Molecular cloning and expression in COS-1 cells of pig kidney aminopeptidase P

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Aminopeptidase P (AP-P; X-Pro aminopeptidase; EC 3.4.11.9), a key enzyme in the metabolism of the vasodilator bradykinin, has been cloned from a pig kidney cortex cDNA library following the use of the PCR to identify sub-libraries enriched in AP-P clones. The complete primary sequence of the enzyme has been deduced from a full-length cDNA clone. This predicts a protein of 673 amino acids with a cleavable N-terminal signal sequence and six potential N-linked glycosylation sites. A stretch of mainly hydrophobic amino acids at the C-terminus is predicted to co-ordinate the attachment of a glycosyl-phosphatidylinositol (GPI) membrane anchor. Although AP-P is a zinc metallopeptidase, the predicted primary sequence does not contain any recognizable zinc-binding motif. Transient expression of AP-P cDNA in COS-1 cells resulted in enzymic activity characteristic of AP-P, namely apstatin- and EDTA-sensitive hydrolysis of bradykinin and Gly-Pro-Hyp. The expressed protein was recognized as a polypeptide of M_r 91000 under reducing conditions, following immunoblotting of COS-1 membranes with a polyclonal antibody raised against purified pig kidney AP-P. The presence of a GPI anchor on expressed AP-P was established by demonstrating release of the enzyme from a membrane fraction following treatment with bacterial phosphatidylinositol-specific phospholipase C and its corresponding conversion from an amphipathic to a hydrophilic form, as assessed by phase separation in Triton X-114. Sequence comparisons confirm that AP-P is a member of the proline peptidase family of hydrolytic enzymes and is unrelated in sequence to other brush-border membrane peptidases.

INTRODUCTION

Aminopeptidase P (AP-P; X-Pro aminopeptidase; EC 3.4.11.9) is a zinc metallopeptidase that releases the N-terminal amino acid residue from peptides with a penultimate proline residue [1]. The mammalian enzyme has been located in renal microvillar membrane fractions from a number of species [2–4] and was first purified from pig kidney [5], but membrane-bound forms have also been identified in rat intestine [6] and lung [7], and bovine lung [8], and soluble forms have been characterized in guinea pig serum [4] and rat brain [9]. AP-P has been shown to cleave several biologically active peptides, including bradykinin, substance P and peptides of the pancreatic polypeptide family [8,10–12], and may act together with angiotensin-converting enzyme (EC 3.4.15.1) in the physiological inactivation of the potent vasodilator bradykinin [8,13,14]. Hence AP-P is a potential target for novel anti-hypertensive agents.

Mammalian AP-P is unusual among cell-surface peptidases in being a glycosyl-phosphatidylinositol (GPI)-linked membrane protein [2], and solubilization of activity by bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) has been exploited as a first step in purification of the enzyme from pig kidney [5,15]. The soluble form of AP-P in serum [4] may be derived from the membrane form through the action of endogenous phospholipases. Purified pig kidney AP-P has an M_r of 91000, with up to 25% by weight being due to N-linked sugars [5], and contains 1 mol of Zn^{2+}/mol of protein [16].

AP-P is atypical among aminopeptidases in its inhibitor profile, being relatively insensitive to inhibition by bestatin, amastatin, actinonin and puromycin [5,7,16]. However, hydrolysis of the synthetic peptide substrate Gly-Pro-Hyp by AP-P is inhibited by a number of inhibitors of angiotensin-converting enzyme, such as enalaprilat, cilazaprilat and ramiprilat [16]. A more specific inhibitor of AP-P, apstatin, has recently been described [7]. The hydrolysis of bradykinin and Gly-Pro-Hyp by AP-P show a number of unexplained differences in sensitivity to inhibitors and to cation activation [15].

AP-P appears to be structurally related to the 'proline peptidase' family of hydrolytic enzymes, which includes *Escherichia coli* AP-P, prolidase, methionine aminopeptidase and creatinase [17,18]. The amino acid sequence of pig kidney AP-P has recently been determined by Edman degradation and MS of the purified protein [19], and sequence comparisons further emphasize the similarity between AP-P and other members of the proline peptidase family.

Knowledge of the amino acid sequence of purified AP-P reveals that it does not possess the His-Glu-Xaa-Xaa-His motif typical of many zinc metallopeptidases, nor any other recognizable zinc-binding motif [20], although chemical modification studies have indicated that the protein contains two essential histidine residues [21]. The amino acid sequence of the mature protein [19] also, of course, lacks any N-terminal signal peptide that may be required for membrane targeting and the C-terminal signal sequence for co-ordinating the attachment of the GPI-anchor moiety. In the present study we report the molecular cloning, expression and characterization of pig kidney AP-P as a prelude to mutagenesis studies aimed at understanding its catalytic mechanism and to aid in the design of potent and selective inhibitors.

EXPERIMENTAL

Materials

Tissue culture media, serum, trypsin-EDTA, penicillin, streptomycin, L-glutamine, transfection reagent (lipofectAmine),

Abbreviations used: AP-P, aminopeptidase P; GPI, glycosyl-phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C.

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The nucleotide sequence data reported have been deposited in the GenBank/EMBL/DDBJ Nucleotide Sequence Databases under accession no. U55039.

Superscript II RNase H$^-$ reverse transcriptase and *Taq* DNA polymerase were purchased from Gibco-BRL (Paisley, U.K.). mRNA separator kits were purchased from Clontech (Cambridge, U.K.). T7 DNA sequencing kit was from Pharmacia Biotech (St. Albans, U.K.). [α-^{35}S]dATP and [α-^{32}P]dCTP (1000 Ci/mmol) were from New England Nuclear (Stevenage, U.K.). Bradykinin, bradykinin-(2–9), Gly-Pro-Hyp and Pro-Hyp were all purchased from Sigma (Poole, Dorset, U.K.). Restriction enzymes, T4 DNA ligase and Nick Translation kit were purchased from Boehringer Mannheim (Lewes, U.K.). The mammalian expression vector pBKCMV was from Stratagene (Cambridge, U.K.). Apstatin was a gift from Professor W. H. Simmons (Loyola University, Chicago, IL, U.S.A.), and enalaprilat was from Merck, Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.). *Bacillus thuringiensis* PI-PLC was a gift from Dr. M. G. Low (Columbia University, New York, NY, U.S.A.). Pig kidney AP-P was purified to homogeneity as described previously [5]. All other reagents were of analytical grade.

Cloning of pig AP-P cDNA

The choice of degenerate oligonucleotide primers was determined from partial peptide sequences of purified pig kidney AP-P obtained in our laboratory [22] and from the protein sequence reported by Vergas Romero et al. [19]. The sequences of the sense primers were: F1, 5′-CARATGGAYTGYAAWTGGG-3′; F2, 5′-GGIGAYGTIAARATHTGGAT-3′. The antisense primers were: R1, 5′-TARTCYTCIARYTGIACRCACAT-3′; R2, 5′-GARTTYTTIACIGCYTTIGTHATCAT-3′; R3, 5′-GTRAACATICCYTCNGCCAT-3′. Total RNA was isolated from pig kidney cortex using the method of Logemann et al. [23]. Poly(A)$^+$ RNA was selected using the Clontech separator system and single-strand cDNA was synthesized using Superscript II. This cDNA was used as a template in PCR reactions with all the degenerate primer pairs. PCR products were subcloned and sequenced by using established protocols [24]. The sequences of these subcloned PCR products corresponded to the expected regions of pig AP-P. By using these sequences, highly specific oligonucleotide primers were synthesized (see Figure 1). These primers were then used in a much more stringent PCR amplification procedure.

Screening of pig kidney cortex cDNA library

Aliquots of a pig kidney cortex cDNA library constructed in λZap (Stratagene) were screened using the specific primer pair FS1/RS1 (see Figure 1). A PCR product of the expected size was amplified from one of the library aliquots. This aliquot was re-titred and serial dilutions were made which were rescreened using the same PCR conditions. During the screen a PCR product of the expected size (591 bp) was amplified from a titre containing 5×10^2 plaque-forming units. This sub-library was re-titred, 5×10^3 plaque-forming units were plated and positive plaques were identified by plaque hybridization. The 702 bp PCR product of the reaction with primers F1/R2 on the single-strand cDNA from pig kidney was labelled with [α-^{32}P]dCTP, using the Nick Translator kit, and used in plaque hybridization following standard procedures [24]. Ten positive plaques were selected and a repeat screen was carried out to check the plaque purity. Several positive clones were plaque purified and sequenced.

Sequencing and analysis

DNA sequences were initially determined by the dideoxynucleotide method using the T sequencing kit (Pharmacia Biotech). One clone (102231A) was sequenced completely on both strands using *Taq* Dye Deoxy Terminator Cycle sequencing chemistry in conjunction with the ABI373A DNA sequencing system. DNA and protein sequences were assembled and analysed using the Wisconsin-GCG programs.

Construction of the expression vector and transient expression in COS-1 cells

The 3.6 kb insert encoding pig kidney AP-P (102231A) was released from the Bluescript SKII$^-$ vector following *Xba*I/*Kpn*I digestion. The expression vector pBKCMV was digested with *Nhe*I and *Kpn*I. Vector and insert were ligated using standard cloning procedures [24], the orientation of the insert being verified by a directional PCR reaction. The resulting construct (pBK-APP) was used for transfection of COS-1 cells, which were cultured as described previously [25]. For transient expression of pBK-APP in COS-1 cells, the cells were plated in 150 cm^2 flasks at approx. 2×10^6 cells per flask. After 24 h of growth, the cells were washed twice with OptI-Mem and transfected (5 μg of DNA/flask) by using lipofectAmine as cationic lipid. The cells were incubated for 2 h at 37 °C and then Dulbecco's modified Eagle's medium containing 10 % (v/v) foetal calf serum was added. After 24 h the medium was replaced with fresh, and transient expression was assayed on the cell surface or in a solubilized membrane preparation 48 h after transfection. Membrane fractions were prepared from COS-1 cells and solubilized in 60 mM n-octyl β-D-glucopyranoside as described previously [25].

PI-PLC treatment and Triton X-114 phase separation

Membrane fractions (54 μg) from COS-1 cells transfected with pBK-APP were made up to 0.2 ml with 10 mM Tris/HC1 buffer, pH 7.4, containing 0.15 M NaCl. To half of this sample was added 1 unit of PI-PLC followed by incubation for 1 h at 37 °C. Pre-condensed Triton X-114 was then added to each sample to a final concentration of 1.0 % (w/v), and samples were subjected to phase separation at 30 °C for 3 min as detailed in [26]. The upper (detergent-depleted) phase was separated from the lower (detergent-rich) phase and both phases were brought to the same volume. Aliquots (10 μl) from each phase were taken for determination of AP-P activity assayed by the hydrolysis of bradykinin. The results are expressed as the percentage of total activity recovered and are the means $\pm$ S.E.M. for triplicate determinations.

SDS/PAGE and immunoelectrophoretic blot analysis

SDS/PAGE was performed with a 7–17 % (w/v) polyacrylamide gradient as described previously [27]. Immunoelectrophoretic (Western) blot analysis [28] was carried out with Immobilon P poly(vinylidene difluoride) membranes, and the detection of protein was performed by using the enhanced chemiluminescence detection method (ECL kit; Amersham) in accordance with the manufacturer's instructions. The primary antibody was a rabbit polyclonal antibody raised against purified pig kidney AP-P [15]. M_r standards were run in parallel and visualized by Coomassie Blue staining.

Enzyme assays

The hydrolysis of Gly-Pro-Hyp (1 mM) and bradykinin (0.1 mM) by detergent-solubilized COS-1 cell membranes (3.0 μg) was assayed by HPLC methods, as described previously [2,15]. Incubations were performed in triplicate under each set of conditions or in duplicate when inhibitors were present.

Protein determination

Protein concentrations were determined using the bicinchoninic acid assay [29], with BSA as standard. The method was adapted for use in 96-well microtitre plates [30].

RESULTS AND DISCUSSION

Predicted coding sequence

A clone (102231A) was isolated and sequenced completely on both DNA strands. The clone contains an insert of 3539 bp, excluding the poly(A) tail. The sequence comprises 345 nucleotides of 5′-untranslated region, an open reading frame of 2019 nucleotides, and 1199 nucleotides of 3′-untranslated region (Figure 1). The full-length cDNA sequence encodes a protein of 673 amino acids with a calculated M_r of 75755. A hydropathy plot of this protein sequence reveals potential transmembrane-spanning hydrophobic regions at both termini (results not shown). There is a typical cleavable signal sequence at the N-terminus. When the N-terminal region is analysed using the weight-matrix method of von Heïjne [31], two possible cleavage sites are identified, both of which differ from the N-terminus reported in [19]. The position that generates the highest 'score' (6.70) places Lys^{24} at the N-terminus (Figure 1). However, this puts Pro at the −1 position, which is unusual but not unique in eukaryotic signal sequences. When the His at position 22 (Figure 1) is considered as the N-terminal residue (score of 6.15), more favourable amino acids are aligned at the important signal sequence positions of −1 and −3. As the weight-matrix method cannot distinguish between cleavage positions whose scores are similar, we cannot deduce with any certainty which of the two predicted residues represents the true N-terminus of the mature protein. However, these data do support the contention of Vergas Romero et al. [19] that their previously reported N-terminal sequence does not represent the N-terminus of the mature protein due to partial proteolysis. Our previous attempts

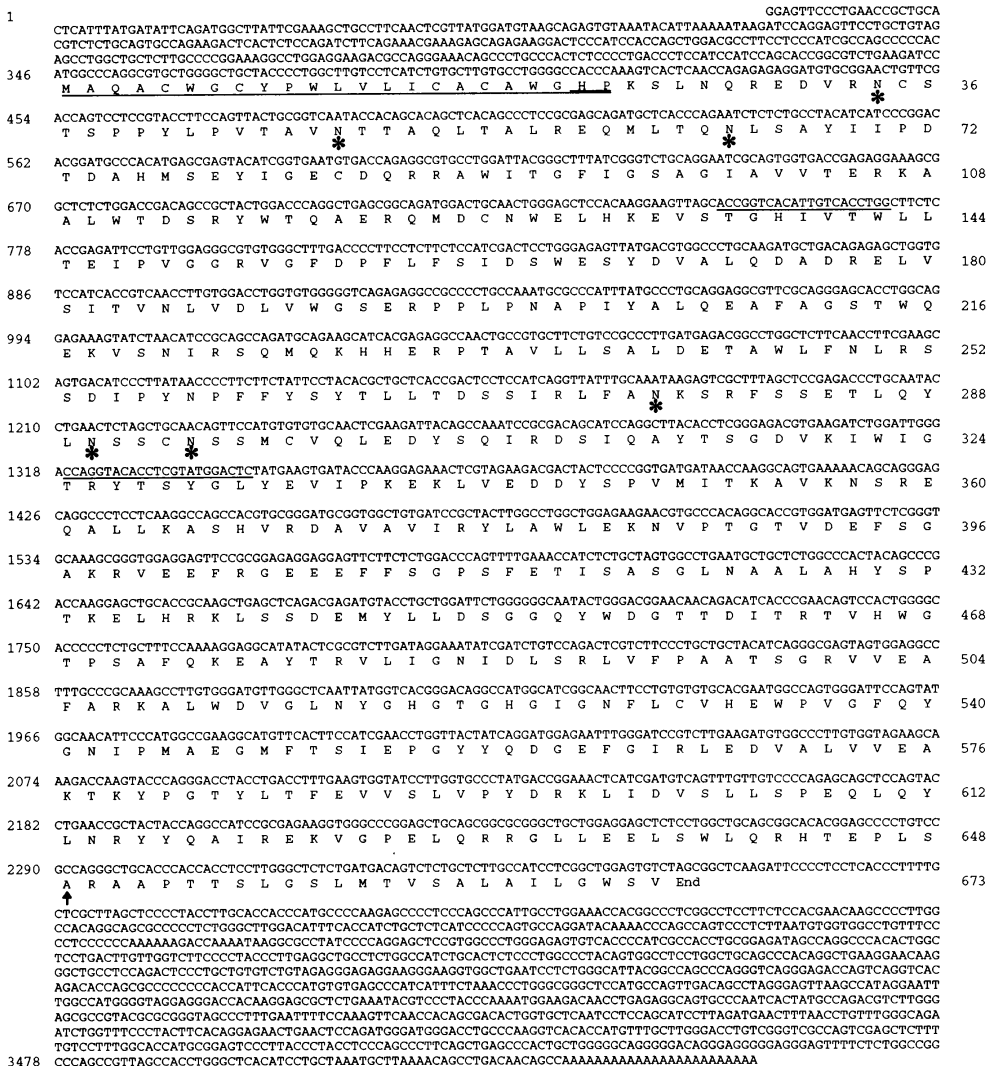

Figure 1 Nucleotide and deduced amino acid sequence of pig kidney AP-P

The nucleotide sequence of clone 102231A isolated from a pig kidney cDNA library, and the deduced amino acid sequence for the encoded AP-P, are provided. The amino acid sequence is numbered from the deduced N-terminal methionine residue. The N-terminal signal sequence is underlined. Weight-matrix predictions [31] identify two possible cleavage points for the signal sequence, either at Gly^{21} or at Pro^{23} (double underlined). The underlined nucleotide sequences represent the two specific PCR primers (FS1/RS1) used in the selection of the enriched cDNA sub-libraries. The predicted attachment point of the GPI anchor (Ala^{649}) is indicated by an arrow. The six possible N-linked glycosylation sites are indicated by asterisks.

Table 1 Effects of apstatin and EDTA on AP-P activity expressed in COS-1 cells

Solubilized cell membranes from COS-1 cells transfected with pBK-APP were assayed as described in the Experimental section. Results from incubations with inhibitors are expressed as the means of duplicate readings which differed by $< 7\%$. Values in parentheses represent percentage activity relative to control in the absence of inhibitor. In the absence of any inhibitors, approx. 20% breakdown of substrate was observed.

	Specific activity	
Inhibitor	Bradykinin [nmol of bradykinin-(2–9)/min per mg]	Gly-Pro-Hyp (nmol of Pro-Hyp/min per mg)
None	4.06 ± 0.02	7.99 ± 0.09
Apstatin (0.26 mM)	2.72 (67%)	0
EDTA (1 mM)	2.13 (53%)	0

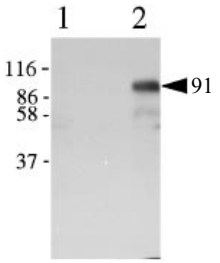

Figure 2 Western blot analysis of pig kidney AP-P expressed in COS-1 cells

Samples were prepared and analysed as described in the Experimental section. Lane 1, solubilized membranes from COS-1 cells transfected with pBKCMV vector alone (5 μg of protein); lane 2, solubilized membranes from COS-1 cells transfected with pBK-APP (5 μg of protein). The positions of M_r markers (left) and of AP-P (arrowhead) (all $10^{-3} \times M_r$) are indicated.

to obtain N-terminal sequence data for purified pig kidney AP-P have been unsuccessful due to blockage of the N-terminus [5,22]. The amino acid sequence in Figure 1 also differs from that in [19] at one additional position: Cys^{530} in Figure 1 was reported as a glutamine residue in [19].

At the C-terminus there is a stretch of mainly hydrophobic amino acids, which is typical of a signal sequence for attachment of a GPI anchor. Based on the cleavage prediction criteria established by Udenfriend and Kodukula [32], Ala^{649} is predicted as the ω-residue to which the preformed GPI moiety is attached, with Arg and Ala in the $\omega+1$ and $\omega+2$ positions. This prediction is consistent with the determination of Ala^{649} as the C-terminus of mature AP-P [19]. There are six putative N-linked glycosylation sites in the AP-P sequence, which are all located in the N-terminal half of the protein. Pig kidney AP-P has sequence similarities with *E. coli* and *Streptomyces lividans* AP-Ps [33,34] (44.8% and 50.3% similarity respectively using the GAP program [35]), especially in the C-terminal half of the protein, as well as with other members of the proline peptidase family [17,18], as previously noted [19].

Expression of AP-P cDNA in COS-1 cells

Transient expression of the pig kidney AP-P cDNA in COS-1 cells using the expression vector pBKCMV produced enzyme activity consistent with the properties of AP-P purified from pig kidney brush-border membranes [5,15]. The first indication that the correct cDNA had been expressed was provided by the observation that intact transfected COS-1 cells could hydrolyse Gly-Pro-Hyp at the Gly–Pro bond and that this activity was activated by 4 mM Mn^{2+} and inhibited by enalaprilat (results not shown). Subsequent experiments were performed with a solubilized membrane preparation from transfected cells which was able to hydrolyse both bradykinin and Gly-Pro-Hyp appropriately (Table 1). With Gly-Pro-Hyp as substrate, activity was stimulated approx. 11-fold in the presence of 4 mM Mn^{2+}, similar to the purified enzyme [15]. In the case of bradykinin, HPLC analysis confirmed that hydrolysis occurred exclusively at the Arg^1–Pro^2 bond (results not shown). No significant hydrolysis of Gly-Pro-Hyp or bradykinin was detected in either intact cells or membrane preparations from cells transfected with the control plasmid (pBKCMV). Hydrolysis of bradykinin and Gly-Pro-Hyp was inhibited by apstatin and EDTA (Table 1), although the inhibition of bradykinin hydrolysis was only partial in both cases. This is consistent with previous reports showing marked differences in the sensitivity of bradykinin and Gly-Pro-Hyp hydrolysis by purified AP-P to various inhibitors [15]. The present work establishes that these differences are an intrinsic property of the expressed enzyme rather than being due to the presence of a contaminating bradykinin-hydrolysing activity in the previously purified preparations of AP-P.

Phase separation in Triton X-114 of membrane preparations from transfected cells showed that the AP-P activity partitioned exclusively ($99.2 \pm 0.7\%$) into the detergent-rich phase, indicating amphipathic character, consistent with its occurrence as an integral membrane protein. However, when membranes were preincubated with PI-PLC before phase separation, AP-P activity now partitioned virtually completely ($98.5 \pm 1.3\%$) into the detergent-depleted phase, indicating that cleavage of a GPI anchor had occurred, causing release of a hydrophilic form of the enzyme.

AP-P expressed in COS-1 cells was analysed by Western blotting with a polyclonal antibody raised to pig kidney AP-P. Under reducing conditions a single polypeptide of approx. M_r 91000 was detected, which corresponds to the size of purified AP-P (Figure 2). The antibody did not detect any equivalent band in membranes prepared from COS-1 cells transfected with the pBKCMV vector alone (Figure 2).

General conclusions

The predicted amino acid sequence encoded by the 2019 bp open reading frame of clone 102231A matched the amino acid sequence of mature AP-P deduced by Edman degradation and MS of the purified protein, with one exception [19]. In addition, hydrophobic signal sequences were detected at both the N- and C-termini of the protein, consistent with its occurrence as a GPI-anchored cell-surface ectoenzyme. The precise N-terminus could not be deduced with certainty but the previously reported N-terminus was clearly in error as a result of proteolytic degradation during purification [19]. Transient expression of the cDNA clone in COS-1 cells has confirmed that it encodes a fully functional AP-P protein. Biochemical analysis has shown that the expressed protein possesses the expected characteristics of substrate specificity, inhibitor profile and antigenic recognition. The present work represents the first example of the cloning of AP-P from a eukaryotic source. However, the sequence fails to reveal likely zinc-binding or catalytic residues, although chemical modification studies on the purified enzyme have indicated the presence of two critical histidine residues [21] whose location has yet to be

determined. We are now in a position to initiate site-directed mutagenesis studies to identify these and other residues involved in the action of this new class of proteolytic enzyme.

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