Human recombinant membrane-bound aminopeptidase P: production of a soluble form and characterization using novel, internally quenched fluorescent substrates

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APP (aminopeptidase P) has the unique ability to cleave the N-terminal amino acid residue from peptides exhibiting a proline at P₁'. Despite its putative involvement in the processing of bioactive peptides, among them the kinins, little is known about the physiological roles of both human forms of APP. The purpose of the present study is first to engineer and characterize a secreted form of hmAPP (human membrane-bound APP). Our biochemical analysis has shown that the expressed glycosylated protein is fully functional, and exhibits enzymic parameters similar to those described previously for mAPP purified from porcine or bovine lungs or expressed from a porcine clone. This soluble form of hmAPP cross-reacts with a polyclonal antiserum raised against a 469-amino-acid hmAPP fragment produced in *Escherichia coli*.

Secondly, we synthesized three internally quenched fluorescent peptide substrates that exhibit a similar affinity for the enzyme than its natural substrates, the kinins, and a higher affinity compared with the tripeptide Arg-Pro-Pro used until now for the quantification of APP in biological samples. These new substrates represent a helpful analytical tool for rapid and reliable screening of patients susceptible to adverse reactions associated with angiotensin-converting enzyme inhibitors or novel vasopeptidase (mixed angiotensin-converting enzyme/neprilysin) inhibitors.

Key words: adverse reaction, aminopeptidase P, glycosylphosphatidylinositol anchor, internally quenched fluorescent substrate, kinin, screening test.

INTRODUCTION

Bioactive peptides comprise an important and diverse class of extracellular chemical messengers that mediate a wide range of intercellular interactions. Several of these peptides (hormones, neuropeptides and neurotransmitters) escape non-specific protease degradation because of a Xaa-Pro motif at their N-termini. By its cyclic nature, proline confers resistance on these peptides to broad specificity aminopeptidases. APP (aminopeptidase P, also known as X-Pro aminopeptidase, proline aminopeptidase; EC 3.4.11.9) has the unique ability to cleave the N-terminal amino acid residue from peptides exhibiting a proline at P₁'. APP activity is ubiquitous, and has been found in a wide variety of organisms, including bacteria, yeast and vertebrates. *Escherichia coli* APP and all mammalian APPs share homologous blocks of sequences common to members of the 'pita bread-fold' family, of which *E. coli* methionine aminopeptidase is a prototype [1]. APPs are classified as members of the peptidase MG clan on the basis of sequence and structural similarities with other exopeptidases [2,3]. They are metallopeptidases whose activity is abolished by chelators of bivalent metal ions, and they are inhibited selectively by apstatin (see [4] for a review). Despite its putative involvement in the processing of bioactive peptides, little is known about the physiological roles of the human APPs. Human APPs exist in two forms: a membrane-bound form (hmAPP, human membrane-

bound APP) is encoded by the *XPNPEP2* gene localized to Xq25-26.1 [5], whereas a distinct cytosolic homologue is encoded by the *XPNPEPL* (or 1) gene sublocalized on chromosome 10pter-q23.33 [6].

mAPP is a glycosylated, Zn²⁺-containing, multimeric protein, anchored to the surface of the plasma membrane of endothelial and epithelial cells through a GPI (glycosylphosphatidylinositol) bridge. This anchor is added post-translationally to the C-terminal residue of the protein generated after cleavage of a short hydrophobic peptide that acts as the GPI-addition signal. A soluble form of mammalian APP found in plasma probably results from cleavage of the membrane enzyme at the GPI anchor by a phospholipase. Although little is known about cytosolic APP in mammals, it is assumed that the intracellular location of the enzyme limits its involvement in the degradation of circulating peptide hormones.

In human plasma, we have shown that APP plays an important role in the metabolism of kinins [7], a group of inflammatory and vasoactive peptides. BK (bradykinin), the prototype of kinins, is generated from its precursor high-molecular-mass kininogen during activation of the plasma contact system, and is rapidly metabolized by three metallopeptidases. ACE (angiotensin-I converting enzyme) and APP inactivate BK, whereas CPN (carboxypeptidase N) transforms BK into its active metabolite BK₁₋₈ (des-Arg⁹-BK). In turn, this active metabolite is inactivated by APP and ACE [7]. In this case, however, APP is the main metabolic

Abbreviations used: Abz, *o*-aminobenzoic acid; ACE, angiotensin I-converting enzyme; ACE_i, ACE inhibitor; APP, aminopeptidase P; BK, bradykinin; BK₁₋₈, des-Arg⁹-BK; CPN, carboxypeptidase N; DMEM, Dulbecco's modified Eagle's medium; Dnp, 2,4-dinitrophenyl; endo H, endoglycosidase H; GPI, glycosylphosphatidylinositol; HEK-293 cells, human embryonic kidney 293 cells; mAPP, membrane-bound APP; hmAPP, human mAPP; PNGase F, peptide N-glycosidase F; RPP, Arg-Pro-Pro tripeptide; secNEP, soluble secreted neprilysin.

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pathway of BK₁₋₈, and the only degrading enzyme in the presence of an ACE_i (ACE inhibitor).

APP deficiency states have been reported earlier [8]. Our own studies have associated low plasma APP activity with slow degradation of BK₁₋₈ in angioedema, anaphylactoid reactions in haemodialysis and severe hypotension during blood products transfusion [9–14], three rare, but potentially life-threatening, ACE_i-linked side-effects, which could be mediated by kinins. In these studies, plasma APP activity was measured with RPP (Arg-Pro-Pro tripeptide) serving as a substrate [15]. More recently, this low plasma APP activity was linked to a single nucleotide polymorphism in the promoter region of the *XPNPEP2* gene [16]. These results suggest major, if not exclusive, participation of the membrane-bound form of the enzyme in plasma APP activity.

The purpose of the present study is first to produce and characterize fully functional hmAPP. To this end, and for simplicity in downstream purification processes, we generated a secreted form of this normally GPI-anchored protein by introducing a translational stop codon upstream of the C-terminal hydrophobic peptide acting as the GPI-addition signal [17]. Secondly, on the basis of the BK structure, we designed intramolecularly quenched, fluorescent substrates to monitor APP activity. Finally, we deployed these substrates to measure APP activity in plasma samples for which we have previously characterized the metabolism of kinins [7].

MATERIALS AND METHODS

Materials

Reagents

BK (BK₁₋₉), BK₁₋₈, BK₂₋₉ and BK₂₋₈ were obtained from Bachem (King of Prussia, PA, U.S.A.). The ACE_i enalaprilat was from Merck Frosst Canada (Kirkland, QC, Canada). The general aminopeptidase inhibitor bestatin and purified porcine kidney APM (aminopeptidase M) were from Roche (Laval, QC, Canada). Restriction enzymes were purchased from New England Biolabs (Mississauga, ON, Canada). Serum, trypsin-EDTA, penicillin/streptomycin, DMEM (Dulbecco's modified Eagle's medium), Advance DMEM and PBS were purchased from Gibco/Life Technologies (Burlington, ON, Canada). APA (aminopeptidase A) was a generous gift from Dr C. Llorens-Cortes (Collège de France, Paris, France), secNEP (soluble secreted neprilysin) was obtained as described previously [18] and CPM (carboxypeptidase M) was a generous gift from Dr R. Skidgel (University of Illinois at Chicago, U.S.A.). All other fine chemicals, inhibitors (apstatin, phenanthroline, phosphoramidon, G418), purified rabbit ACE and peptides were purchased from Sigma (Montreal, QC, Canada). All chemicals and reagents used were of molecular biology grade.

Nucleotide sequences

All primers were made by Biocorp (Montreal, QC, Canada). Sequencing was performed by the Institut Armand-Frappier DNA Sequencing Facility (Laval, QC, Canada) in an automated ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.).

Sequence analysis tools

GPI anchor attachment sites were predicted by the scheme of Udenfriend and Kodukula [19]. The tool ProtParam (for the calculation of precursor protein molecular masses) was accessed at <http://us.expasy.org/tools/>.

Synthesis and characterization of hmAPP

Construction of hmAPP₁₃₁₋₅₉₉ fragment and antibody production

DNA manipulations were performed in accordance with standard procedures [20]. A plasmid for the production of a glutathione S-transferase-hmAPP fusion protein in *E. coli* was constructed with pGEX4T-3 expression vector (Amersham Biosciences, Baie d'Urfé, QC, Canada). Vent polymerase (New England Biolabs) and human kidney poly(A)⁺ (polyadenylated) RNA as mRNA sources (ClonTech Laboratories, Palo Alto, CA, U.S.A.) served to amplify by reverse transcriptase-PCR a 1447 bp fragment with sense primer 5'-GAGTCGAGCGGCCGCTCCATAAGGAAG-TTGGCACCAC-3' and antisense primer 5'-GCTGACTACGATGCGCGGCCGCTTAGTTCCGGTCATAGGGC-3' (underlined are *NotI* restriction sites included in the primers). The PCR product was cloned in-frame with glutathione S-transferase sequences in the bacterial expression vector, and the recombinant plasmid was sequenced. Production of the recombinant fusion protein in *E. coli* strain AP 401 and purification of the 469-amino-acid hmAPP moiety (hmAPP₁₃₁₋₅₉₉) were undertaken according to the manufacturer's instructions (Amersham Biosciences).

The recombinant fusion protein purified from *E. coli* was used to raise polyclonal antibodies in rabbits under standard methods.

Cloning of a secreted form of hmAPP

A secreted form of human APP (secreted hmAPP) was engineered by introducing, immediately upstream from the hydrophobic peptide acting as the GPI-addition signal [17,18,21], a translational stop codon (TGA) instead of the normal Trp codon (TGG) at position 658. This was done by reverse transcriptase-PCR with Vent polymerase (New England Biolabs) and human kidney poly(A)⁺ RNA as mRNA sources (ClonTech Laboratories). Oligonucleotides 5'-TCTGGCGGCCGCGCACCATGGCCCGGGCT-3' and 5'-CTGGCGGCCGCTCAGGAGGCGGTGTCT-3' were utilized as forward and reverse PCR primers respectively (underlined are *NotI* restriction sites included in the primers). The PCR product was purified, digested with *NotI* restriction endonuclease, and cloned in the pCDNA3/RSV expression vector digested with the same enzyme [22].

Transfection of HEK-293 cells (human embryonic kidney 293 cells)

The expression vector containing secreted hmAPP cDNA was employed to establish stable HEK-293 cells by transfection according to the calcium phosphate-DNA co-precipitation method [23]. HEK-293 cells were maintained in DMEM containing 10% (v/v) fetal bovine serum and supplemented with 60 µg/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml fungizone. To establish permanent cell lines, G418 (Geneticin) selection was initiated 48 h after transfections at 400 µg/ml for 12 days, and gradually decreased to 100 µg/ml.

Isolation of recombinant secreted hmAPP

For protein synthesis and purification, transfected HEK-293 cells were incubated for 18 h in Advance DMEM containing 2 mM sodium butyrate, 1× L-glutamine, 0.5× antibiotic-antimycotic (from 100× stock; Gibco BRL) and 100 µg/ml G418. Culture media containing the secreted hmAPP were processed as follows. First, the media were filtered (0.45 µm), dialysed versus loading buffer (20 mM Tris/HCl, pH 8.0) and concentrated approx. 20-fold in a Centricon Plus-80 system (30 000 Da cut-off). Then, the samples were loaded on a 10 ml quaternary ammonium fast flow ion-exchange column (Amersham Biosciences), and eluted with a stepwise salt gradient (62.5–500 mM NaCl). Fractions

containing APP activity and immunoreactivity (Q125) were submitted to a second chromatographic step in a DEAE fast flow ion-exchange column, under the same conditions. Fractions containing APP activity and immunoreactivity (Q125/D125) were collected and concentrated, and immunoreactive protein levels were assessed by immunoelectrophoretic-blot analysis with the recombinant hmAPP_{131–599} fragment produced from *E. coli* as standard.

Immunoelectrophoretic-blot analysis and glycosylation studies

All collected chromatographic fractions were tested for immunoreactivity. Fractions were resolved by SDS/PAGE on a 4–15 % gel gradient. Molecular mass standards were electrophoresed in parallel and visualized by Coomassie Blue staining. Protein transfer was performed on nitrocellulose membranes. The membranes were then incubated overnight with the polyclonal antibody (1/1000 dilution) raised against the hmAPP_{131–599} fragment, followed by 2 h incubation with appropriate horseradish peroxidase-conjugated (1/3000 dilution) IgG (Bio-Rad, Mississauga, ON, Canada). Protein was quantified with the ChemiGlow West kit (Alpha Innotech Corporation, San Leandro, CA, U.S.A.) according to the manufacturer's instructions.

For the glycosylation studies, secreted hmAPP was incubated with endo H (endoglycosidase H) or PNGase F (peptide N-glycosidase F) as recommended by the distributor (Roche) before immunoelectrophoretic-blot analysis as described above.

Characterization of recombinant secreted hmAPP

Kinetics parameters were measured with GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, U.S.A.; www.graphpad.com). For K_m determination, the data were plotted to fit the Michaelis–Menten equation by non-linear regression. Substrate hydrolysis was limited to ≤ 10 % product formed (initial velocity conditions). Assay mixtures without recombinant enzymes served as controls. For inhibition studies, the data were plotted to fit the 1-site competition equation by non-linear regressions, with imposed constraints (-5.0 to 110 % activities).

(a) Kinetics of secreted hmAPP using RPP. Secreted hmAPP activity was measured by fluorimetric assay adapted by the method of Simmons and Orawski [15]. A Q125/D125 chromatographic elution fraction containing 125 pg of secreted hmAPP was preincubated in 0.1 M Hepes buffer (pH 7.4), and then incubated with the substrate at concentrations ranging from 12 to 2000 μ M (0.2 ml of total reaction volume) at 37 °C for 10 min. The reaction was stopped with 1 ml of ethanol and centrifuged (1000 *g*, 4 °C), the supernatant was decanted and mixed with 3 ml of *o*-phthalaldehyde/2-mercaptoethanol reagent, and fluorescence was read (excitation wavelength of 340 nm, emission wavelength of 455 nm) on a TD-700 fluorometer (Turner Designs, Sunnyvale, CA, U.S.A.). A calibration curve was obtained with successive dilutions of an arginine solution (0.05–0.5 mM).

(b) Kinetics of secreted hmAPP using kinins. BK and BK_{1–8} were applied as substrates. A Q125/D125 chromatographic elution fraction containing 1 ng of secreted hmAPP was preincubated in 0.1 M Hepes buffer (pH 7.4), and then incubated with the substrate at concentrations of 20–1000 μ M (50 μ l of total reaction volume) at 37 °C for 30 min. The reaction was stopped by heating to 99 °C for 5 min, and the addition of phosphoric acid [1 % (v/v) final]. BK, BK_{1–8}, BK_{2–9} and BK_{2–8} were separated on a poly(2-sulphoethyl aspartamine) strong cation exchange column (Poly-SULFOETHYL ATM; The Nest Group, Southborough, MA, U.S.A.) within 35 min, with a linear gradient from 100 % solvent

A [5 mM KH₂PO₄, pH 3, with 25 % (v/v) acetonitrile] to 100 % solvent B (solvent A plus 300 mM KCl) at a constant flow rate of 1 ml · min⁻¹. The eluted peptides were detected by absorbance at 220 nm. With this method, peptides elute in the order of increasing net-positive charge at pH 3.

Internally quenched fluorescent substrates

Internally quenched fluorescent substrate synthesis

The substrates K(Dnp)PPGFSPK(Abz)NH₂, K(Dnp)PPGK(Abz)NH₂ and K(Dnp)PPK(Abz)NH₂ containing the fluorescent group Abz (*o*-aminobenzoic acid) and the quencher Dnp (2,4-dinitrophenyl) were synthesized by solid-phase methodology, with Fmoc-Lys(Dnp)-OH. The sequences chosen are derived from the structure of the nonapeptide BK (RPPGFSPFR), a natural substrate for APP. The enzyme cleaves BK at the R–P bond. In the present study, the quencher group substitutes the R at the N-terminal. The peptides were shortened to avoid non-specific hydrolyses.

Synthesis was undertaken in an automated solid-phase peptide synthesizer, Shimadzu Model PSSM-8, as described previously [24]. All the peptides obtained were purified by semi-preparative HPLC. The molecular mass and purity of the synthesized peptides were checked by amino acid analysis and by molecular mass determination with matrix-assisted laser-desorption/ionization–time-of-flight mass spectrometry, using TofSpec E from Micromass (Manchester, U.K.).

Kinetics of hmAPP using internally quenched fluorescent substrates

Peptides K(Dnp)PPGFSPK(Abz)NH₂, K(Dnp)PPGK(Abz)NH₂ and K(Dnp)PPK(Abz)NH₂ were dissolved in 100 % DMSO as 10 mM stocks and stored at -20 °C. Reactions were in 96-well flat bottom plates (Costar UV plate 3635; Corning, Corning, NY, U.S.A.). The total reaction volume was 150 μ l, consisting of 100 mM Hepes buffer (125 μ l), pH 7.4, 15 μ l substrate (final concentrations of 0.1–60 μ M) and a Q125/D125 elution fraction containing secreted hmAPP (10 μ l, 1 ng). Reactions were duplicated. Fluorescence (excitation wavelength of 340 nm, emission wavelength of 420 nm) was assessed kinetically in a FL600 microplate fluorescence plate reader (BioTek, Winooski, VT, U.S.A.). Fluorescent units were converted into pmol of substrate hydrolysed based on a standard fluorescence curve of complete hydrolysis of each substrate at eight different concentrations. The enzyme concentration for the initial rate determination was chosen at a level intended to hydrolyse < 10 % of the amount of added substrate. The inner-filter effect was corrected using an empirical equation as described previously [24]. Errors did not exceed 5 % for any of the kinetic parameter values obtained.

Specificity of internally quenched fluorescent substrates

The specificity of the newly synthesized substrates for secreted hmAPP was tested. These analytical criteria were assessed with pure recombinant (APA, CPM and secNEP) or purified (rabbit ACE and porcine APM) metalloproteinases known to be present in human plasma with susceptibility to hydrolyse kinin-like substrates. The experimental conditions were as described above for secreted hmAPP, with final substrate concentrations of 15 μ M, over a 60 min fluorescence acquisition period. The enzyme concentrations tested were 1 and 2.5 m-units/ml for APA, 15 and 40 ng/ml for CPM, 1.5 and 5 μ g/ml for secNEP, 1 and 2.5 m-units/ml for rabbit ACE, and 20 m-units and 1 unit/ml for porcine APM.

Optimum pH determination, Zn^{2+} influence on catalytic activity, and inhibition studies

These characterizations were conducted with K(Dnp)PPGK-(Abz)NH₂ under the aforementioned conditions. For pH optimization, 50 mM phosphate buffer over a pH range from 5.0 to 8.0 and 50 mM Tris buffer over a pH range from 7.4 to 9.0 were used. To check the effect of Zn^{2+} ions and various inhibitors (apstatin, phenanthroline, bestatin and phosphoramidon), the enzyme was preincubated with different concentrations of the metal ion or inhibitors at 37 °C for 15 min before substrate addition.

Application of novel, quenched fluorescent substrates to the quantification of APP in human plasma

Origin of plasma samples

For the present study, we tested 47 plasma samples described previously to determine the reference values for APP activity, measured by a modification of the fluorimetric method published earlier by Simmons and Orawski [15] with the RPP substrate. The metabolism of endogenous BK and its active metabolite BK₁₋₈ was also measured kinetically for these samples during the activation of the contact system of plasma in the presence of an ACE_i [7]. The relationship between the time and the concentrations of endogenous kinins released in plasma was expressed according to the mathematical model $y = kt^\alpha e^{-\beta t}$. In this model, y is the concentration of the kinin (BK or BK₁₋₈), t the time of measurement, and α and β characterize the formation and the degradation of each kinin respectively. Specifically, for small (large) values of β , the speed at which the kinin concentration decreases is slow (fast). This parameter is, therefore, directly dependent on enzyme activity responsible for the catabolism of kinins. In the presence of an ACE_i, the parameter depends solely on APP activity for BK₁₋₈ profiles, whereas it combines APP and CPN activity for BK.

Plasma APP activity measurement with quenched fluorescent substrates

Plasma APP activity was measured with three internally quenched, novel fluorescent substrates, with the ACE_i enalaprilat (400 nM). For a 150 μl final volume, 10 μl of plasma was used. Final substrate concentrations were 10 μM . Fluorescence was read as described above.

Statistical analysis

To validate further the novel, internally quenched fluorescent substrates, Pearson correlations were calculated between APP activities obtained with RPP or kinetic parameter β [7] and the activities were measured with the three quenched fluorescent substrates.

Sensitivity and reproducibility of APP activity assessment in human plasma with internally quenched fluorescent substrates

To quantify the sensitivity of APP activity measurements in human plasma to the internally quenched fluorescent substrates, a pool of 40 plasmas from healthy donors was successively diluted with Hepes buffer (10 dilutions, from 100% plasma, 0% buffer to 5% plasma, 95% buffer), and APP activity was assessed five times for each dilution, for each substrate, as described. For statistical analysis purposes, this corresponds to 10 groups of five samples for each substrate. This pool is currently the control in our laboratory, and its APP activity, as assessed with RPP, is $24 \pm 4 \text{ nmol} \cdot \text{min}^{-1} \cdot (\text{ml of plasma})^{-1}$.

For each fluorescent substrate, one-way ANOVA followed by Dunnett contrasts with respect to the blank value was performed to determine the highest dilution (corresponding to the lowest activity detectable) for which the fluorescent signal is different from the blank value.

The reproducibility of the assays with the novel, internally quenched fluorescent substrates was tested in two pools of human plasma from healthy donors ($n = 20$), whose APP activity, as assessed with RPP, was $< 15 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$ or $> 20 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$. APP activities with the novel, internally quenched fluorescent substrates were measured five times on five different days.

RESULTS

Synthesis and characterization of secreted hmAPP

Expression of secreted hmAPP in HEK-293 cells

HEK-293 cells were transfected with a pCDNA3/RSV expression vector containing secreted hmAPP cDNA, and a permanent cell line was established. This secreted form of hmAPP was engineered by introducing a translational stop codon (TGA) instead of the normal Trp codon (TGG at position 658) upstream from the hydrophobic peptide acting as the GPI-addition signal. Immunoblotting with a polyclonal antibody raised in rabbit against fragment L131–N599 of hmAPP produced in *E. coli* showed that, after 18 h of culture, secreted hmAPP was present in the unprocessed culture medium from transfected cells but not in the medium of control cells (Figure 1A, lanes 1 and 2). Small amounts of the enzyme were also detected in the transfected cell extract, but not in mock transfected cells (Figure 1A, lanes 3 and 4). Secreted hmAPP had an apparent molecular mass of approx. 85 kDa.

To characterize the glycosylation state of secreted hmAPP, we next submitted the recombinant protein to deglycosylation by PNGase F and endo H. PNGase F removes high mannose as well as most of the complex N-linked oligosaccharides added in the Golgi complex. In contrast, endo H removes N-linked oligosaccharide side-chains of the high-mannose type found on proteins in the rough endoplasmic reticulum but which have not yet transited through the Golgi complex; thus, resistance to endo H can be used as an indication that the protein has travelled through the Golgi complex. PNGase F treatment showed that the secreted hmAPP was N-glycosylated, since electrophoretic mobility increased after digestion (Figure 1B, lane 2). This protein was, however, resistant to digestion with endo H, indicating true secretion of the enzyme (Figure 1B, lane 3).

Culture media from HEK-293 and HEK/hmAPP cells were tested for enzymic activity with RPP as substrate. Activity was detected in the culture medium of HEKhmAPP-transfected cells but not in that of HEK-293 mock-transfected cells. Activity increased linearly with the amount of recombinant enzyme (Figure 1C) and with the incubation period (results not shown), indicating that the degradation of the substrate was due to hmAPP enzyme activity.

Isolation and characterization of recombinant secreted hmAPP using RPP and kinins as substrates

Secreted hmAPP was isolated from concentrated culture media of transfected HEK-293 cells by two-step chromatographic separation. The sample was first loaded on a quaternary ammonium fast flow ion-exchange column. The 125 mM NaCl elution fraction (Q125) contained APP activity and immunoreactivity, but an ACE or ACE-like contaminating activity, inhibited by enalaprilat,

Figure 1 Expression of secreted hmAPP in HEK-293 cells

(A) Immunoblot analysis of secreted hmAPP-transfected HEK-293 cells. Proteins from culture medium and from cell extracts (corresponding to $\sim 1/25$ of a 75 mm flask) were separated by SDS/PAGE (4–15% gels), transferred on to nitrocellulose membranes and revealed with a hmAPP-specific polyclonal antibody. Lane M, molecular mass marker; lane 1, medium from hmAPP-transfected cells; lane 2, medium from mock-transfected cells; lane 3, cell extracts from hmAPP-transfected cells; lane 4, cell extracts from mock-transfected cells. (B) Analysis of secreted hmAPP processing in HEK-293 cells. Proteins from culture medium with hmAPP-transfected cells were separated as described above. Lane 1, untreated hmAPP; lane 2, hmAPP treated with PNGase F; lane 3, hmAPP treated with endo H. (C) hmAPP enzymic activity as a function of the amount of protein. Decreasing amounts of hmAPP protein from transfected cell media were tested for peptidase activity using RPP as substrate. The amount of hmAPP in the assays, as detected by immunoblotting, is shown in the panel above the graph.

could also be detected, using the substrate Abz-YRK(Dnp)P as in [24]. The fraction was submitted to a second chromatographic step, in a DEAE fast flow ion-exchange column, under the same conditions. This was done to separate APP activity from contaminating ACE-like activity. The 125 mM NaCl elution fraction (Q125/D125), showing APP activity and immunoreactivity, was concentrated, and the secreted hmAPP, as determined by immunoelectrophoretic blotting, was 315 ng/ml. Assays with up to 1 ng of immunoreactive secreted hmAPP showed no contaminating ACE-like activity.

We characterized the enzymic parameters of recombinant secreted hmAPP with RPP and the natural substrates of the enzyme, the kinins BK and BK₁₋₈. The K_m values were 0.837 ± 0.075 mM, 75 ± 15 and 56 ± 13 μ M for RPP, BK and BK₁₋₈ respectively (Figures 2A and 2B). With BK as substrate, secreted hmAPP activity was completely inhibited by prior incubation with 10 μ M apstatin (results not shown).

Figure 2 Michaelis–Menten plots for secreted hmAPP hydrolysis of peptides

Initial velocity data for secreted hmAPP hydrolysis of RPP (A), BK and BK₁₋₈ (B) and the internally quenched fluorescent substrates K(Dnp)PPGFSPK(Abz)NH₂, K(Dnp)PPGK(Abz)NH₂ and K(Dnp)PPK(Abz)NH₂ (C) were determined as described in the Materials and methods section. Data were fit to the Michaelis–Menten equation ($v = V_{max}[S]/K_m + [S]$) using GraphPad Software. K_m values are summarized in the text.

Internally quenched fluorescent substrate synthesis and characterization

Characterization of secreted hmAPP activity with novel fluorescent substrates

The enzymic parameters of recombinant secreted hmAPP were characterized with the novel substrates K(Dnp)PPGFSPK(Abz)NH₂, K(Dnp)PPGK(Abz)NH₂ and K(Dnp)PPK(Abz)NH₂ (Figure 2C). The K_m values of 18 ± 3 , 20 ± 5 and 19 ± 2 μ M respectively, for these substrates compared favourably with those obtained for the kinins BK and BK₁₋₈, and were much lower than those obtained for RPP.

Specificity of internally quenched fluorescent substrates

Various peptidases (secNEP, CPN, ACE, APM and APA) were compared for their ability to hydrolyse the novel fluorescent

Figure 3 Inhibition study of secreted hmAPP

Effect of increasing concentrations of various inhibitors on secreted hmAPP-catalysed hydrolysis of K(Dnp)PPGK(Abz)NH₂ as described in the Materials and methods section. The data were plotted to fit the one-site competition equation by non-linear regression with GraphPad Software and imposed constraints (–5.0 to 110 % activities). The IC₅₀ values obtained were 2.3 μM for apstatin (APS), 10.6 μM for phenanthroline (PHE), and >1 mM for phosphoramidon (PHO) and bestatin (BES).

substrates under the aforementioned conditions. Substrates K(Dnp)PPGK(Abz)NH₂ and K(Dnp)PPK(Abz)NH₂ were highly selective for secreted hmAPP, as none of the tested enzymes hydrolyse them in any noteworthy way under these conditions. The longer substrate K(Dnp)PPGFSPK(Abz)NH₂ was, however, partially hydrolysed by ACE (up to 17 pmol of substrate hydrolysed · min^{–1} · well^{–1} under the described conditions, inhibited by enalaprilat). The presence of proline in the penultimate position of this substrate precludes the carboxypeptidase activity of ACE. However, the carboxytripeptidase activity of ACE has been described previously [25,26]. This activity is stimulated by the presence of proline in the penultimate position of the substrate. We suggest that ACE cleaves K(Dnp)PPGFSPK(Abz)NH₂ at the F–S bond.

Effects of inhibitors on secreted hmAPP activity towards K(Dnp)PPGK(Abz)NH₂. The effect of various inhibitors on the hydrolysis of K(Dnp)PPGK(Abz)NH₂ by secreted hmAPP was studied (Figure 3). The chelating agent 1,10-phenanthroline suppressed the enzyme, as is expected for a metalloenzyme (IC₅₀ of 10.6 μM). The specific APP inhibitor apstatin also depressed hmAPP activity with an IC₅₀ of 2.3 μM. The general aminopeptidase inhibitor bestatin did not significantly block the enzyme, nor did phosphoramidon (IC₅₀ in the mM range).

Effects of Zn²⁺ and pH on secreted hmAPP activity towards K(Dnp)PPGK(Abz)NH₂

The influence of increasing concentrations of Zn²⁺ on the hydrolysis of K(Dnp)PPGK(Abz)NH₂ on secreted hmAPP was examined (Figure 4A), and inhibitory effects were noted as for 10 μM. With the same substrate, the optimum pH for hmAPP was found to be 7.4 (Figure 4B).

Plasma studies

Correlations between novel, internally quenched fluorescent substrate activities, RPP activities and the β parameter

As shown in Table 1, the Pearson correlations for APP activities in plasma in the presence of an ACE_i, measured with RPP as

Figure 4 Dependence of secreted hmAPP-catalysed hydrolysis of K(Dnp)PPGK(Abz)NH₂ on Zn²⁺ (A) and pH (B)

Conditions are described in the Materials and methods section; ●, 50 mM phosphate buffer; ▲, 50 mM Tris buffer.

Table 1 Pearson correlations between activities assessed with RPP and internally quenched fluorescent substrates

APP activity was measured in plasma samples from 47 healthy Caucasians using RPP, K(Dnp)PPGFSPK(Abz)NH₂, K(Dnp)PPGK(Abz)NH₂ and K(Dnp)PPK(Abz)NH₂ as substrates, in the presence of an ACE_i. The experimental conditions were as described in the Materials and methods section. Pearson correlations were calculated between activities obtained with RPP and those obtained with the three quenched fluorescent substrates; *P* < 0.001 in all cases.

	Pearson correlation		
	K(Dnp)PPGFSPK(Abz)	K(Dnp)PPGK(Abz)	K(Dnp)PPK(Abz)
RPP	0.913	0.921	0.891

substrate and three novel substrates, were highly significant (*P* < 0.001).

Correlations between kinetic parameter β and APP activities, obtained with the three fluorescent substrates and RPP, are presented in Table 2 [7]. With similar significance for both kinins

Table 2 Pearson correlations between APP activities and kinetic parameter β [7] characterizing the plasma catabolism of kinins BK and BK₁₋₈

APP activity was measured in the presence of an ACE_i in 47 human plasma samples using RPP, K(Dnp)PPGFSPK(Abz)NH₂, K(Dnp)PPGK(Abz)NH₂ and K(Dnp)PPK(Abz)NH₂ as substrates. In the same samples, the β parameter was calculated for BK and BK₁₋₈ catabolism. Significant Pearson correlations were calculated between enzyme activities and β parameter; $P < 0.001$ in all cases.

β value	Pearson correlation			
	RPP	K(Dnp)PPGFSPK(Abz)	K(Dnp)PPGK(Abz)	K(Dnp)PPK(Abz)
BK	0.397	0.499	0.489	0.475
BK ₁₋₈	0.766	0.839	0.822	0.813

Table 3 Sensitivity of APP activity assessment in human plasma with internally quenched fluorescent substrates

The fluorescence measured for each dilution and each substrate was compared with the blank sample (substrate and plasma, with 10 μ M apstatin). For each substrate, a one-way ANOVA followed by Dunnett contrasts with respect to the blank value was performed to determine the highest dilution (corresponding to the lowest activity detectable) for which the fluorescent signal is different from the blank value. P values are for Dunnett contrasts. This statistical test shows that the dilution statistically different from the blank value is 1/5 for RPP, 1/3 for K(Dnp)PPGFSPK(Abz)NH₂, 1/3 for K(Dnp)PPGK(Abz)NH₂ and 1/10 for K(Dnp)PPK(Abz)NH₂.

Dilution (% plasma)	P value			
	RPP	K(Dnp)PPGFSPK(Abz)	K(Dnp)PPGK(Abz)	K(Dnp)PPK(Abz)
5	0.999	1.000	0.928	0.345
10	0.656	0.923	0.285	0.015
20	0.049	0.758	0.602	<0.001
30	0.057	0.006	0.014	<0.001
40	<0.001	0.071	0.029	<0.001
50	<0.001	<0.001	<0.001	<0.001
66	<0.001	<0.001	<0.001	<0.001
80	<0.001	<0.001	<0.001	<0.001
100	<0.001	<0.001	<0.001	<0.001

($P < 0.001$), a higher correlation could be obtained for BK₁₋₈ for the four substrates. Moreover, the correlation was higher between the β parameter and APP activity measured with the three quenched fluorescent substrates than that calculated previously for RPP. As the β parameter is dependent solely on APP activity for BK₁₋₈ in presence of an ACE_i, the correlation was directly dependent on enzyme activity towards the substrates.

Sensitivity and reproducibility of APP activity assessment in human plasma with internally quenched fluorescent substrates

Table 3 details the sensitivity limit as defined by statistically different fluorescence from that measured in a blank sample (substrate and plasma, with 10 μ M apstatin). One-way ANOVA first determined $F(9,36) = 42.45$, $P \leq 0.001$; $F(9,36) = 20.54$, $P \leq 0.001$; $F(9,36) = 20.75$, $P \leq 0.001$ and $F(9,36) = 25.07$, $P \leq 0.001$ for RPP, K(Dnp)PPGFSPK(Abz)NH₂, K(Dnp)PPGK(Abz)NH₂ and K(Dnp)PPK(Abz)NH₂ respectively. Thus the mean values of activities for the different dilutions were significantly different. To identify the level of dilution that is significantly different from the blank value, Dunnett contrasts were subsequently calculated. This statistical test showed that the highest dilution statistically different from the blank value was 1/5 for RPP ($P = 0.049$), 1/3 for K(Dnp)PPGFSPK(Abz)NH₂ ($P = 0.006$), 1/3 for K(Dnp)PPGK(Abz)NH₂ ($P = 0.014$) and 1/10

for K(Dnp)PPK(Abz)NH₂ ($P = 0.015$). Hence the latter substrate allowed the most sensitive quantification of APP activity in human plasma.

Reproducibility, as assessed by CV% [coefficient of variance % = (s/X) $\times$ 100, where s is the standard deviation and X is the mean (average)] for activity with a given substrate was $\leq 6\%$ on the same day (intra-assay) and $\leq 10\%$ for different days (inter-assay) for both pools of plasma tested.

DISCUSSION

Different purification methods have been described for both cytosolic APP [27,28] and mAPP. In this way, bovine [15,29], porcine [30–32], rat [33] and guinea pig [34,35] mAPP have been obtained from the lungs and/or kidneys, as well as from guinea pig serum [34,36]. These methods usually include several chromatographic steps, detergent use and phospholipase digestion. Although some have reported the cloning and the tissue distribution of hmAPP [37,38], no method has described the synthesis or purification of a soluble form of hmAPP. In the present study, we describe, for the first time, the production of a secreted form of hmAPP, using the expression of a cDNA clone in HEK-293 cells. Biochemical analysis has shown that the expressed glycosylated protein is fully functional and exhibits similar enzymic parameters as described previously for mAPP purified from porcine and bovine lungs [31,33], and from an engineered porcine clone [39]. Similar properties were also obtained for the inhibitory and pH profiles. The availability of a stable source of recombinant enzyme will be useful in preclinical selectivity studies of novel metalloproteinase inhibitors. In fact, we cannot exclude, at this time, that some side-effects of these new drugs, namely mixed vasopeptidase inhibitors, could be caused by non-specific inhibition of APP activity, as described earlier by Hooper et al. [40] with a pig kidney APP preparation and various ACE_i.

Clinical interest in mAPP has increased following the description of partial or total plasma APP deficiency states associated with ACE_i-linked side-effects. In fact, we have previously reported significantly lower plasma APP activity in hypertensive, dialysed and transfused patients, treated with an ACE_i, who subsequently suffered angioedema, anaphylactoid reaction or severe hypotension [9,12–14]. These findings were linked to BK₁₋₈ accumulation in pathological plasmas, and a lower β kinetic parameter measured in the presence of an ACE_i [11–14]. Since that time, an extensive genetic linkage study of 29 families, in which one member suffered an angioedema or an anaphylactoid reaction when treated with an ACE_i, has shown that mutations in *XPNPEP2* gene increase susceptibility to those ACE_i-associated adverse reactions [16]. In this genetic analysis, no linkage could be found between chromosome 10 (hence, cytosolic APP) and plasma APP activity measured with RPP, indicating that cytosolic APP plays little or no role in plasma peptide metabolism. The exact nature of the enzymic defect (qualitative, quantitative or both) remains to be confirmed. Taken together, these experimental and clinical observations urge the development of analytical methods for rapid and reliable screening of patients susceptible to metalloproteinase inhibitor-associated adverse reactions. With this perspective, we have successfully obtained a polyclonal antibody recognizing a secreted form of hmAPP on immunoblotting that could be instrumental in the development of an immunoassay for the quantification of immunoreactive hmAPP in human plasma.

We have also successfully designed and produced three internally quenched fluorescent peptide substrates for APP that exhibit a similar affinity for the enzyme as its natural substrates. In

this strategy, the peptide substrate design followed the general structure of Abz-peptidyl-EDDnp (where EDDnp stands for ethylenediamine 2,4-dinitrophenyl), in which Abz is the fluorescent group and EDDnp is the quenching group, separated by a peptide sequence containing the peptidase cleavage site. Two of these substrates, [K(Dnp)PPGK(Abz)NH₂ and K(Dnp)PPK(Abz)NH₂] are highly specific for APP. A third one [K(Dnp)PPGFSPK(Abz)NH₂] is hydrolysed by ACE. However, this susceptibility to ACE hydrolysis is not important for application to human plasma. In fact, clinical interest in plasma APP measurement is in the presence of an ACE_i, to mimic the *in vivo* inhibition occurring in patients manifesting ACE_i-associated side-effects.

When tested in plasma, the APP activities obtained with these novel, internally quenched fluorescent substrates significantly correlated with the tripeptide substrate RPP and with kinetic parameter β describing the catabolism of the kinins BK, and more so of BK₁₋₈ in normal human plasma [7,11]. With a similar or higher sensitivity compared with RPP, these substrates offer the advantage of simplicity, speed and continuous measurements without revelation steps. They represent a useful analytical tool for a rapid and reliable screening of patients susceptible to ACE_i or novel vasopeptidase (mixed peptidase) inhibitor-associated adverse reactions.

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