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Expression, purification and characterization of a Kunitz-type protease inhibitor domain from human amyloid precursor protein homolog

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Abstract

The Kunitz-type protease inhibitor domain from a recently identified homolog of the Alzheimer amyloid precursor protein (APPH KPI) was expressed in yeast, purified and characterized. Its inhibition profile towards several serine proteases was studied and compared to that of APP KPI, the Kunitz domain from the Alzheimer amyloid precursor protein. APPH KPI was shown to inhibit proteases with trypsin-like specificity with an inhibitor profile resembling that of the APP KPI domain. The KPI domains from APP and APPH inhibited trypsin (K_i = 0.02 nM), and plasma kallikrein (K_i = 86 nM) with approximal equal affinity. In comparison to APP KPI (K_i = 82 nM) the KPI domain of the homolog, APPH KPI, (K_i = 8.8 nM) was a more potent inhibitor of glandular kallikrein. APPH KPI was a less potent inhibitor of chymotrypsin than APP KPI (K_i = 78 nM as compared to K_i = 6 nM), plasmin (K_i = 81 nM as compared to 42 nM), and factor XI_a (K_i = 14 nM as compared to K_i = 0.7 nM). The affinity of factor XI_a for APPH KPI is sufficiently high to allow for an interaction in the blood. It is, however, well possible that the physiological protease ligand for the receptor-like APPH protein has yet to be identified.

Key words: Amyloid precursor protein homolog; Kunitz-type protease inhibitor; Factor XIa; Alzheimer; Serine protease

1. Introduction

The amyloid β-protein which accumulates in senile plaques of patients with Alzheimer's disease is a 42-amino-acid peptide which is derived by proteolysis from a much larger precursor known as the Amyloid Precursor Protein (APP). Several alternative molecular forms of the precursor exist, apparently due to a combination of alternative mRNA splicing and proteolytic cleavage. Recent studies have shown that the full-length APP cDNA can be processed by mammalian cells in culture to produce both the amyloid peptide and also a truncated, secreted form of the extracellular protein domain [1,2].

The cDNA for a similar molecule, called Amyloid Precursor Protein Homolog (APPH), has recently been identified and cloned in this laboratory from human placenta [3]. Like APP, APPH appears to have a domain organization resembling a cell-surface receptor with a very large extracellular segment, a single trans-membrane domain, and a short intracellular domain. The extracellular segment of both proteins contains a 'Cys-rich' and a Kunitz-type protease inhibitor (KPI) domain. The highly conserved regions of the protein suggest similarity of function for APP and APPH within their relative biological contexts, although the normal functions of these proteins are presently unknown. Some aspects of possible function can be inferred from the inhibitory properties of the KPI domain of APP, which has been studied both as the truncated, secreted form of the native molecule [4–8], and also as an individual KPI domain expressed in COS-1 cells [9], bacterial [10,11] and yeast [12] systems.

In order to study the potential protease inhibitor properties of the APPH KPI domain, we have employed a yeast expression system shown previously to be highly efficient in the synthesis and secretion of KPI domains [13]. The APPH KPI domain was expressed in high yield, purified and the inhibition profile compared to that of APP KPI domain and BPTI produced by the same expression system.

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Abbreviations: APP, amyloid precursor protein; APPH, amyloid precursor protein homolog; KPI, Kunitz-type protease inhibitor; BPTI, bovine pancreatic trypsin inhibitor.

2. Materials and methods

2.1. Substrates

Chromogenic substrates *H*-D-Val-Leu-Lys-pNA; MeO-Suc-Arg-Pro-Tyr-pNA; *H*-D-Val-Leu-Arg-pNA; <Glu-Gly-Arg-pNA; *H*-D-Ile-

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Pro-Arg-pNA; <Glu-Pro-Arg-pNA; *H*-D-Pro-Phe-Arg-PNA; *H*-D-Phe-Pip-Arg-pNA were purchased from Chromogenix (Moelndal, Sweden), MeO-CO-CHA-Gly-Arg-pNA was from NycoMed (Oslo, Norway), and MeO-Suc-Ala-Ala-Pro-Val-pNA; Suc-Ala-Ala-Pro-Phe-pNA were from Sigma (St.Louis, Mo). The fluorogenic substrate Boc-Glu(OBzl)-Ala-Arg-MCA was from Peptide Institute (Osaka, Japan).

2 2. Proteases

Porcine trypsin, chymotrypsin, and human thrombin was from Novo Nordisk (Bagsværd, Denmark). Glandular kallikrein was from Sigma (St.Louis, MO). Human plasmin and plasma kallikrein was from Kabi (Stockholm, Sweden) and uPA was from Serono (Freiburg, Germany). Recombinant proteins of human tPA, protein C_a and factor VII_a were from Novo Nordisk (Bagsværd, Denmark). Human leukocyte elastase and cathepsin G were purified according to the procedure described by Baugh and Travis [14]. Human factor X_a, factor XI_a and factor XII_a were generous gifts from Dr W. Kisiel (Albuquerque, NM), Dr K. Fujikawa (Seattle, WA) and Dr I. Schousboe (Copenhagen, Denmark), respectively.

2.3. General methods

Standard DNA techniques were carried out as described [15]. Synthetic oligonucleotides were prepared on an automatic DNA synthesizer (380B, Applied Biosystems) using commercially available reagents. DNA sequence determinations were performed by the dideoxy chain-termination technique [16]. Polymerase chain reactions (PCR) were performed on a DNA Thermal Cycler (Perkin-Elmer-Cetus) using a commercial kit (GeneAmp, Perkin-Elmer-Cetus).

2 4. Construction of the APPH KPI secreting yeast strain

1 μg of human genomic DNA (Clontech, Palo Alto, CA) was used as a template in a PCR reaction containing 100 pmol each of the primers 1 (GCTGAGAGATTGGAGAAGAGAGATGTCAAAGCTGTCTGCTCCC) and 2 (AGTTGGTCTAGATTACGCTTTACACACAGCCATAC) (Fig. 1). The 3′ half of primer 1 is identical to the N-terminal encoding part of the APPH KPI gene [3], and the 5′ half is identical to the C-terminal encoding part of the previously described hybrid yeast leader [17]. Primer 2 is complementary to the C-terminal part of the APPH KPI domain and carries a 5′ extension containing a translation stop codon followed by an *Xba*I site.

The PCR reaction was performed in a 100 μl volume and the following cycle: 94°C for 20 s, 50°C for 20 s, and 72°C for 30 s. After 19 cycles a final cycle was performed in which the 72°C step was maintained for 10 min. The PCR product, a 210 bp fragment, was isolated by electrophoresis on a 2% agarose gel.

The APPH KPI DNA fragment was fused in-frame to the hybrid leader gene by overlay extension PCR [18]. The product was digested with *Eco*RI and *Xba*I and isolated as a 412 bp DNA fragment. This fragment was ligated to the 9.3 kb *Nco*I–*Xba*I fragment and the 1.6 kb *Nco*I–*Eco*RI fragment both from the *S. cerevisiae–E. coli* shuttle vector pMT-636 [17]. The ligation mixture was transformed into *E. coli* strain MT-172, and the APPH KPI expression plasmid, KFN-1953, was isolated. Plasmid pKFN-1953 was transformed into *S. cerevisiae* strain MT-663 by selection for growth on glucose as the sole carbon source. One transformant, KFN-1963, was selected for fermentation.

2 5. Construction of the APP KPI secreting yeast strain

PCR cloning of APP KPI 3–58 (BPTI numbering) was performed essentially as described above from human genomic DNA by substituting primer 1 with (GCTGAGAGATTGGAGAAGAGAGAGGTGTGCTCTGAAC) and primer 2 with (CTGCTATCTAGATTAGGCGCTGCCACACACGGC). The expression plasmid was transformed into *S. cerevisiae* and one transformant, KFN-1653, was selected for fermentation.

2.6. Fermentation

The transformants described above were cultivated at 30°C for 4 days in yeast peptone dextrose (YPD) medium [19] to an OD 650 nm value of 30. At the end of the fermentation the pH was adjusted to 3.0 by addition of conc. H_3PO_4. The supernatants were isolated after centrifugation.

2.7. Purification

A total of approximately 1 litre fermentation supernatant was adjusted to pH 8.0 by addition of solid Tris-HCl to 50 mM and titration with 4 M NaOH. The supernatant was filtered before it was applied to a column of bovine trypsin immobilized to CNBr-activated Sepharose (350 mg bovine trypsin pr 35 ml gel). The column was subsequently washed with 150 ml 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.0 and 150 ml 0.01 M Tris-HCl, pH 8.0 before bound material was eluted with 200 ml 0.2 M glycine-HCl pH 3.0. Fractions of 10 ml was collected and analyzed by reverse phase HPLC. Protein containing fractions were combined. This pool was applied to a preparative reverse-phase HPLC column, (Vydac 214TP510, The Separations Group, Hesperia CA) equilibrated with 5% B and 95%A, where eluent A was 0.1% TFA in H_2O and eluent B was 0.07% TFA in acetonitrile. Flow rate was maintained at 4 ml/min. Following the application, the column was washed with 5% B until baseline was achieved at 214 nm. Gradient elution with fraction collection was then performed from 5 to 85% B over 80 min. Fractions containing UV absorbing material were analyzed by reverse phase HPLC (Vydac 214TP54) and combined to give pools of chromatographically pure material. Removal of solvent was obtained by vacuum centrifugation. The concentration and total yield of inhibitor in the major pools was estimated by reverse phase HPLC analysis and by comparison to a BPTI standard. The final preparations were characterized by electrospray mass spectrometry (SCIEX API III).

2.8. Trypsin titration by APPH KPI, APP KPI and BPTI

Trypsin (8 nm) was incubated with various concentrations of inhibitor for 15 min. Substrate (0.6 mM H-D-Val-Leu-Lys-pNA) was then added, and residual activity was measured. The reaction took place in microtiter wells in 100 mM NaCl, 50 mM Tris-HCl, 0.01% Tween 80, pH 7.4 at 25°C in a total volume of 300 μl. Amidolytic activity was measured as the change in absorbance at 405 nm.

2.9. Inhibition kinetics

Measurements of inhibition kinetics were performed in microtiter wells essentiall as described for the trypsin inhibition experiment. Amidolytic activity was measured as the change in absorbance at 405 nm or as the change in fluorescence Em. at 460 nm (Ex. 380 nm). The apparent inhibition constant, K_i', was determined using the non-linear regression data analysis program Enzfitter (Biosoft, Cambridge, UK). K_i values were obtained by correcting for the effect of substrate according to the equation.

$$K_i = K_i'/(1+[S]/K_m)$$

3. Results

Two inhibitor pools were obtained from the final step of the APPH KPI purification procedure. The major pool contained 12.1 mg inhibitor. Electrospray mass

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EcoRI
GAATTCCATTCAAGAATAGTTCAAACAAGAAGATTACAAACTATCAATTTCATACACAAT

ATAAACGACCAAAAGAATGAAGGCTGTTTTCTTGGTTTTGTCCTTGATCGGATTCTGCTG
                  M  K  A  V  F  L  V  L  S  L  I  G  F  C  W

GGCCCAACCAGTCACTGGCGATGAATCATCTGTTGAGATTCCGGAAGAGTCTCTGATCAT
 A  Q  P  V  T  G  D  E  S  S  V  E  I  P  E  E  S  L  I  I

CGCTGAAAACACCACTTTGGCTAACGTCGCCATGGCTGAGAGATTGGAGAAGAGAGATGT
 A  E  N  T  T  L  A  N  V  A  M  A  E  R  L  E  K  R↑ D  V
    1→
CAAAGCTGTCTGCTCCCAGGAGGCGATGACGGGGCCCTGCCGGGCCGTGATGCCTCGTTG
 K  A  V  C  S  Q  E  A  M  T  G  P  C  R  A  V  M  P  R  W

GTACTTCGACCTCTCCAAGGGAAAGTGCGTGCGCTTTATATATGGTGGCTGCGGCGGCAA
 Y  F  D  L  S  K  G  K  C  V  R  F  I  Y  G  G  C  G  G  N
                                   ←2          XbaI
CAGGAACAATTTTGAGTCTGAGGATTATTGTATGGCTGTGTGTAAAGCGTAATCTAGA
 R  N  N  F  E  S  E  D  Y  C  M  A  V  C  K  A
```

Fig. 1. Nucleotide sequence and corresponding amino acid sequence of the 412 bp *Eco*RI–*Xba*I fragment encoding the leader - APPH KPI fusion protein. The Kex 2 processing site is indicated by a vertical arrow. The amino acid sequence of the secreted APPH KPI domain is shown in bold letters. The underlined sequences correspond to the PCR primers with horizontal arrows indicating the direction

spectrometry analysis revealed a molecular mass of 6459.0 Da in agreement with the theoretical calculated mass of the inhibitor (6458.4 Da) were obtained. However, mass spectrometry analysis also demonstrated that a molecular form with an additional 162 mass unit was present in the preparation. This is the molecular weight of a hexose unit and strongly indicated that a proportion, approximately 20%, of the inhibitor was O-glycosylated with a single mannose residue. This partially glycosylated preparation was used for characterisation of the APPH KPI inhibition. Glycosylations with typically 1 to 4 mannose residues in O-glycosidic linkage to serine or threonine is a common posttranslational modification in yeast [20]. Both serine (3 residues) and threonine (1 residue) are present in APPH KPI. However, potential glycosylation sites are not present in the protease contact region of APPH KPI, and therefore probably of no significance to inhibitory interactions.

A molecular mass of 6477.0 Da was found for the dominating molecular species in the smaller, second pool from the APPH KPI purification procedure (not shown). The result suggests that one of three methionine residues in APPH KPI was oxidized to its sulfoxide. One methionine is present in the protease contact region (P4′) and preliminary results indicated that this specific residue was relatively susceptible to oxidation. The modification might affect the functional properties of the inhibitor, and the preparation was not used for the inhibition experiments reported in the present paper. A molecular form with an additional 162 mass units, probably representing an oxidized- and O-glycosylated form of the inhibitor, was also present as a fraction of this inhibitor pool.

APP KPI was expressed in higher yield than APPH KPI and about 100 mg was purified from one litre of yeast fermentation supernatant. Only a single molecular species of 6152.2 Da was found in the final preparation in accordance with a calculated molecular mass of 6153.8 Da for APP KPI. No glycosylation of APP KPI was observed by mass spectrometric analysis. As was the case for APPH KPI, a separate, smaller pool obtained from the HPLC fractionation contained, however, mainly oxidized APP KPI. This preparation was not further characterized.

BPTI was also expressed in high yield, and the final preparation was homogenous when analyzed by reverse-phase HPLC and mass spectrometry. A molecular mass of 6512.0 Da was obtained for the recombinant BPTI in accordance with the theoretical value of 6511.5 Da.

All three KPI proteins inhibited trypsin with a high affinity. This property could be used to obtain a functional inhibitor concentration from a trypsin inhibition (titration) experiment (results not shown). These results, and the presence of a lysine/arginine in the P1 position, indicated that the target enzyme should be found among the serine proteases with trypsin-like substrate specificity.

Fig. 2 shows a direct comparison of the inhibition profiles for the KPI domains of APP, APPH and BPTI with proteases involved in digestion, coagulation, fibrinolysis and immune system reactions. The figure shows per cent inhibition at $1.0 \cdot 10^{-6}$ M of the inhibitor. Pro-

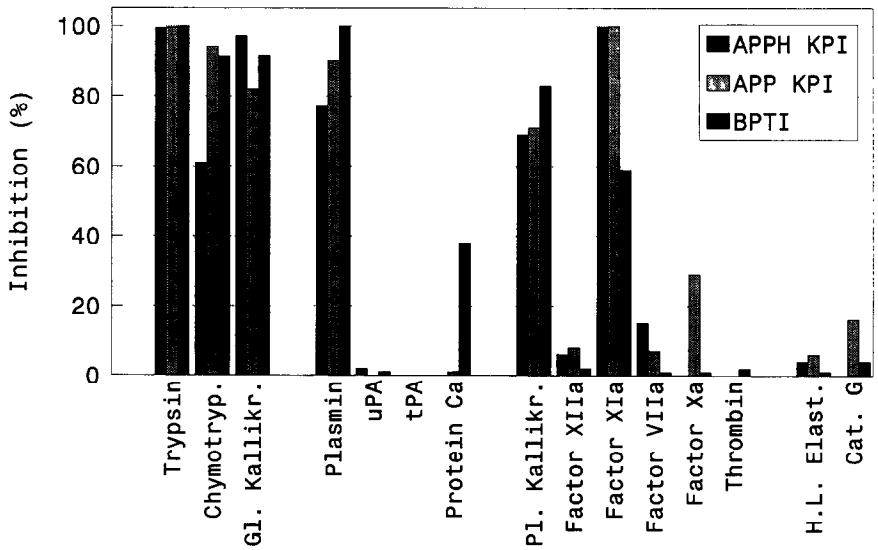

Fig. 2. Inhibition profile of APPH KPI, APP KPI and BPTI: inhibition in% at 1 μM inhibitor. The inhibition was measured as the residual activity obtained after incubation of the protease with inhibitor for 30 min. The protease and its respective substrate were present in the reaction mixture as follows: 8 nM trypsin, 0.6 mM D-Val-Leu-Lys-pNA; 2.5 nM chymotrypsin, 0.6 mM MeO-Suc-Arg-Pro-Tyr-pNA; 1 U/ml glandular kallikrein, D-Val-Leu-Arg-pNA; 10 nM plasmin, 0.6 mM D-Val-Leu-Lys-pNA; 5 nM uPA, 0.6 mM <Glu-Gly-Arg-pNA; 5 nM tPA, 0.6 mM D-Ile-Pro-Arg-pNA; 5 nM protein C_a, 0.6 mM <Glu-Pro-Arg-pNA; 3 nM plasma kallikrein, 0.6 mM D-Pro-Phe-ArgpNA; 30 nM factor XII_a, 0.6mM D-Pro-Phe-Arg-pNA; 1 nM factor XI_a, 0.12 mM Boc-Glu(OBzl)-Ala-Arg-MCA; 300 mM factor VII_a, 0.6 mM D-Ile-Pro-Arg-pNA; 0.2 NIHU/ml thrombin, 0.6 mM D-Phe-Pip-Arg-pNA: 10 nM leukocyte elastase, MeO-Suc-Ala-Ala-Pro-Val-pNA; 50 nM cathepsin G, 0.6 mM Suc-Ala-Ala-Pro-Phe-pNA.

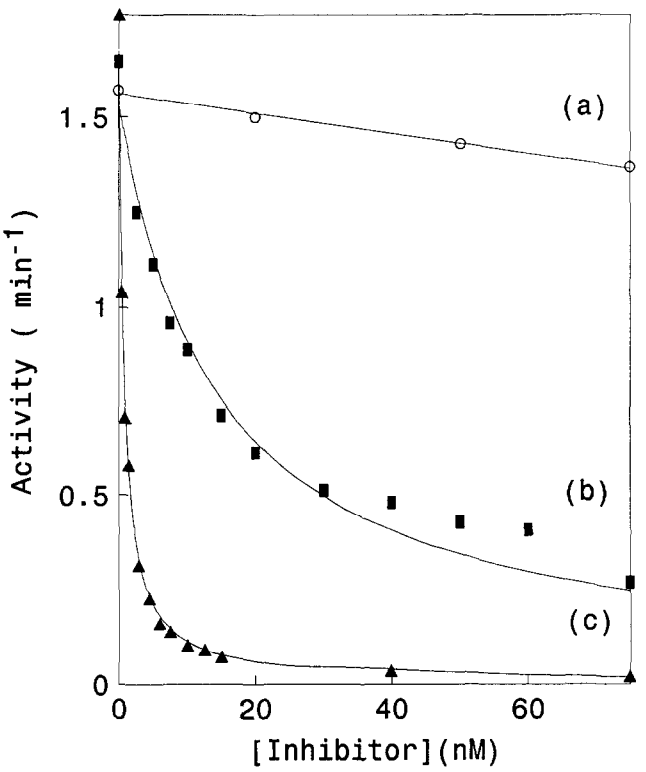

Fig. 3. Factor XI_a inhibition by APPH KPI, APP KPI and BPTI. Factor XI_a (1 nM) was incubated with various concentrations of inhibitor for 15 min. Substrate (0.12 mM Boc-Glu(OBzl)-Ala-Arg-MCA) was then added, and the residual activity was measured by fluorometry (*Ex*: 380 nm; Em: 460 nm). The activity was measured in arbitrary fluorescence units per min. Curve a: BPTI; curve b: APPH KPI; curve c: APP KPI.

found inhibition by APPH KPI under these conditions was found with trypsin, chymotrypsin, plasmin, factor XI_a, and plasma- and glandular kallikrein.

The inhibition kinetics with these proteases were measured, and the results (K_i values) are summarized in Table 1. APPH is a more potent inhibitor of glandular kallikrein than APP and binds with a ten fold higher affinity. APP, on the other hand, binds to chymotrypsin with a tenfold higher affinity than that of APPH.

APP is a very potent inhibitor of factor XI_a, and this property may be a clue to its physiological function [5,6]. It was therefore of interest to study the inhibition of this protease by APPH KPI in further detail. Fig. 3 shows the inhibition of factor XI_a by APP, APPH and BPTI. The KPI domain of the homolog was clearly less potent as an inhibitor of factor XI_a than that of APP. Compared to BPTI, however, the affinity of APPH KPI was still appreciable.

Fig. 4 shows an experiment in which factor XI_a was added to a reaction mixture containing fluorogenic substrate plus inhibitor. Curve b and c show the resulting transient inhibition curves with 20 nM APPH KPI, and curve d and e the results with 20 nM APP KPI. The activity of factor XI_a in the absence of inhibitor (curve a) is shown for comparison. As has been previously reported [6], heparin increased the rate of factor XI_a inhibition by APP KPI (curve e). The results also indicated that heparin induced a similar increase in the rate of factor XI_a inhibition by APPH KPI (curve c) and by BPTI (results not shown).

4. Discussion

Unlike seemingly non-inhibitory KPI domains such as the domain 3 from TFPI and the domain from the α(3) chain of collagen type VI (unpublished results), the KPI domain from APPH is clearly capable of interacting with several proteases. In its inhibition profile it resembles somewhat that of the KPI domain from APP.

Previous studies on APP have revealed that the inhibitory properties of the truncated, secreted form of the native molecule [4–8] were similar to those of the individual domain expressed in bacteria, yeast and COS-1 cells [9–12]. We may assume that the same is true for APPH.

The observation that the APP KPI domain was a potent inhibitor of trypsin, chymotrypsin, plasmin and notably Factor XI_a, all with inhibitor constants $<10^{-7}$ M [9–12], has been confirmed by the present work (Table 1). In addition we have confirmed the observation [9] that APP KPI interacts weakly ($K_i \sim 10^{-7}$ M) with plasma- and glandular kallikrein.

APPH KPI inhibits glandular kallikrein with a tenfold higher affinity, but inhibits chymotrypsin with a tenfold lower affinity than APP KPI (Table 1). It is interesting to note that the effect of heparin on factor XI_a inhibition is not unique to APP [6], but appears to be a rather general phenomenon which was also observed with the separate KPI domains tested in the present study (Fig. 4).

Table 1
K_i values for the inhibition by APPH KPI and APP KPI

Protease (concentration)	APPH KPI K_i (nM)	APP KPI K_i (nM)	Substrate (concentration)	K_s (mM)
Trypsin (0.11 nM)	0.02	0.02	<Glu-Gly-Arg-pNA (0.6 mM)	0.17
Chymotrypsin (2.5 nM)	78	6	MeO-Suc-Arg-Pro-Tyr-pNA (0.6 mM)	0.082
Plasmin (10 nM)	81	42	*H*-D-Val-Leu-Lys-pNA (0.6 mM)	0.22
Pl. Kallikrein (3 nM)	91	82	*H*-D-Pro-Phe-Arg-pNA (0.6 mM)	0.19
Gl. Kallikrein (1 U/ml)	8.8	82	*H*-D-Val-Leu-Arg-pNA (0.6 mM)	0.21
Factor XIa (1 nm)	14	0.7	Boc-Glu(OBzl)-Ala-Arg-MCA (0.12 mM)	0.86

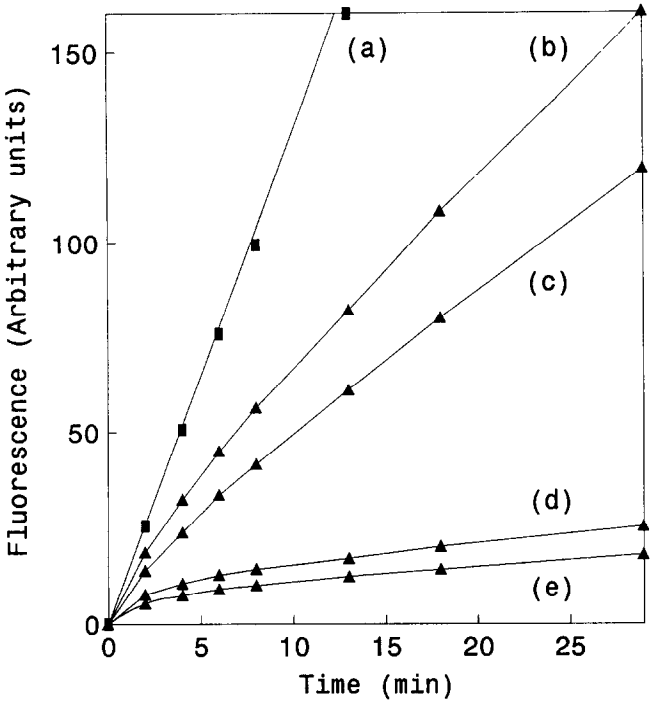

Fig. 4. Transient inhibition of factor XI_a by APPH KPI and APP KPI in the presence and absence of heparin. Factor XI_a (3 nM) was added to a reaction mixture containing 0.25 mM Boc-Glu(OBzl)-Ala-Arg-MCA and 20 nM inhibitor (curve b and c: APPH KPI; curve d and e: APP KPI). Heparin (10 μg/ml) was present in experiments shown as curve c and e. The activity of factor XI_a in the absence of inhibitor is shown for comparison (curve a). The progress curve in fluorescence emission at 460 nm (Ex. 380 nm) was followed as a function of time.

The soluble form of APP was purified from the conditioned media from HepG2 cells as a factor XI_a inhibitory activity [6]. The potency of the inhibitor towards factor XI_a, its activation by heparin, and synthesis in platelets and liver-derived cells [6,7] has prompted several laboratories to speculate that the truncated, secreted form of APP may play a role in regulation of haemostasis. The KPI domain of APPH is highly similar (37/57 identical amino acids) to the KPI domain of APP, including strong homology of residues of the protease contact region. These properties, and also our observation (data not shown) that this molecule is transcribed in human endothelial cells, make it possible that APPH, like APP, could be involved in regulation of the haemostatic balance.

The results of the present study (Table 1, Fig. 3) confirm previous observations [5–7] that the APP KPI domain is indeed a very potent inhibitor of factor XI_a, and that other serine proteases of the coagulation system were not inhibited to an appreciable extend by this domain.

Results obtained with the separately expressed KPI domain (Table 1, Fig. 3) might suggest that APPH, like APP, could in fact function as an inhibitor of factor XI_a in vivo. Although the affinity was 20-fold lower than that of APP, it is still in the nanomolar, physiological range, and factor XI_a might therefore serve as a putative ligand for APPH. It is likely, however, that the true physiological protease ligand for the APPH receptor molecule has still to be identified.

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