Epitope mapping by screening of phage display libraries of a monoclonal antibody directed against the receptor binding domain of human α2-macroglobulin

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Abstract The human proteinase inhibitor, α2-macroglobulin (α2-M), inhibits a large number of proteinases. α2-M-proteinase complexes are rapidly cleared from the circulation by binding to a cellular receptor (α2-M-R/LRP) via the receptor binding domain (RBD) which is made up of a 20 kDa C-terminal stretch of the 180 kDa monomer of the inhibitor. A monoclonal antibody (mab α-1) has been described which reacts with the receptor-recognizable form of the inhibitor, the so called transformed α2-M (α2-Mt). By screening of a phage display library an epitope in the RBD of the inhibitor was identified that reacts with mab α-1. Out of 25 phage clones a heptapeptide sequence (S-x_1-x_2-D-x_3-x_4-K) was obtained containing identical amino acids in three positions. A consensus peptide (S-R-S-D-P-P-K) was synthesized

located at the tip of the H-like structure of the inhibitor molecule [1,5]. The RBD is formed by a 20 kDa C-terminal stretch of the 180 kDa monomer [6,7].

Recently, we described a monoclonal antibody (mab), α-1, reacting with an unknown epitope located at the 20 kDa C-terminal fragment of α2-Mt. This antibody has been used to specifically detect the transformed inhibitor in plasma by ELISA [4].

Screening a random phage display library was employed to identify an epitope in α2-M reacting with mab α-1. This method has been proved to be a powerful technique for detection of peptide ligands for several proteins including mem-

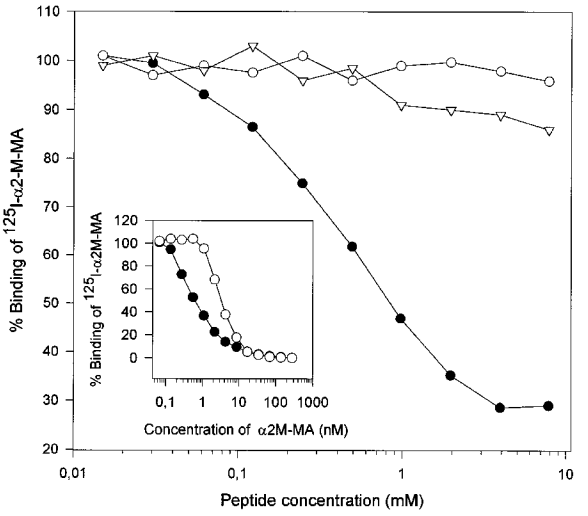

Fig. 1. Competition of consensus peptide SRSDPPK and reversed peptide KPPDSRS for binding of ^{125}I-α2-M-MA to immobilized mab α-1 and α-11. Titer plates were coated with mab α-1 or mab α-11. The wells were incubated with 0.6 nM [^{125}I]α2-M-MA (500 000 cpm/cavity) and increasing concentrations of consensus peptide and r-peptide, respectively, in phosphate buffer (50 mM sodium phosphate, 150 mM NaCl, 0.05% Tween 20, pH 7.4) for 1 h at 37°C. After washing bound radioactivity was eluted by incubation with 1 M NaOH/0.7% SDS for 1 h at 37°C. ●, consensus peptide and mab α-1; ○, consensus peptide and mab α-11; ▽, r-peptide and mab α-1. The r-peptide which had no competitive effect on binding of α2-M to α-11 is not shown in the figure. Inset: Competition of α2-M-MA for binding of [^{125}I]α2-M-MA to immobilized mab α-1 and mab α-11. Conditions were as described above with the exception that no peptide but increasing concentrations of α2-M-MA were applied for competition. ●, binding to mab α-1; ○, binding to mab α-11.

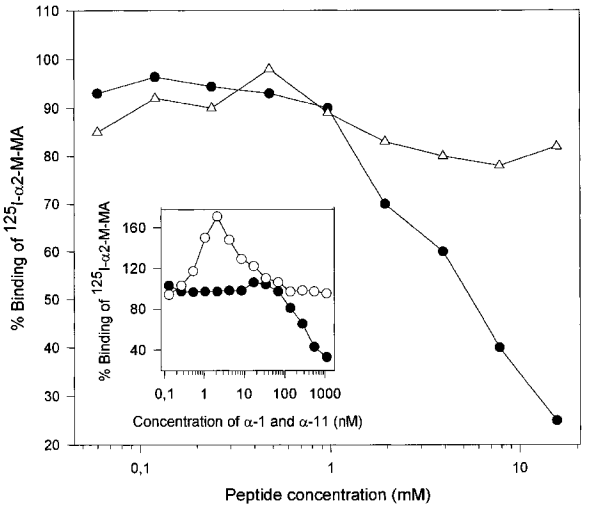

Fig. 2. Effect of consensus peptide and reversed peptide on binding to α2-M-R/LRP of [^{125}I]α2-M-MA. Titer plates were coated with purified receptor (2 μg/ml). The wells were incubated with 0.6 nM [^{125}I]α2-M-MA (500 000 cpm/cavity) and increasing concentrations of consensus peptide (●) or reversed peptide (△) in 20 mM HEPES, 150 mM NaCl, 5 mM Ca^{2+}, 0.05% Tween 20, pH 7.4 for 3 h at 37°C. After washing bound radioactivity was eluted by incubation with 1 M NaOH/0.7% SDS for 1 h at 37°C. The degree of non-specific binding to the immobilized receptor evaluated by binding in the presence of 20 mM EDTA instead of Ca^{2+} was subtracted. Inset: Effect of mab α-1 and mab α-11 on binding of [^{125}I]α2-M-MA to immobilized α2-M-R/LRP. Conditions were as described above with the exception that increasing concentrations of mab α-1 (●) and mab α-11 (α) were used instead of peptides.

shared the common motif S-x_1-x_2-D-x_3-x_4-K. The most common amino acids intervening in this motif were R, S, P, P for x_1, x_2, x_3, x_4, respectively, yielding the consensus sequence of R-S-R-D-P-P-K. This consensus peptide was not found among the phage sequences.

A peptide with this sequence was synthesized and its interaction with mab α-1 and mab α-11, used as control, was evaluated by ELISA.

Mab α-1, used for biopanning, recognizes an epitope which is only present in α2-Mt, whereas mab α-11 binds to an unknown epitope common in both the native and the transformed inhibitor. As seen in Fig. 1 the heptapeptide S-R-S-D-P-P-K competitively inhibited the binding of α2-Mt to mab α-1, while binding of α2-Mt to mab α-11 was not affected by the peptide. The inhibition constant (IC_{50}) of the heptapeptide was estimated to be 300 μM. However, this inhibitory effect is lower than the IC_{50} of α2-Mt binding to mab α-1 and α-11, which were 0.5 nM and 3 nM, respectively (Fig. 1, inset).

A reversed peptide (r-peptide) with the sequence K-P-P-D-S-R-S was synthesized to confirm that the sequence obtained from the phage library was specifically bound to mab α-1. The r-peptide displayed only a weak competitive effect on binding of radiolabelled α2-Mt to α-1 and no influence on interaction between mab α-11 and the inhibitor was observed (data not shown). These findings indicate that the phage-derived peptide specifically binds to the antigen binding site in mab α-1, most probably by mimicking a certain structural motif in the C-terminal part of the α2-M polypeptide chain.

The C-terminal part of the α2-M subunit is known to harbor the receptor recognition sites. In a second type of experiments we studied the influence of the consensus peptide on binding of α-2Mt to its receptor. First, we tested the effect on α2-Mt binding to immobilized α2-M-R/LRP of the mabs α-1

Table 1
Epitope sequence of phages isolated after three rounds of biopanning with mab α-1

1	**Ser**	Arg	Ile	**Asp**	Pro	Pro	**Lys**	(*n*=9)
2	**Ser**	Phe	Ser	**Asp**	Gln	Pro	**Lys**	(*n*=6)
3	**Ser**	Thr	Ser	**Asp**	Pro	Val	**Lys**	(*n*=6)
4	**Ser**	Phe	Ser	**Asp**	Ala	Trp	**Lys**	(*n*=2)
5	**Ser**	Thr	Leu	**Asp**	Pro	Ile	**Lys**	(*n*=1)
6	**Ser**	Arg	Leu	**Asp**	Pro	Pro	**Lys**	(*n*=1)
	Ser (25)	Arg/Thr (10/7)	Ser/Ile (14/9)	**Asp** (25)	Pro/Gln (17/6)	Pro/Val (16/6)	**Lys** (25)	

The phage epitope was deduced from the DNA sequence.

and α-11 (Fig. 2, inset). At low concentrations of mab α-11 an increased binding of α2-Mt to α2-M-R/LRP was observed which may be due to an antibody-mediated dimerization of the inhibitor but no competition was found at higher antibody concentrations. In contrast, mab α-1 caused a clear inhibition of receptor binding at concentrations higher than 100 nM. The consensus peptide S-R-S-D-P-P-K was found to inhibit receptor binding of α2-Mt, too (Fig. 2). Despite the high inhibitory concentration needed (IC_{50}=4 mM), specificity is demonstrated by the absence of inhibition in the case of r-peptide.

4. Discussion

A heptamer peptide library displayed on the N-terminus of protein III of bacteriophage M13 was used for epitope mapping. Out of 25 clones six different subsets of clones were isolated, all containing the common motif S-x_1-x_2-D-x_3-x_4-K. By alignment and calculation of the most frequently occurring amino acids at the intervening positions the consensus structure, S-R-S-D-P-P-K, was deduced. Subsequent peptide synthesis and inhibition studies clearly demonstrated competition between radiolabelled α2-Mt and this peptide for binding to monoclonal antibody α-1 used for panning. However, the competitive effect of the consensus peptide was much less pronounced when compared with the whole transformed inhibitor. This likely represents suboptimal sequences which may be due to the limited size of the library or to the shortness of the peptide with missing flanking sequences crucial for optimal binding. Recently, it was claimed that antibodies may make direct contact with up to 22 amino acid residues of the antigen [12]. Thus, a heptapeptide may only comprise most essential amino acid residues for binding. Furthermore, a discontinuous epitope may be difficult to mimic by a short peptide compared with a continuous epitope. Finally, the consensus sequence which itself is not displayed by the phage may be a worse binder than one of the six phage sequences found.

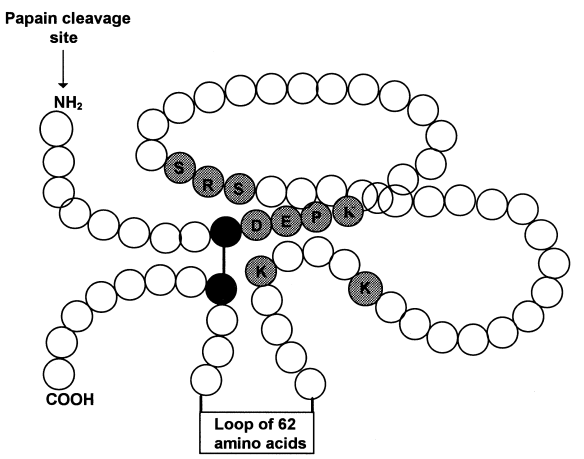

Fig. 3. Proposed spatial arrangement of discontinuous epitope in α2-M recognized by phage selected from the library. Model of spatial structure of the C-terminal peptide of α2-M. Amino acids are symbolized by circles. Single letter code of amino acids is used. A disulfide bridge connects Cys-1329 with Cys-1444 (black circles). Downstream of C-1329, a loop is formed to bring DEPK and SRS close together. Lys(K)-1370 and Lys(K)-1374, which have been shown to be crucial for receptor binding, are indicated. For the sake of clarity, a loop of a further 62 amino acids is omitted (box).

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          1321          *
h-α2-M  GVQTLPQTCDEPKAHTSFQISLSVSYTGSRSASNMA
b-α2-M  EVQTLPQTCDGPKAHTSFQISLSV SYIGSRPASNMA
h-PZP   KVQTVPQTCDGHKAHTSFQISLTI SYTGNRPASNMV

          1357
h-α2-M  IVDVKMVSGFIPLKPTVKMLERSNHVSRTEVSSNH
b-α2M   IVDVKMVSGFIPLKPTVKMLERSN- VSRTEVSNNH
h-PZP   IVDVKMVSGFIPLKPTVKMLERSSSVSRTEVSNNH

          1392
h-a2-M    VLIYLDKVSNQTLSLFFTVLQDVPVRDLKPAIVK
b-a2-M    VLIYLDKVSNETLTLF FTVLQDI PVRDLKPAIVK
h-PZP     VLIYVEQVTNQTLSFSFMVLQDIPVGDLKPAIVK

          1426                *
h-α2-M    VYDYYETDEFA IA EYNAPCSKDLGNA
b-α2M     VYDYYETDEFAVAEYNAPCSKDTGNA
h-PZP     VYDYYETDESVVAEY I APCSSDTEHGNY
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Fig. 4. Alignment of amino acid sequence of the receptor binding domains of human α2-macroglobulin, human pregnancy zone protein and bovine α2-macroglobulin. Sequence alignment of the RBDs of human α2-M (upper line) [16] with the corresponding domain of bovine α2-M (middle line) [17] and with human PZP (lower line) [18]. The residues are numbered according to their position in human α2-M. Bold printed amino acids represent the proposed epitope fragments recognized by phage display. Asterisks indicate Cys residues forming the intra-chain disulfide bridge responsible for folding the C-terminal part of the polypeptide chain.

However, comparable inhibition constants have been described recently when a hexapeptide library was applied [13] and somewhat lower constants when a larger (30-mer) peptide library was used [12]. Nevertheless, tight binding of phage to mab α-1 sufficient for biopanning was observed, which could be attributed to multivalency of binding sites during the panning process [14,15].

Looking for a peptide sequence in the α2-M structure analogous to the phage-derived consensus peptide we could identify two peptide stretches which probably represent the common motif found in the diverse phage-derived peptides, namely D-E-P-K (aa 1330–1333) and S-R-S (aa 1349–1351) in the C-terminal sequence of the inhibitor. No continuous linear sequence was found that coincided with any of the phage-derived peptides. Thus, the epitope defined by mab α-1 binding may be made up of two different parts in different locations within the polypeptide chain and indicates the recognition of a conformation epitope. This implies that the polypeptide chain of the C-terminal fragment has to be folded in such a way as to bring the two parts of the epitope close together. The proposed folded structure is shown in Fig. 3. The formed loop is stabilized by a disulfide bond extending between Cys-1329 and Cys-1444 [1]. The recent finding that mab α-1 does not react with the purified 20 kDa C-terminal fragment after reduction and carboxymethylation supports our hypothesis [4].

Interestingly, the proposed epitope lies in a highly conserved region of the human inhibitor [16] which shows strong homology to related proteins such as bovine α2-M [17] and human pregnancy zone protein (PZP) [18] (Fig. 4). The motif D-x_3-x_4-K is conserved in all three proteins, whereas the motif S-x_1-x_2 is found only in the two macroglobulins but not in PZP. This might explain why mab α-1 reacts strongly with the bovine inhibitor but not with pregnancy zone protein [4]. The nearness of Cys-1329 to the epitope may be the cause of the

high sensitivity toward reducing agents of epitope recognition by mab α-1.

Attempts were made by several authors to identify amino acids in the RBD involved in binding of α2-M to its receptor by chemical modification experiments and mutational analysis [19,20]. Two lysine residues (Lys-1370 and Lys-1374) spaced by three amino acids were found to be crucial for receptor binding. This is supported by the finding that ovomacroglobulin lacking a lysine residue equivalent to Lys-1374 in α2-M exhibited no affinity to the receptor. However, mutational insertion of lysine in this protein did not reconstitute receptor binding indicating that in addition to the two lysine residues other sites may contribute to receptor binding, too.

Several discontinuous epitopes have been mapped already using the phage display technique. However, either libraries comprising larger random peptides were used for that purpose [12] or if libraries with smaller peptides were screened, sequences found after panning could not be assigned to the sequence in proteins [13,21], or did not reflect sequences in different regions of the protein directly [22]. Our results represent an example that different parts of a discontinuous epitope can be detected as two linear sequences within one peptide by the phage display technique even if libraries comprising short random heptapeptides are screened.

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