Human plasma kininogens are identical with α-cysteine proteinase inhibitors

Evidence from immunological, enzymological and sequence data

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Human high- and low-M_r kininogens were shown to be potent inhibitors of cysteine proteinases such as cathepsin L and papain (K_i = 17–48 pM). A strong immunological cross-reaction between the kininogens and low-M_r α-cysteine proteinase inhibitor from human plasma was found. Comparison of partial amino acid sequences from high- and low-M_r kininogen and low-M_r α-cysteine proteinase inhibitor demonstrated sequence identity for all segments analyzed. These findings suggest that the kininogens and the α-cysteine proteinase inhibitors from human plasma are identical proteins.

Kininogen *Kallikrein-kinin system* *α-Cysteine proteinase inhibitor* *Cysteine proteinase* *Human plasma protein*

1. INTRODUCTION

Two types of kininogens, a high- and a low-M_r form, are present in human plasma and secretions [1,2]. The kininogens are the large precursor molecules of the vasoactive kinins involved in blood pressure regulation [2]. Beyond its role as a kinin carrier, high-M_r kininogen in a complexed form with prokallikrein serves as an assembly factor in the intrinsic clotting system [1]. Inhibitory functions of the kininogens have not yet been recognized.

Mammalian kininogens are single-chain glycoproteins [1]. They share the heavy chains preformed at the N-terminus and the consecutive kinin segments, but differ considerably in their light chain portions preformed at the C-terminus [1,2].

Cysteine proteinase inhibitors are found in human body fluids and tissues [3,4]. Two major plasma forms have been identified, commonly referred to as low- (α_2) and high-M_r (α_1) cysteine proteinase inhibitor (αCPI) [5]. They inhibit thiol proteinases with high affinity and specificity [4]. Functions apart from the inhibitory capacity of the αCPIs have not yet been reported.

Here, we present evidence from immunological cross-reactivity, kinin releasability, inhibitory activity, and amino acid sequence identity, that kininogens and αCPIs from human plasma are identical sets of proteins.

Abbreviation: αCPI, cysteine proteinase inhibitor from the α-fraction of human plasma

2. MATERIALS AND METHODS

Kininogens were isolated from human plasma by ion-exchange chromatography on DEAE- and

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CM-Sephadex followed by immunoaffinity chromatography on immobilized anti-kininogen IgGs [2,6]. Tryptic cleavage of human kininogens, isolation and sequence analysis of the resultant fragments was as described in [7]. Antisera against the two kininogens were raised in sheep following conventional immunization schemes. Double immunodiffusion tests were performed according to Ouchterlony [8].

Low-M_r αCPI was purified from human plasma by affinity chromatography on CM-papain-Sepharose and gel filtration on Sephacryl S-200 (in preparation). Smaller fragments were separated from the virgin molecule by gel chromatography on Sephacryl S-200 before and after oxidative cleavage of the disulfide bonds. Amino acid sequence analysis of the isolated low-M_r αCPI fragments was by automated solid-phase Edman degradation [9].

The purity of the resultant proteins was assessed by analytical SDS electrophoresis [10]. For Western blotting [11] and immunoprinting, a ^{125}I-labelled monoclonal antibody (from mouse) directed against the heavy chain of human high-M_r kininogen was used [12]. Kinin was released from the proteins by tissue kallikrein from porcine pancreas and quantitated by radioimmunoassay [13]. Inhibition constants were determined essentially as described in [4]. Papain was from Sigma, and cathepsin L was isolated from human spleen (in preparation).

3. RESULTS

To test for the immunological cross-reactivity of kininogens and low-M_r αCPI, we applied the double immunodiffusion test (fig.1). High-M_r kininogen, low-M_r αCPI and low-M_r kininogen were delivered to the upper wells, and sheep antiserum against high- (fig.1A) and low-M_r kininogen (fig.1B) to the bottom wells. Application of the anti-low-M_r kininogen immune serum yielded a pattern of fused precipitin lines indicating that low-M_r αCPI and the two kininogens share antigenic sites recognized by the anti-low-M_r kininogen-directed antibodies. The antiserum against high-M_r kininogen formed lines of partial identity with low-M_r αCPI and high-M_r kininogen (fig.1A, arrowhead) indicating that the anti-high-M_r kininogen-directed IgGs recognize both

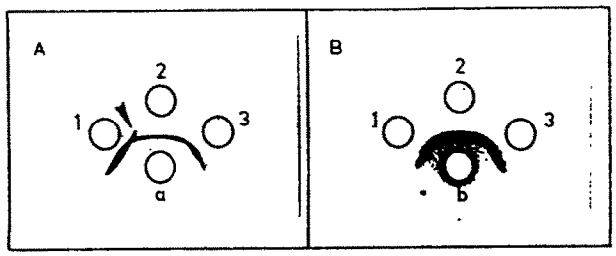

Fig.1. Double immunodiffusion test [8] of high-M_r kininogen (1), low-M_r αCPI (2), and low-M_r kininogen (3) with antiserum against high-M_r kininogen (a) and low-M_r kininogen (b).

epitopes common to high-M_r kininogen and low-M_r αCPI as well as antigenic sites unique for high-M_r kininogen. Immunoprinting revealed that a monoclonal antibody directed against the heavy chain portion of the human high-M_r kininogen also recognized human low-M_r αCPI (not shown).

In view of the extensive immunological cross-reactivity of low-M_r αCPI and the human kininogens, we tested the kininogens for their inhibitory activity against cysteine proteinases. The thiol proteinases cathepsin L (from human spleen) and papain (from Papaya latex) were used. Table 1 lists the inhibition constants (K_i ranging from 17 to 48 pM) for the two enzymes and the human kininogens thus classifying the kininogens as potent inhibitors of cysteine proteinases. For comparison, the inhibition constants for human low-M_r αCPI [4] are included in the table. Both sets of K_i values are in the same order of magnitude. Hence, these data stress the similarity, if not identity of the αCPIs and the kininogens.

To further evaluate this possibility, we have probed for releasable kinin from low-M_r αCPI.

Table 1

Inhibition constants K_i (pM) of low- and high-M_r kininogen with papain and cathepsin L

Cysteine proteinase	CPI	Low-M_r kininogen	High-M_r kininogen
Cathepsin L	62	48	nd
Papain	34	17	20

For comparison, the data for low-M_r αCPI are included [4]. nd, not determined

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                    270                 280                 290                 300
(a)                 D I P T N S P E L E E T L T H T I T K L N A E N X A T F Y F K I
(b) K I C V G C P R D I P T N S P E L E E T L T H T I T K L N A E N N A T F Y F K I
(c) R L C A G C P K P I P V D S P D L E E P L S H S I A K L N A E H D G A F Y F K I

                    310                 320
(a) D N V K K A R V Q V V A G K R Y F I D F V A R E T
(b) D(N,V,K,K,A)R V Q V V A G X R Y F I D F V A R E T T C S K
(c) D T V K K A T V Q V V A G L K Y S I V F I A R E T T C S K
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Fig.2. Alignment of the partial amino acid sequences from (a) the N-terminal portion of the 15 kDa fragment from human low-M_r αCPI with the C-terminal parts of the heavy chains from human [7] and bovine [15] high-M_r kininogens. Numbers indicate the positions of the residues in the cDNA-derived amino acid sequence of bovine high-M_r kininogen [15].

The inhibitor was subjected to limited proteolysis by tissue kallikrein, and liberated Lys-bradykinin was quantitated by radioimmunoassay. A small, though distinct quantity of lysyl-bradykinin amounting to some 4% of the theoretically releasable kinin was present in our low-M_r αCPI preparation. This suggests that major part of the low-M_r αCPI had already undergone proteolytic cleavage, possibly during its isolation from human plasma. These results were corroborated by findings from SDS-gel electrophoresis indicating that the predominant part of the low-M_r αCPI was present in a two-chain form, with a heavy chain (62 kDa) connected to a light chain (5 kDa) by disulfide-bridging (not shown). In addition, a minor fragment of 15 kDa was found.

Amino acid sequence analysis of human kininogens was done with tryptic fragments derived from the heavy chain portion of high-M_r kininogen (in preparation) and the light chain of low-M_r kininogen [7]. Partial amino acid sequence determination of low-M_r αCPI was carried out with smaller fragments of apparent molecular mass 15 and 5 kDa prepared from the purified inhibitor (in preparation). Comparison of the resulting sequence data indicated that the fragment of M_r 15000 exhibited total sequence identity to the known sequence portions of the high-M_r kininogen heavy chain (fig.2). Similarly, the alignment of the sequences of the 5 kDa fragment of low-M_r αCPI and the light chain of low-M_r kininogen demonstrated total identity of the analyzed protein portions (fig.3).

4. DISCUSSION

Our results indicate that kininogens are strong inhibitors of cysteine proteinases; probably they are the most abundant inhibitors of thiol proteinases in plasma (total kininogen concentration 210 mg/l) and urine (1–6 mg/l) [14]. Comparison of the sequences of several low-M_r cysteine proteinase inhibitors of the cystatin- and stefin-type reveals a well conserved stretch of amino acids located in the center portion of the inhibitor molecules (fig.4). This segment has been suggested

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     390                 400                 410                 420                 430
(a)        I G E I K E E T T S H L R S C E Y K G R P P K A G A E P A S E R G V S
(b) S S R I G E I K E E T T S H L R S C E Y K G R P P K A G A E P A S E R G V S
(c) S V Q V M K T E G S T T T H V K S C E Y K G R P Q E A G A E P A P Q G E V S L P A E S P Q L A R
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Fig.3. Alignment of the partial amino acid sequences from (a) the 5 kDa fragment of the human low-M_r αCPI with the light chains from (b) human [7] and (c) bovine [17] low-M_r kininogens. Numbers indicate the positions of the residues in the cDNA-derived amino acid sequence of bovine low-M_r kininogen [16].

to contain the reactive site of the inhibitors [3]. Interestingly, a highly homologous segment is also present in the heavy chain of the human kininogens some 110 amino acids upstream from the kinin segment (fig.4). This suggests that the reactive site mediating the inhibitory function of the kininogens might reside in their heavy chain.

At present we do not know the precise effect of kinin release on the inhibitory function of the kininogens. However, preliminary evidence suggests that kinin-free low-M_r kininogen still inhibits cysteine proteinases. Our findings support the emerging idea [2] of the kininogens being multifunctional, multi-domain molecules which serve such different functions such as kinin delivery, surface attachment, kallikrein binding, and inhibition of cysteine proteinases.

The results from the partial amino acid sequencing of human kininogens and low-M_r αCPI strongly suggest – though they do not entirely prove – that low-M_r kininogen and low-M_r αCPI on one hand and high-M_r kininogen and high-M_r αCPI on the other hand are identical proteins. Additional evidence for this fact comes from cDNA sequencing [18] demonstrating that human low-M_r kininogen and low-M_r αCPI are identical on the cDNA level.

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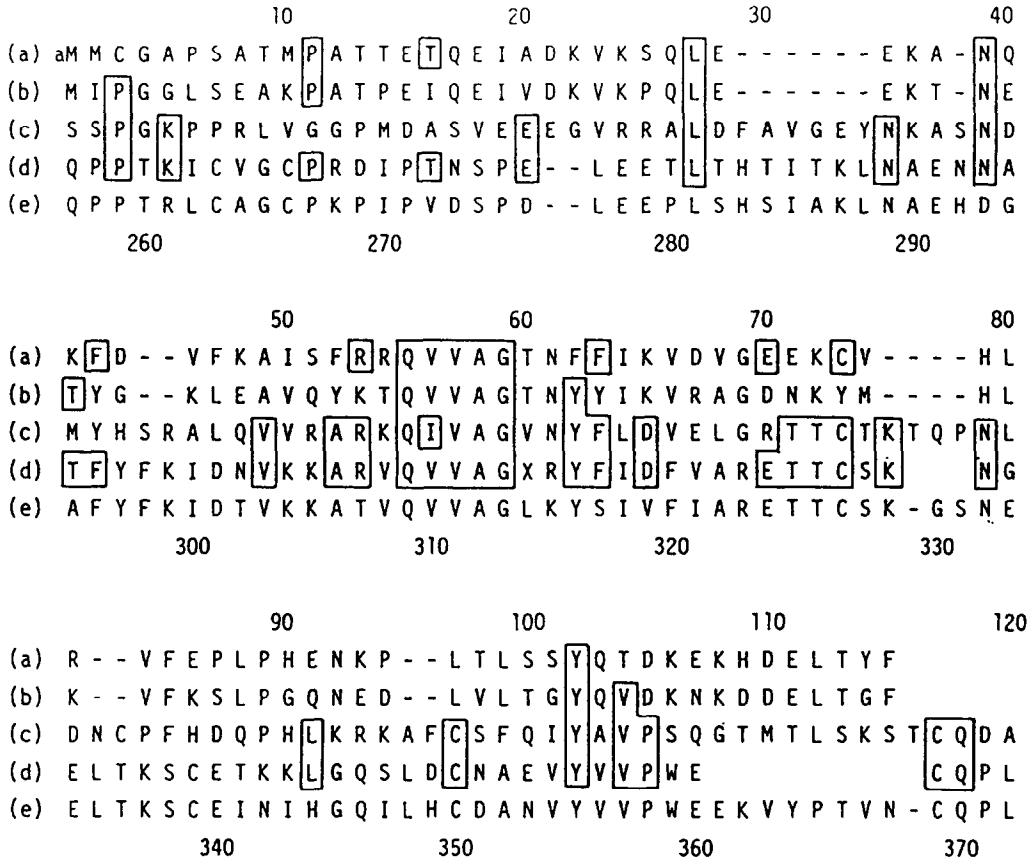

Fig.4. Comparison of the complete amino acid sequences of (a) the thiol proteinase inhibitor (TPI) from rat liver [17], (b) human stefin [9], and (c) human cystatin [3] with (d) partial sequences of human high-M_r kininogen and (e) bovine low-M_r kininogen [16]. Allowance for gaps (indicated by dashes) has been made to improve the alignment. Residues identical in human kininogen and at least one of the inhibitors are boxed.

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