A recombinant bait region mutant of human α 2-macroglobulin exhibiting an altered proteinase-inhibiting spectrum

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Abstract

Alpha 2-macroglobulin (α 2M), a plasma glycoprotein produced in the liver, inhibits a variety of proteinases and thus considered to play important homeostatic roles in the body. This broad inhibitory spectrum has been explained by the trapping theory by which a proteinase recognizes a region of 25–30 amino acid peptide in α 2M called bait region and cleaves it, leading to the conformational change of α 2M, and to the subsequent entrapment and inhibition of the proteinase.

We constructed α 2M cDNAs with mutated DNA sequences in the bait region, and obtained recombinant CHO cell lines producing either wild type α 2M, or mutant α 2Ms, i.e., α 2M/K692 and α 2M/K696, each with substitution of Arg with Lys at codons 692 and 696, respectively. We tested if lysyl endopeptidase is not inhibited by wild type α 2M, but could be inhibited by these engineered mutant α 2Ms. Thus, recombinant α 2M/K696 protein successfully inhibited lysyl endopeptidase activity, while recombinant α 2M/K692 protein was not sensitive to lysyl endopeptidase, suggesting that not all bait region peptide bonds can equally be accessible and susceptible to proteinases. The present results not only provided the trapping theory with additional supportive evidence, but the first experimental evidence for the value of engineered α 2M-derived proteinase inhibitor with an artificial proteinase inhibitory spectrum of potential industrial and/or therapeutic usefulness.

Abbreviations: α 2M, alpha 2-macroglobulin; h α 2M, human α 2M; rh α 2M, recombinant h α 2M; rh α 2M/K692, rh α 2M with substitution of Arg with Lys at codon 692; rh α 2M/K696, rh α 2M with substitution of Arg with Lys at codon 696.

Introduction

Alpha 2-macroglobulin (α 2M) is a protein that is capable of inhibiting enzymatic activities of many different kinds of proteinases including those of thiol, serine, metallo and aspartic acid type enzymes either of endogenous or foreign origins. Human alpha 2-macroglobulin (h α 2M) is one of the major plasma glycoproteins with four identical 180 kDa subunits. It is produced by cells of several types including hep-

atocytes, macrophages, fibroblasts, glioblastoma cells, sertoli cells and adrenocortical cells (For reviews see Borth, 1992; Chu and Pizzo, 1994).

Proteins of similar molecular structure with capability to inhibit proteinases, e.g., ovomacroglobulin, have been found widely in animals, and are considered to play important homeostatic, antibacterial, and antiviral roles (Starkey and Barrett, 1982; Freedman and Pizzo, 1985; Arakawa et al., 1986; Osada et al., 1986; Chhatwal et al., 1987; Pritchett and Paulson, 1989; Feldman, 1991; Miyagawa et al., 1991; Araujo-Jorge et al., 1992; Ryan-Poirier and Kawaoka, 1993).

A 25–30 amino acid peptide stretch called bait

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A	675											681											689							
	His	Gly	Pro	Glu	Gly	Leu	Arg	Val	Gly	Phe	Tyr	Glu	Ser	Asp	Val															
	690					692													696											704
	Met	Gly	<u>Arg</u>	Gly	His	Ala	<u>Arg</u>	Leu	Val	His	Val	Glu	Glu	Pro	His															
B																								692						
	Primer 692:		5'C		ATG	CAC	CAG	GCG	TGC	ATG	GCC	<u>TTT</u>	TCC	CAT	TAC	ATC3'														
	Primer 696:		5'C		ATG	CAC	CAG	<u>TTT</u>	TGC	ATG	GCC	TCT	TC3'																	

Figure 1. Primary sequence of the human $\alpha 2M$ bait region peptide (A) and the design of two mutant $\alpha 2M$ bait region sequences with amino acid substitution at 692 and at 696 (B) (see Materials and Methods section).

region and a thioester bond both located within the middle of each of the subunits, have been shown to play important roles for $\alpha 2M$ to inhibit proteinases. The primary structure of the bait region is unique in that it is susceptible to virtually all common proteinases (Sottrup-Jensen et al., 1989; Chu and Pizzo 1994; see Figure 1).

It has been hypothesized that the bait region proteolysis by a target proteinase triggers the autocleavage of the thioester bond in each subunit, leading to the conformational change of the molecule and to the subsequent entrapment and inhibition of the target proteinase, and further to the exposure of a receptor binding site which has otherwise been hidden within the molecule (Barrett and Starkey, 1973).

Experimental evidence that supports the trap hypothesis has been accumulated: In an electrophoregram, native $\alpha 2M$ (slow form) moves slower than the thioester-cleaved $\alpha 2M$ (fast form) (Barrett et al., 1979; Bjork et al., 1985). The interaction of $\alpha 2M$ and a wide variety of proteinases have been successfully explained by the theory (Mortensen et al., 1981; Borth, 1992). It was also shown that the protein-proteinase complex is internalized into receptor-bearing cells like hepatocytes and macrophages (Pastan et al., 1977; Willingham et al., 1979).

Although a vast majority of foreign, as well as endogenous, proteinases are inhibited by $\alpha 2M$, there are still some, which are not, or incompletely inhibited by the molecule: among them are proteinases produced by *Porphyromonas gingivalis* (Bedi and Williams, 1994), *Clostridium perfringens* (Wolf et al., 1992) and *Serratia liquefaciens* (Wolf et al., 1991). The lack of inhibitory activity toward these proteinases has been explained either by the fact that some of these proteinases, such as *Clostridium collagenase*

with its molecular weight of more than 100 kDa, are too large to be encaged in $\alpha 2M$, or that they are incapable of cleaving the bait region peptide (Robert and Hall, 1983; Bjork, 1984; Sottrup-Jensen and Birkedal-Hansen, 1989).

The study of the mechanism of proteinase inhibition, as outlined above, suggests the possibility that, by introducing an artificial peptide sequence in the bait region using genetic engineering and recombinant production technologies, we could further test the trapping theory, and in addition, obtain new artificial proteinase inhibitor molecules each with an altered inhibition spectrum of potential industrial and/or therapeutic usefulness.

To test the possibility, we paid special attention to *Achromobacter lyticus* lysyl endopeptidase (Sekiyama and Masaki, 1994), since its molecular weight, ca. 20 kDa as a dimer molecule, is not too large to be encaged in $\alpha 2M$, while the bait region of $\alpha 2M$ does not have lysyl residues, a prerequisite for the enzymatic action of lysyl endopeptidase.

Here we report some of the characteristics of genetically engineered recombinant $\alpha 2Ms$ ($rh\alpha 2Ms$) with Lys substitution in its bait region. Detailed procedures of the genetic engineering and recombinant production of wild type and altered $\alpha 2Ms$ are described (Nakamichi et al., 1998).

Materials and methods

Construction of production systems for $rh\alpha 2Ms$

The procedure for the construction of recombinant wild type- and mutant $rh\alpha 2M$ production systems are described in details separately (Nakamichi et al.,

1998). Briefly, cultured HepG2 cells (ATCC CCL61) were used as the source of $\alpha 2\text{M}$ cDNA. From the poly (A)⁺ RNA obtained from a culture of HepG2 cells, double strand cDNA (ds-cDNA) was synthesized, and it was cloned into λ gt10 phage vector. Using ³²P-labeled 5'- and 3'-end fragment probes of $\alpha 2\text{M}$ cDNA, wild type cDNA clones were isolated.

We designed two mutant $\alpha 2\text{M}$ s with modified peptide sequences of bait region (Fig. 1). Thus, one, called $\alpha 2\text{M}/\text{K692}$, was designed to contain Lys, in place of Arg at position 692, and the other, $\alpha 2\text{M}/\text{K696}$, to contain Lys, again in place of Arg at position 696. We chose Arg for substitution with Lys, intending to minimize chemical and structural changes of the whole molecule due to this substitution.

Corresponding to each of the above peptide substitutions, a modified nucleotide sequence was designed, i.e., TTT for Arg 692 substituted by TCT for Lys, and TTT for Arg 696 by GCG for Lys, respectively (Fig. 1). cDNAs with these bait region substitutions were obtained, as integrated in the plasmid vector pUC19, using the technique of unique site elimination (U. S. E.) mutagenesis kit (Pharmacia Biotech, Tokyo, Japan). The prototype and the two engineered cDNAs were further cloned into the mammalian cell expression vector, pcDSR α -neo Δ 11 (Noda et al., 1994). The constructs were designated as pcDSR α Δ -neo Δ 11/ $\alpha 2\text{M}$, pcDSR α Δ -neo Δ 11/K692 $\alpha 2\text{M}$ and pcDSR α Δ -neo Δ 11/K696 $\alpha 2\text{M}$, respectively for wild type rh $\alpha 2\text{M}$, rh $\alpha 2\text{M}/\text{K692}$ and rh $\alpha 2\text{M}/\text{K696}$.

The expression vectors were introduced into Chinese hamster ovary (CHO) K1 cells (ATCC CCL61), selected for producer clones with G418. The recombinant CHO cells were grown in Eagle's MEM (GIBCO BRL, Maryland, USA) supplemented with non-essential amino acids and 10% fetal bovine serum (FBS, JRH Biosciences, Lenexa, Kansas, USA) following the method described previously (Nakamichi et al., 1997). In this study only one cell clone for the recombinant production of each of the three proteins was used, which will be referred to as rCHO/ $\alpha 2\text{M}$, rCHO/ $\alpha 2\text{M}/\text{K692}$, and rCHO/ $\alpha 2\text{M}/\text{K696}$, respectively.

DNA sequencing

The prototype and the two engineered cDNAs were thoroughly sequenced, and were confirmed to fit the designed sequences. For the sequence analyses, template plasmids were purified by a published method (Hattori and Sakaki, 1986). M13/M4 universal and

M13/RV reversal sequencing primers were purchased (Takara Shuzo, Shiga, Japan). Other primers shown in the figure legends were synthesized using an automated DNA synthesizer (Perkin Elmer, Chiba, Japan). Sequencing was carried out using a 7-DEAZA sequencing kit (Takara Shuzo) or an automated DNA sequencer (Perkin Elmer).

Production and purification of $\alpha 2\text{M}$ s

For the production of wild-type and mutant $\alpha 2\text{M}$ proteins, the recombinant cells were transferred to roller bottles each with 1750 cm² surface area, and grown in a serum-free medium CHO-SFM-2 (GIBCO) for two days before harvesting spent culture medium. In some experiments a proteinase inhibitor Pefabloc SC (Merck, Darmstadt, Germany) and Leupeptin (Sigma, St. Louis, Missouri, USA) was added to the serum-free medium at the concentration of 0.05 mM and 1 $\mu\text{g}/\text{ml}$, respectively, in order to avoid non-specific proteolytic decay of the product during cultivation and downstream procedures.

For the purification of rh $\alpha 2\text{M}$ s, spent culture medium was treated with hydrochloric acid to adjust its pH to 6.0, and passed through a Chelating Sepharose Fast Flow column saturated with zinc ion (Pharmacia Biotech). Bound $\alpha 2\text{M}$ was eluted with an elution buffer (0.1 M acetate buffer containing 0.15 M NaCl pH 5.0).

Eluate was concentrated by reducing its volume and the elution buffer was replaced with PBS buffer. Spent medium collected from the culture of rCHO/K692 and rCHO/K696 cell lines was treated with hydrochloric acid to adjust its pH to 6.0 and passed through the Chelating Sepharose Fast Flow column saturated with zinc ion (Pharmacia Biotech). Bound $\alpha 2\text{M}$ was eluted with the same elution buffer as above. Eluate was concentrated by reducing its volume and the elution buffer was replaced by PBS buffer (Dangott and Cunningham, 1982). Human plasma $\alpha 2\text{M}$ was purified by the published method (Kan et al., 1985).

Methylamine treatment and electrophoresis

Obligatory conformational change from the slow form to the fast form was induced by the treatment of $\alpha 2\text{M}$ with 200 mM methylamine (Tokyo Kasei, Tokyo) by overnight treatment in a Tris-HCl buffered solution (pH 8.0), essentially following the method of Barrett et al. (1979). To detect the slow form and the fast form, electrophoresis was performed using Phast

Gel Gradient 4–15 on a Phast System (Pharmacia Biotech).

Enzyme inhibition assay

Inhibition of proteinase with $\alpha 2M$ was assayed according to Arakawa et al. (1989) except that the incubation buffer was changed to 0.145 M sodium chloride at 8 mM sodium phosphate, 1.4 mM potassium phosphate, 2.7 mM potassium chloride, pH 7.4. Various amounts of $\alpha 2M$ were incubated with or without 0.4 mg of trypsin (swine, and Type III, bovine pancreas; Sigma, St. Louis, Missouri, USA), or 0.7 mg of lysyl endopeptidase (*Achromobacter lyticus* M-497-1, Wako, Japan) in 250 μ l of the above incubation buffer. The reaction mixture was first incubated for 10 min. at 37 °C, and then 2 ml suspension of 1.0% hide powder azure (Sigma) as chromogenic substrate for proteinases was added and incubated for further 30 min. at 37 °C. The supernatant was collected by centrifugation after the incubation period and absorbancy at 595 nm was measured. In some experiments, TLCK (Na-p-tosyl-L-lysine chloromethyl ketone, Sigma) was used to inhibit lysyl endopeptidase.

Results

Sequence analyses of cDNAs

The DNA sequence in the range around 692 to 696 was determined for each of the $\alpha 2M$ cDNAs of the wild-type, K692 and K696 as described in Materials and Methods, and the results shown in Figures 2 and 3. It was clearly shown that in K692, 5'AGA3' for Arg at codon 692 was substituted with 5'AAA3' for Lys, and in K696, 5'CGC3' for Arg with 5'AAA3' for Lys. Separately, we sequenced the wild-type $\alpha 2M$ cDNA, and confirmed that its entire sequence was identical to the previously reported sequence of $\alpha 2M$ cDNA of HepG2 origin in amino-acid codon level (Kan et al., 1985; Boel et al., 1990; Nakamichi et al., 1998).

Interaction of $\alpha 2M$ s with proteinases

Expression vectors, and recombinant CHO cells thereof, were constructed using the wild type and mutant cDNAs, and preparations of $\alpha 2M$ proteins were obtained from the culture spent medium as described in Materials and Methods and in a separate paper (Nakamichi et al., 1998). The proteins were reacted with trypsin and lysyl endopeptidase.

Figure 2. The bait region nucleotide sequence of wild type $\alpha 2M$ cDNA and $\alpha 2M/K692$ cDNA.

In Figure 4, residual proteinase activity in the presence of the wild type $\alpha 2M$ and $\alpha 2M/K696$ for trypsin and for lysyl endopeptidase are shown. Both $\alpha 2M$ s inhibited trypsin above the $\alpha 2M$ /proteinase ratio of 2. $\alpha 2M/K696$ inhibited lysyl endopeptidase, but wild type $\alpha 2M$ did not. For comparison, an inhibition profile of trypsin by plasma-derived natural $\alpha 2M$ is shown. We confirmed the inability of plasma-derived $\alpha 2M$ to inhibit lysyl endopeptidase in a separate experiment (Figure not shown). Although $\alpha 2M/K696$ inhibited lysyl endopeptidase, the inhibitory efficacy was not as high as that for trypsin: It required a 5 to 10 times molar excess of the engineered protein over the proteinase (Fig. 4). This low efficacy may be due to the instability of the proteinase/ $\alpha 2M$ complex in this case (see below).

Native $\alpha 2M$ and its genetically engineered derivatives reacted with lysyl endopeptidase differently

Figure 3. The bait region nucleotide sequence of wild type $\alpha 2M$ cDNA and $\alpha 2M/K696$ cDNA.

Figure 4. Residual activity of proteinases in the presence of various $\alpha 2M$ s at molar ratios given on the abscissa. Plasma $\alpha 2M$ and trypsin (▲—▲), mutant $\alpha 2M/K696$ and trypsin (■—■), wild type $\alpha 2M$ and trypsin (□—□), mutant $\alpha 2M/K696$ and lysyl endopeptidase (●—●), and wild type $\alpha 2M$ and lysyl endopeptidase (○—○).

from each other. For native $\alpha 2M$ which gives a 180 kDa band in SDS-PAGE (Fig. 5A lane 1 and Fig. 5B lane 1), a 90 kDa band always appeared in SDS-PAGE after a proteinase specific cleavage of bait region, as shown in Figure 5A (lane 4) and Figure 5B (lane 2) for trypsin-treated $\alpha 2M$. For $\alpha 2M/K696$, a 90 kDa

band was also noted after lysyl endopeptidase treatment, indicating that it is cleaved within its bait region (Figure not shown). For $\alpha 2M/K692$, however, no distinct band appeared except for broad staining around 90 kDa together with numerous small bands indicating that $\alpha 2M/K692$ was degraded in the pres-

Figure 5. A. SDS PAGE of plasma derived hα2M after various treatments. Lane 1: Untreated α2M, lane 2: treated with 0.2 molar ratio of lysyl endopeptidase, lane 3: same as lane 2 but in the presence of TLCK, lane 4: treated with trypsin. B. SDS PAGE of plasma derived hα2M and α2M/K692 after proteinase treatments. Lanes 1-4: plasma derived hα2M. Lane 1: untreated (the three minor bands are contaminants), lane 2: treated with trypsin, lanes 3 and 4: treated with lysyl endopeptidase with molar ratio over hα2M of 0.2 (lane 3), 1 (lane 4). Lanes 5 and 6: α2M/K692 treated with lysyl endopeptidase with molar ratio over α2M/K692 of 0.2 (lane 5), 1 (lane 6). C. Native PAGE of mutant α2M/K692 after treatment with proteinases. Lane 1: α2M/K692 without proteinase treatment, lane 2: treated with trypsin, lane 3-6: treated with lysyl endopeptidase with molar ratio over α2M of 0.1 (lane 3), 0.2 (lane 4), 1 (lane 5), and 2 (lane 6).

ence of lysyl endopeptidase (Fig. 5B lanes 5 and 6). Therefore it was not conclusive whether rhα2M/K692 was cleaved within its bait region or not.

Lysyl endopeptidase reacted with hα2M and genetically engineered derivative of the latter in regions other than the bait region. Lysyl endopeptidase-treated hα2M showed a distinct 150 kDa band in SDS-PAGE indicating that a peptide bond outside of its bait region was cleaved without resulting in the inhibition of the proteinase (Fig. 5A lane 2, and Fig. 5B lane 3). We noted a similar 150 kDa band in lysyl endopeptidase-treated rhα2M/K696 (Fig. not shown) indicating that this engineered molecule is cleaved both inside and outside of its bait region. As to rhα2M/K692, only a faint band at 150 kDa was seen in an early stage of lysyl endopeptidase treatment, probably because it was quickly degraded by the proteinase in a short period of time (Fig. 5B lanes 5 and 6).

We confirmed that the transition from the slow form to the fast form, as triggered either by the methylamine-cleavage of thioester bond or by the trypsin-cleavage of bait region peptide, happens in the genetically engineered derivatives as well as in the native hα2M. Thus, trypsin-treated rhα2M/K692 showed the fast form in PAGE under a reducing condition (Fig. 5C lane 2 in comparison with Fig. 5C lane 1 for untreated rhα2M/k692), as well as trypsin-treated hα2M as shown separately (Nakamichi et al., 1998). A similar transition was also noted for rhα2M/K692 after methylamine treatment (Nakami-

chi et al., 1998). A fast form band was also noted for rhα2M/K696 after treatment either with methylamine or with lysyl endopeptidase (Figure not shown). The change from the slow form to the fast form, however, was not clearly shown for lysyl endopeptidase-treated rhα2M/K692 (Fig. 5C lanes 3-6). These results indicate that the thioester-bond-mediated machinery for conformational change is maintained in both of the genetically engineered hα2Ms, and suggest that, although Lys at 692 is located within the bait region, it is not easily accessible to lysyl endopeptidase.

Discussion

In the present study using recombinant mutant α2Ms, we provided additional evidence supporting the trapping theory: We presented that, by introducing a peptide sequence susceptible to a proteinase into the bait region of α2M, a recombinant α2M protein capable of inhibiting the proteinase, which is otherwise not inhibited, was obtained. To our knowledge, this is the first experiment to show this, and also to extend the value of engineered α2M-derived proteinases of potential industrial and/or therapeutic usefulness.

It should be noted, however, that the new inhibitory function was confirmed only for one of the two engineered α2Ms, i.e., rhα2M/K696, and even in that case the inhibitory efficiency was not as high as we expected: rhα2M/K696 required a 5 to 10 times molar

excess of the protein over lysyl endopeptidase. We noted that when rh α 2M/K696 was reacted with lysyl endopeptidase, enzymatic degradation of the protein proceeded further, than in the case of wild type (data not shown). This is in contrast to the case where h α 2M is complexed with trypsin or chymotrypsin: h α 2M becomes resistant against further attack of proteinases including that of lysyl endopeptidase. We speculate either that the structural transition of h α 2M after the bait region cleavage due to lysyl endopeptidase is not quite as complete as in the case of other proteinases, or that the trapped proteinase continues to degrade h α 2M from inside of the latter. We think the latter possibility more likely because there has been an observation that trapped enzymes would later be freed from α 2M as an active molecule (Borth and Teodorescu, 1986; Molla et al., 1987).

Although rh α 2M/K692 contains Lys in its bait region, it was not split at the bait region by lysyl endopeptidase as detected by 90 kDa fragment. Instead, a treatment with the proteinase yielded a 150 kDa fragment, showing that rh α 2M/K692 was cleaved at a site different from its bait region. It may be relevant to mention here that, proteinase cleavage sites are frequently located between 681 to 687 and 696 and 700, but scarcely between 688 to 695 (Sottrup-Jensen et al., 1989). The results may suggest that within the bait region some peptide bonds are inaccessible to a proteinase.

Inhibition of lysyl endopeptidase has clearly not been in the program of h α 2M evolution. Molecular engineering of α 2M, as extension of this study will not only further practical aspects of technological utilization of recombinant proteins, but also evolutionary aspects of this interesting protein.

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