinity receptor binding domain from rat α_1 -macroglobulin

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Abstract A recombinant version of the receptor binding domain of rat α_1 -macroglobulin (RBDv) consisting of residues 1319–1474 has been expressed in *E. coli*. Competition experiments with ¹²⁵I-labelled methylamine treated human α_2 -macroglobulin reveal that the α_1 -macroglobulin-RBDv exhibit the same high affinity for the α_2 -macroglobulin receptor as the entire 40 kDa light chain from rat α_1 -macroglobulin. It is therefore concluded, that all determinants for receptor interaction reside in the C-terminal approx. 150 residues of the α -macroglobulin subunit.

Key words: α -Macroglobulin; Domain structure; Protein expression; α_2 -Macroglobulin receptor; Refolding, in vitro

1. Introduction

Rat α_1 -macroglobulin (α_1 M) belongs to the α -macroglobulins (α Ms), most of which are large tetrameric proteinase inhibitors present in the blood of vertebrates and invertebrates and in bird and reptile egg-white. Upon interaction of proteinases with α Ms a large structural rearrangement, known as transformation is initiated by cleavage within the exposed 'bait regions', resulting in entrapment and inhibition of the proteinase (for reviews, see [1,2]). Transformation of α Ms exposes a previously concealed receptor binding domain, important for the rapid clearance from the circulation [3–5]. The receptor for transformed α Ms termed α_2 MR/LRP [6–8] consists of a 515 kDa ligand binding α -chain and a 85 kDa membrane spanning β -chain [9–11]. α_2 MR/LRP is present in many tissues, including fibroblasts, adipocytes, macrophages and hepatocytes [3,5,12,13]. In addition to α M-proteinase complexes the receptor binds ligands of several classes, such as plasminogen activator inhibitor–plasminogen activator complexes, lipoprotein lipases and α_2 -macroglobulin receptor associated protein (α_2 M-RAP) [6,10,11,14–18].

The receptor binding domain of α Ms reside in their C-terminal region, and from several α Ms an approx. 138-residue C-terminal fragment can be released by limited proteolysis (RBD) [19–21]. The affinity of the α_2 M-RBD fragment for α_2 MR/LRP is approx. 100 nM, i.e. only 0.1–0.2% of that of intact α_2 M-proteinase complexes [19–21].

The subunit of rat α_1 M is processed to a 1200 residue N-terminal heavy chain and a 250 residue C-terminal light chain [22], and since the α_1 M light chain seems to bind to α_2 MR/LRP with higher affinity than RBD [21] it has been questioned whether RBD contains complete information for receptor binding [21].

In a previous paper [23] we showed that a human α_2 M-RBD variant binding α_2 MR/LRP with high affinity (8 nM) could be produced by including 15 upstream residues from the α_2 M sequence (α_2 M-RBDv). The K_d of the α_2 M-RBDv is of the same order of magnitude as for the interaction of one monomer of intact activated α_2 M with α_2 MR/LRP [10]. In the present study we extend this result by demonstrating that a corresponding construct based on the rat α_1 M sequence produces a rat α_1 M-RBD variant which has significantly higher affinity for α_2 MR/LRP than a rat α_1 M construct corresponding to human α_2 M-RBD [24]. This result shows that the proper structural domain boundary of both α M-RBDs is located within the upstream 15-residue segment.

2. Materials and methods

2.1. Synthesis of glutathione-Sepharose

Glutathione-Sepharose (GSH-Sepharose) was synthesised from 25 g of drained Sepharose CL-6B, activated with 1,1'-carbonyldiimidazole [25]. 2.5 g of reduced glutathione was dissolved in 0.1 M Na-phosphate (pH 7.0) and oxidised on ice with 0.9 ml of 30% hydrogen peroxide. Oxidised glutathione was added to the gel suspension and mixed overnight. After blocking of remaining groups with 1 M ethanolamine for 4 h and extensively washing of the gel with 0.1 M Na-phosphate (pH 7.0), the gel was washed with degassed water and treated with 20 mM dithiothreitol. The reduced immobilised glutathione was then treated with 20 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). After extensive washing of the matrix with water it was packed into a column and kept refrigerated in the dark until use.

2.2. Fusion protein design and *E. coli* expression vector construction

To allow for initial affinity purification of fusion protein, immobilisation of fusion protein to glutathione-Sepharose by disulfide links and final liberation of α_1 M-RBDv by factor X_a cleavage, we devised a four segment fusion protein by joining sequences encoding a hexahistidine tag, a GSCTGS hexapeptide, a factor X_a recognition sequence and the α_1 M-RBDv cDNA fragment. Vector assembly strategy and methods paralleled those given in [23], except that the linker inserted at the vectors BamHI site was designed to encode the GSCTGS peptide segment. The construction was verified by DNA sequencing using SEQUENASE ver. 2.0.

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Abbreviations: RBD, receptor binding domain (approx. 138 C-terminal residues); RBDv, receptor binding domain variant (approx. 153 C-terminal residues); α_1 M, rat α_1 -macroglobulin; α_2 M, human α_2 -macroglobulin; α_1 M-MA, α_2 M-MA, methylamine treated rat α_1 -macroglobulin or human α_2 -macroglobulin, respectively; α_2 MR/LRP, α_2 -macroglobulin receptor/low density lipoprotein receptor-related protein; α_2 M-RAP, α_2 -macroglobulin receptor associated protein; α_1 M-LC, α_1 -macroglobulin light chain; FX_a, activated blood coagulation factor X; GSH, reduced glutathione.

2.3. Purification, refolding and processing of α_1 M-RBDv fusion protein

Following solvation of the crude protein pellet in guanidinium chloride and buffer exchange into 50 mM Tris-HCl, 8 M urea, 0.5 M NaCl, 10 mM 2-mercaptoethanol (pH 8.0), by gel filtration on Sephadex G-25 the protein extract was applied to a 40 ml Ni²⁺-NTA-Sepharose column [26]. After washing the column with 50 mM Tris-HCl, 6 M guanidinium chloride, 10 mM 2-mercaptoethanol (pH 8.0), the column was washed with 50 mM Tris-HCl, 8 M urea, 0.5 M NaCl and the bound protein was eluted with 50 mM Tris-HCl, 8 M urea, 0.5 M NaCl, 10 mM EDTA (pH 8.0). This pool was applied to a glutathione-Sepharose column and refolded while immobilised on the column using an iterative refolding procedure. The fusion protein exhibited a tendency to aggregate, so in order to achieve maximum yield of α_1 M-RBDv, the cleavage with FX_a to liberate the domain from the fusion tail was performed while immobilised on the GSH-sepharose, by applying 1 mg of FX_a dissolved in 2 ml of 50 mM Tris-HCl, 0.5 M NaCl, 2 mM CaCl₂ (pH 8) to the column and circulating this pool for 16 h. Gel filtration into a buffer of low ionic strength and ion-exchange chromatography on Q-Sepharose (Pharmacia) yielded pure α_1 M-RBDv. The concentration of the α_1 M-RBDv was determined by amino acid analysis.

2.4. Preparation of α_1 M-LC

Rat α_1 M was first treated with 0.2 M methylamine to cleave its internal thioesters, and then fully reduced with dithiothreitol followed by treatment with iodoacetamide to block all SH-groups. Heavy and light chains of carboxamidomethylated α_1 M were separated by gel filtration on a Superose 12 column equilibrated and eluted with 50 mM Tris-HCl, 6 M guanidinium chloride (pH 8.0) essentially according to [21]. The denaturant was removed by dialysis against 50 mM Tris-HCl (pH 8.0).

2.5. Amino acid composition analysis

After hydrolysis for 20 h at 110°C in vacuo in 6 M HCl, 0.1% phenol, 5% thioglycolic acid the amino acid composition was determined by cation-exchange chromatography [27] modified as in [28].

2.6. Determination of affinity of binding to the α_2 MR/LRP

Competition assays using 10 pM [¹²⁵I] α_2 M-MA as competitor were performed as in [23]. 100% binding was 400–600 cpm above a background of 5–10 cpm. Non-specific binding to wells not coated with α_2 MR/LRP was less than 0.5% of binding of the added tracer.

3. Results

Expression in *E. coli* and protein purification, refolding and processing were monitored by SDS-PAGE and the final prepa-

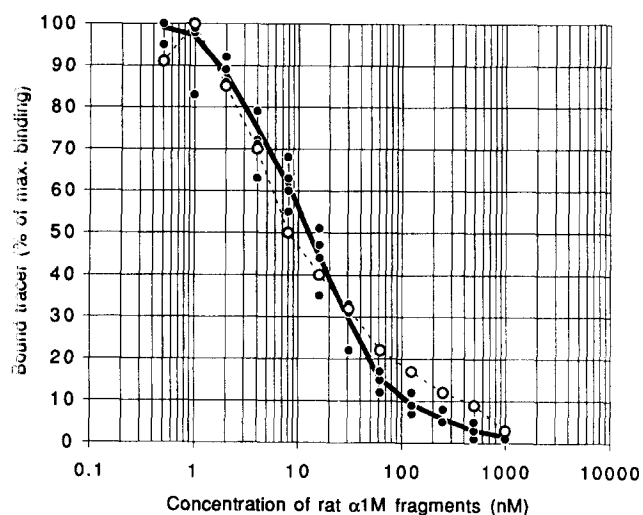


Fig. 1. Competition of α_1 M-RBDv (closed circles) and α_1 M light chain (open circles) with [¹²⁵I] α_2 M-MA for binding to placental α_2 MR/LRP immobilised to microtiter wells. The thick line represents an average of four independent determinations for α_1 M-RBDv, while the dotted line shows the result of a single determination of α_1 M light chain.

	1287	RBDv	RBD	1335
human α_2 m	GEYSMKVTGE	GCYVLTQSLK	YNILPEKE.E	FPFALGVQTL PQTCDEPKAH
rat α_1 m	GNVITKVSQS	GCYVLTQSLK	YNILPEAEQE	APFTLKVNLT PLNFQKAEHH
rat α_{113}	GNVTVSVSG	GCYVLTQTLR	YNVPLEKQ.Q	PAPALKVQTV PLTCNNPKGQ
rat α_2 m	GDYTKVVTGE	GCYVLTQSLK	YSVLPREE.E	FPFAVWVQTL PGTCBDPKAH
murinoglob. 2	GDYTVSVSG	GCYVLTQTLR	YNHLEKQ.L	SAPALRVQTV PLTCNNPKGH
murinoglob. 1	GDYTVSVSG	GCYVLTQTLR	YNHLEKQ.Q	SAPALRVQTV PLTCNNPKGH
mouse α_2 M	GNVITKVSQS	GCYVLTQSLK	YNILPVADGK	APFALQVNTL PLNFQKAEHH
human P2P	GEYVITVTGE	RCYVLTQSMK	YNILPEKE.D	SPFALKVQTV PQTCDEGKHAH
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Fig. 2. Alignment of the N-terminal border of the receptor binding domains of 8 macroglobulins. Asterisks indicate the presence of 6 or more identically located residues. The beginning of authentic RBD and recombinant RBDv is indicated. The numbering of human α_2 M is used.

ration produced one band consistent with a molecular mass of approx. 17 kDa, in agreement with the expected mass of 17783 Da.

The amino acid composition of α_1 M-RBDv was in excellent agreement with that expected. By gel filtration on a Superose 12 column under non-denaturing condition α_1 M-RBDv eluted as a monomeric protein of approx. 18 kDa even after several rounds of freezing and thawing. In contrast, α_1 M-LC eluted in the void volume of the column under the same conditions, showing that the product, although soluble, was extensively aggregated [30].

Fig. 1 shows the concentration dependence of inhibition of binding of 10 pM [¹²⁵I] α_2 M-MA to immobilised α_2 MR/LRP by unlabelled α_1 M-RBDv and α_1 M-LC. Half-maximal binding is observed at 12 and 8 nM, respectively. In contrast, the proteolytic RBD fragment isolated from α_2 M-MA produces half-maximal binding at approximately 100 nM [19–21]. The affinity of α_1 M-MA is higher, consistent with the suggestion that tetrameric α_1 M-MA is able to bind to adjacent receptor molecules [10].

4. Discussion

The molecular events resulting in binding, endocytosis and degradation of the α M-proteinase complex to the α_2 MR/LRP are not well understood. We have developed a system in which we are able to refold a variant of the receptor binding domain, (residues 1299–1451; RBDv) from human α_2 M and have shown that the affinity of this fragment is approx. 10 times higher than the affinity of the proteolytically derived RBD-fragment (residues 1314–1451).

Expression in *E. coli* of a fragment from α_1 M (MetGlu¹³³⁴-Ala¹⁴⁷⁷) with receptor binding properties was recently reported [22]. This fragment corresponds to human α_2 M-RBD and the apparent K_d for the interaction with α_2 MR/LRP was reported to 20 nM. The determination of K_d was, however, based on an $E^{1\%,1\text{cm}}_{280}$ of 11.5. In contrast, we find that our longer fragment has an $E^{1\%,1\text{cm}}_{280}$ of 6.0. The calculated value [31] of $E^{1\%,1\text{cm}}_{280}$ is 5.2 for residues 1322–1477 (RBD), so the actual dissociation constant is likely to be higher than 40 nM, approaching that determined for human α_2 M-RBD [19–21]. The α_1 M-RBDv expressed in this study ($E^{1\%,1\text{cm}}_{280}$ determined to be 6.0) appears to be correctly folded since it, once purified and liberated from the fusion protein construct, appears monomeric in gel filtration under non-denaturing conditions.

K_d for in vitro binding of α_1 M-RBDv to α_2 MR/LRP was determined to be 12 nM, similar to the value estimated for the binding of α_1 M-LC to α_2 MR/LRP. Hence recombinant α_1 M-RBDv represents a domain with considerably higher affinity

for the receptor than recombinant Met- α_1 M-RBD and our data do not support the previous suggestion [21] that the approx. 100 residues upstream of RBD in the α_1 M light chain contain additional determinants for receptor binding.

An alignment of the sequences of the N-terminal border of the receptor binding domains is shown in Fig. 2. It is evident that the additional 15-residue segment included in our RBDv construct is part of a highly conserved segment comprising residues 1292–1314. A variant comprising residues 1292–1451 of human α_2 M, where Cys¹²⁹⁸ was replaced with a serine has also been expressed and refolded but no additional increase in affinity was obtained (data not shown).

We have therefore now located the proper domain boundary for both aMs to the region 1299–1313 (human α_2 M numbering) by generating authentic recombinant fragments accounting for the receptor binding affinity in quantitative terms.

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