Identification of Critical Residues in the Active Site of Porcine Membrane-Bound Aminopeptidase P[†]

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ABSTRACT: The membrane-bound form of mammalian aminopeptidase P (AP-P; EC 3.4.11.9) is a mono-zinc-containing enzyme that lacks any of the typical metal binding motifs found in other zinc metalloproteases. To identify residues involved in metal binding and catalysis, sequence and structural information was used to align the sequence of porcine membrane-bound AP-P with other members of the peptidase clan MG, including *Escherichia coli* AP-P and methionyl aminopeptidases. Residues predicted to be critical for activity were mutated and the resultant proteins were expressed in COS-1 cells. Immunoelectrophoretic blot analysis was used to compare the levels of expression of the mutant proteins, and their ability to hydrolyze bradykinin and Gly-Pro-hydroxyPro was assessed. Asp449, Asp460, His523, Glu554, and Glu568 are predicted to serve as metal ion ligands in the active site, and mutagenesis of these residues resulted in fully glycosylated proteins that were catalytically inactive. Mutation of His429 and His532 also resulted in catalytically inactive proteins, and these residues, by analogy with *E. coli* AP-P, are likely to play a role in shuttling protons during catalysis. These studies indicate that mammalian membrane-bound AP-P has an active-site configuration similar to that of other members of the peptidase clan MG, which is compatible with either a dual metal ion model or a single metal ion in the active site. The latter model is consistent, however, with the known metal stoichiometry of both the membrane-bound and cytosolic forms of AP-P and with a recently proposed model for methionyl aminopeptidase.

The metabolism of the circulating peptide bradykinin is due to the activities of the two zinc metallopeptidases angiotensin-converting enzyme (ACE; EC 3.4.15.1) and aminopeptidase P (AP-P; X−Pro aminopeptidase, EC 3.4.11.9) (*1*). Since bradykinin exhibits potent vasodilatory and cardioprotective effects, there may be a therapeutic benefit to inhibiting both of these enzymes. Inhibitors of ACE are widely used clinically in the treatment of hypertension and other cardiovascular disorders (*2*, *3*), and more recently apstatin, a selective inhibitor of AP-P, has been shown to block the cleavage of the Arg^{1}−Pro^{2} bond of bradykinin in the rat pulmonary and coronary circulations (*4*, *5*).

AP-P exists in two forms: a membrane-bound form encoded by a gene on the human X chromosome (*6*) and a cytosolic form encoded by a gene on human chromosome 10 (*7*). The membrane-bound form, which is located as an ectoenzyme on the plasma membrane of endothelial and epithelial cells, is the form presumed to be responsible for the extracellular degradation of bradykinin. This form of AP-P was first purified to homogeneity from porcine kidney following cleavage of its glycosylphosphatidylinositol (GPI) anchor by bacterial phospholipase C (*8*). Subsequently the protein was purified from other sources (*9*, *10*). Initial studies on the enzyme revealed that it was a metallopeptidase (*11*), and this was confirmed following its isolation. The activity of AP-P toward the synthetic substrate Gly-Pro-hydroxyPro was inhibited by the chelating agents EDTA and 1,10-phenanthroline (*8*), and the purified porcine kidney enzyme was found to contain zinc (*12*). However, the complete chemical sequencing of the protein (*13*) and isolation of its cDNA (*14*) revealed the absence of any of the typical zinc binding motifs found in other zinc metallopeptidases (e.g., His-Glu-Xaa-Xaa-His or His-Xaa-Xaa-Glu-His) (*15*).

AP-P is now classified as a member of the peptidase clan MG (*16*) on the basis of sequence and structural similarities with other exopeptidases, which include the methionyl aminopeptidases type I and II (EC 3.4.11.18), and the X−Pro dipeptidases (prolidases; EC 3.4.13.9). The overall sequence identity between these proteins is quite low. However, following the structural determination of methionyl aminopeptidase from *Escherichia coli* (*17*), these peptidases were predicted to have a similar C-terminal "pita-bread" fold (*18*)

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[1] Abbreviations: ACE, angiotensin-converting enzyme; AP-P, aminopeptidase P; ECL, enhanced chemiluminescence; GPI, glycosylphosphatidylinositol; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

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first observed in the non-metal-dependent creatinase from *Pseudomonas putida* (*19*). The proteins are predicted to be di-metal-containing enzymes on the basis of the observations that there were two Co^{2+} ions in the crystal structure of *E. coli* methionyl aminopeptidase (*17*) and two Mn^{2+} ions in *E. coli* AP-P (*20*). However, recent data suggest that only one metal ion is required for catalytic activity in these enzymes (*21*, *22*).

Using sequence and structural information, we have aligned the sequences of mammalian AP-P with the methionyl aminopeptidases and other AP-Ps in order to identify amino acid residues involved either in coordinating the active-site metal ion(s) or in catalysis. The predicted residues have been mutated and the enzymic properties of the expressed proteins have been determined. These studies reveal that, like the other members of clan MG, mammalian membrane-bound AP-P has a similar active-site configuration that is compatible with either a single bound metal ion or a dual metal ion model.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from New England Biolabs (Hitchin, U.K.) The mammalian expression vector pBKCMV and *Pfu* DNA polymerase were from Stratagene (Cambridge, U.K.). The enhanced chemiluminescence (ECL) Western blotting kit was from Amersham International plc (Amersham, U.K.). Tissue-culture medium, serum, trypsin−EDTA, penicillin/streptomycin, OptiMEM, phosphate-buffered saline (PBS), the transfection reagent (lipofectAMINE), T4 DNA ligase, and oligonucleotide primers were purchased from Gibco−BRL, Life Technologies Ltd. (Paisley, U.K.).

Site-Directed Mutagenesis. Mutations were introduced into AP-P in the mammalian expression vector pBKCMV according to the method of Landt (*23*) or by use of the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's guidelines. The mutants made were as follows: H429K/L, D449A/N, D460A/N, H519K/L, H523K/L, H532K/L, E554A/Q, E568A/Q, and E588A/Q. In each case a DNA fragment containing the relevant mutation was subcloned into wild-type AP-P. The subcloned region was then sequenced on both strands by use of an ABI 371 automated DNA sequencer.

Transient Expression of Porcine AP-P in COS-1 Cells. Transient expression was performed as previously described (*24*). LipofectAMINE-mediated transfection of COS-1 cells with plasmid DNA was performed, and the cells were then harvested 48 h posttransfection. The COS-1 cells were then washed three times with 5 mL of PBS at room temperature and scraped into 2 mL of ice-cold 50 mM HEPES/NaOH, pH 7.4. The cells were lysed by repeated freeze/thawing.

SDS−PAGE and Immunoelectrophoretic Blot Analysis. Whole-cell lysates (10 μg) were analyzed by SDS−PAGE on a 7−17% polyacrylamide gradient gel as described previously (*25*). Immunoelectrophoretic (Western) blot analysis (*26*) was carried out with Immobilon P poly(vinylidene) difluoride membranes, and the detection of protein was performed by using the 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 (*27*). Molecular weight standards were electrophoresed in parallel and visualized by Coomassie Blue staining.

Enzyme Assays. The hydrolysis of bradykinin (0.1 mM) and Gly-Pro-hydroxyPro (1 mM) by cell lysates (10 μg) was determined by reverse-phase HPLC as described previously (*27*, *28*). Incubations were performed in triplicate from triplicate transfections for each set of conditions. For kinetic analysis of wild type and the H519 mutants, the amount of AP-P present in the lysates was determined by densitometric analysis of Western blots.

Protein Determination. Protein concentrations were determined by the bicinchoninic acid assay (*29*), with bovine serum albumin as the standard. The method was adapted for use in 96-well microtiter plates (*30*).

Immunocytochemistry. COS-1 cells were plated in 24-well plates containing sterile cover slips and transfected with wild-type or mutant AP-P cDNAs. Cells were prepared for immunofluorescence 48 h posttransfection as described previously (*24*). The cells were washed three times at 37 °C with warmed PBS and fixed at room temperature in 4% paraformaldehyde for 20 min. For permeabilization of the cells, a subsequent incubation step of 2 min in 0.2% Triton X-100 was employed. Following fixation, the cells were washed three times in Tris-buffered saline (TBS) and blocked for 30 min at room temperature in the same buffer containing 0.2% gelatin and 1% normal goat serum. Incubation with a primary affinity-purified anti-porcine AP-P antibody (1:50 dilution) (*27*) was performed for 2 h at room temperature. The cells were washed three times in TBS before incubation with the secondary goat anti-rabbit IgG conjugated with fluoroscein isothiocyanate (1:100 dilution) for 30 min. After the cells were washed three times with TBS, the cover slips were mounted on microscope slides in Vectashield (Vector Laboratories Inc., Peterborough, U.K.) and viewed with a Leitz confocal microscope.

RESULTS

Active-Site Modeling. By use of CLUSTALW (*31*), an initial alignment was produced of the amino acid sequences of the catalytic domains of AP-Ps from pig (membrane-bound), human (both membrane-bound and cytosolic) and *E. coli*, as well as methionyl aminopeptidases from *E. coli* and human. Examination of the crystal structures of *E. coli* AP-P (PDB code 1AZ9) (*20*) and methionyl aminopeptidase (PDB code 1C21) (*17*) allowed the identification of regions of structure, which superimpose closely (rms fit of all main-chain atoms of 1.3 Å). These regions include not only the main strands of the β-sheet and the two pairs of α-helices and their connecting loops but also two other loop regions (242−245 and 352−362 in *E. coli* AP-P; 78−81 and 169−179 in *E. coli* methionyl aminopeptidase). The strong structural similarity in these regions was used to improve manually the sequence alignment, by using the program XVISTAS. This alignment is shown in Figure 1.

Although the regions of similar structure are connected by loops of different lengths and structures in *E. coli* AP-P and methionyl aminopeptidase, the similarity around the active site was sufficiently clear to allow a three-dimensional model of the active site of porcine AP-P to be constructed (Figure 2). From the alignment, the metal-coordinating ligands of porcine AP-P are predicted to be Asp449, Asp460,

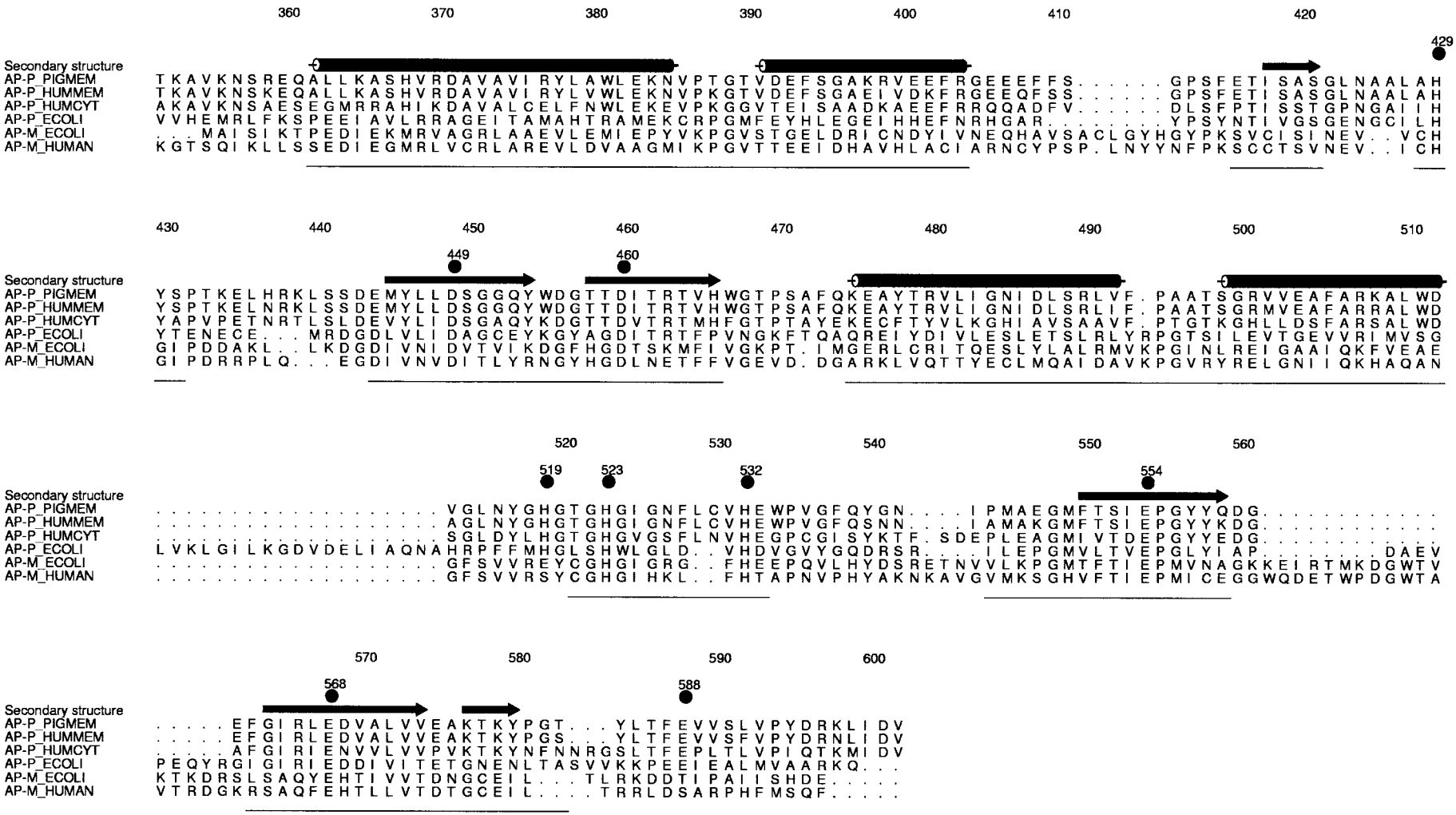

FIGURE 1: Sequence alignment of AP-P and methionyl aminopeptidase from human, pig, and *E. coli*. Substantial regions of the solved *E. coli* structures for AP-P and methionyl aminopeptidase agree closely and form a structural core that is decorated with turns and loops of different lengths. Conservation of this core region was used to aid the alignment of the two sequences, which in turn provided a basis for the inclusion of the other sequences. Core regions are underlined below the sequence. Cylinders above the sequence represent α-helices, and arrows represent β-sheets. The residues highlighted by • above the sequence were mutated in this study (H429, D449, D460, H519, H523, H532, E554, E568, and E588). The numbering refers to the sequence of porcine AP-P. The sequences included in the alignment, their GenBank accession numbers, and amino acid numbering are as follows: AP-P_PIGMEM, membrane-bound porcine AP-P (SSU55039, residues 352−602); AP-P_HUMMEM, membrane-bound human AP-P (HSU90724, residues 353−603); AP-P_HUMCYT, cytosolic human AP-P (AF272981, residues 318−574); AP-P_ECOLI; *E. coli* AP-P (AMPP_ECOLI, P15034, residues 168−440); AP-M_ECOLI; *E. coli* methionyl aminopeptidase (AMPM_ECOLI, P07906, residues 1−264); AP-M_HUMAN; human type I methionyl aminopeptidase (AMP1_HUMAN, P53582, residues 131−394). The figure was prepared with ALSCRIPT (*39*).

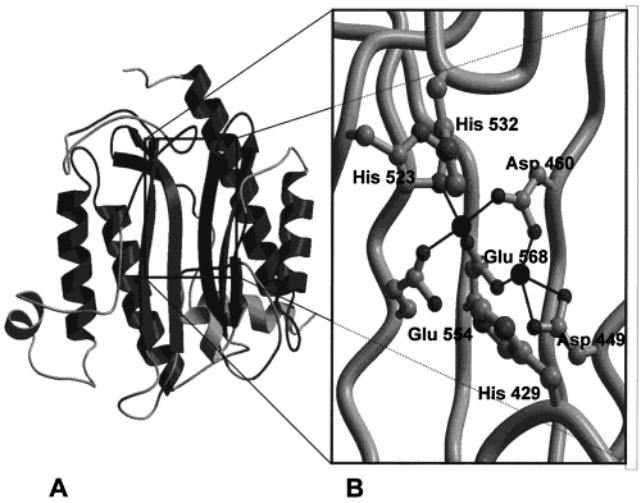

FIGURE 2: Expanded view of a model of the active site of porcine AP-P. (A) Ribbon representation of the pita-bread fold of the aminopeptidase catalytic domain. (B) Model of the structure of the active site of porcine APP, based on the crystal structure of *E. coli* APP (PDB code 1AZ9). The amino acid side chains shown to be critical for enzyme function are shown in ball-and-stick representation, and metal−ligand interactions are indicated. Both parts were produced with the programs MOLSCRIPT (*40*) and RASTER3D (*41*).

His523, Glu554, and Glu568. Two further histidines, His429 and His532, are fully conserved in the alignment and are predicted to lie close to the active site. These residues align with His243 and His361 of *E. coli* AP-P, which are proposed to play a role in shuttling protons from the dinuclear metal center to solvent during catalysis (*20*). The antiangiogenesis agent fumagillin inhibits methionyl aminopeptidases by covalently bonding to a conserved histidine residue. This residue was identified as His231 in the human enzyme (*32*) and is equivalent to His429 in porcine AP-P.

Characterization of Histidine Mutants of Porcine AP-P Expressed in COS-1 Cells. The three conserved histidine residues in the active-site region of porcine AP-P (His429, His523, and His532; see Figures 1 and 2) were each mutated to either lysine or leucine. In addition, a fourth histidine residue in the active-site domain (His519), which is conserved between the mammalian and bacterial AP-Ps but not in the methionyl aminopeptidases, was also mutated. After the mutations were verified by sequencing, the wild-type and mutant cDNAs were expressed in COS-1 cells. The expression level and molecular mass of each of the expressed proteins were assessed by SDS−PAGE followed by immunoblotting with a polyclonal antibody raised against porcine AP-P (Figure 3A). The wild-type porcine AP-P migrated as a single polypeptide of approximately 91 kDa, as previously observed (*14*), which was not observed in mock-transfected cells. The H429K/L, H519K/L, H523K/L, and the H532K mutants were all recognized by the AP-P antibody and the expressed proteins were of a similar M_r to the *N*-glycosylated wild-type protein.

These mutants were all seen to be expressed at the surface of the COS-1 cells with a similar punctate staining and fluorescence intensity as the wild-type AP-P (Figure 4). The H532L mutant showed no fluorescent staining at the cell surface and was expressed at much lower levels and of a lower M_r, indicating instability and degradation of the protein (data not shown). The H532L mutant was therefore not

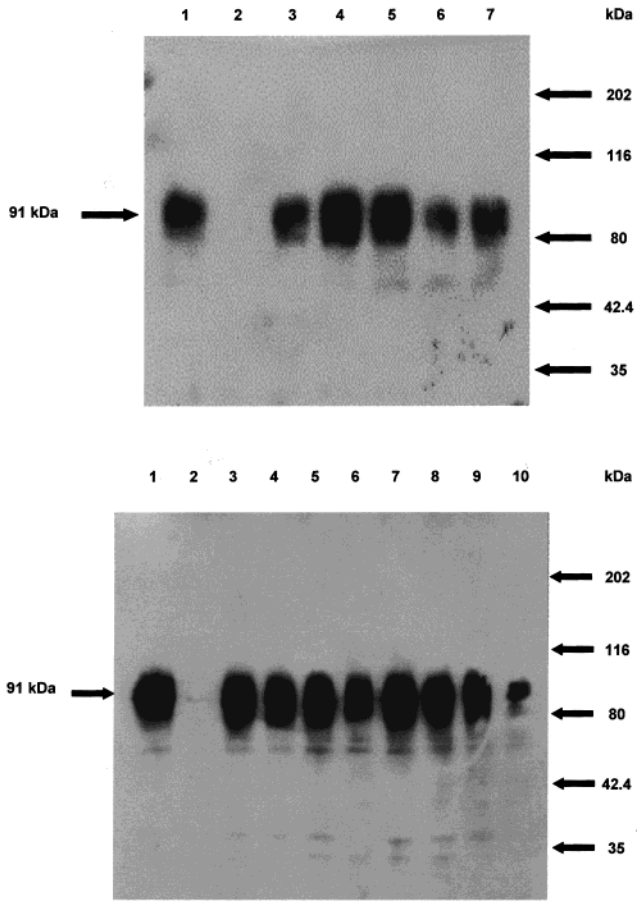

FIGURE 3: Immunoelectrophoretic blot analysis of wild type and mutants of porcine AP-P expressed in COS-1 cells. COS-1 cells were transiently transfected with wild-type AP-P, mutant AP-P, or the control vector pBK−CMV. Whole-cell lysates (10 μg) were subjected to SDS−PAGE and immunoelectrophoretic blot analysis as described under Materials and Methods. (A) Histidine mutants: lane 1, COS-1 cells transfected with wild-type AP-P; lane 2, COS-1 cells transfected with empty vector; lane 3, H429K; lane 4, H429L; lane 5, H523K; lane 6, H523L; lane 7, H532K. (B) Acidic mutants: lane 1, COS-1 cells transfected with wild-type AP-P; lane 2, COS-1 cells transfected with empty vector; lane 3, D449A; lane 4, D449N; lane 5, D460A; lane 6, D460N; lane 7, E554A; lane 8, E554Q; lane 9, E568A; lane 10, E568Q.

investigated further. The specific activity of the expressed wild-type AP-P with bradykinin as substrate was 37.5 nmol min^{-1} mg^{-1}. When the mutant proteins were assayed for AP-P activity, with bradykinin or Gly-Pro-hydroxyPro as substrate, no detectable activity was observed with any of the His429, His523, or His532 mutants. In contrast, the H519K and H519L mutants revealed partial activity, 2.3% $\pm$ 0.6% and 73.8% $\pm$ 4.7% the activity of wild-type AP-P, respectively. A comparison of the kinetic parameters of the H519K and H519L mutants with those of wild-type AP-P (Table 1) indicated that both mutants had a significantly higher K_m than the wild type and that H519K but not H519L had a dramatically lower k_{cat} than wild-type AP-P. Transfections were carried out three times and similar results were seen in all cases.

Characterization of Acidic Mutants of Porcine AP-P Expressed in COS-1 Cells. There are four conserved acidic residues in the active-site region of porcine AP-P: Asp449, Asp460, Glu554, and Glu568 (see Figures 1 and 2). The aspartate residues were mutated to Ala or Asn and the

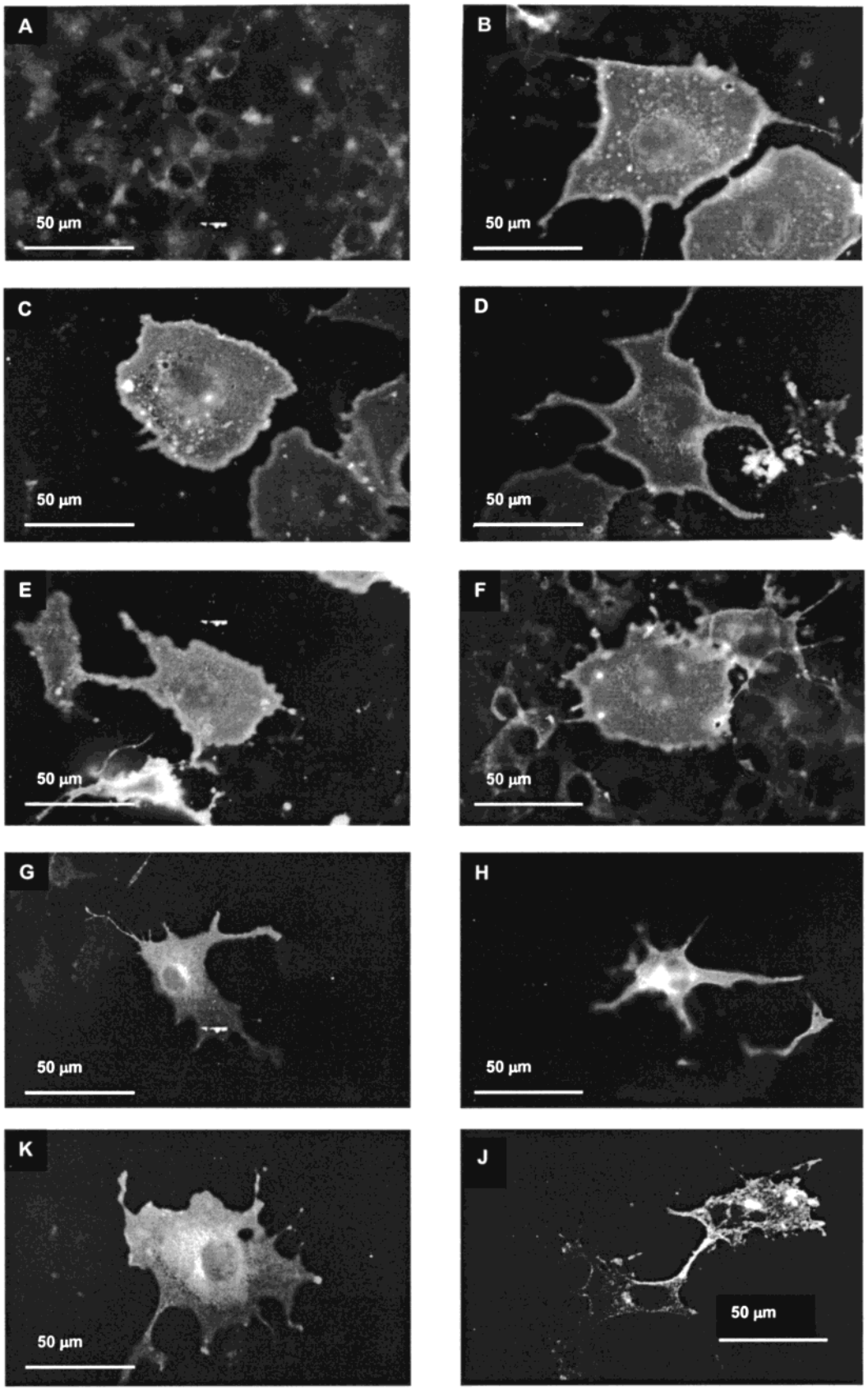

FIGURE 4: Immunofluorescence of wild-type and mutant AP-P using confocal microscopy. Panel A, mock-transfected COS-1 cells; panel B, COS-1 cells transfected with wild-type AP-P; panel C, H429K; panel D, H519K; panel E, H523L; panel F, H532K; panel G, D449A; panel H, D460N; panel I, E554Q; panel J, E568A.

glutamate residues to Ala or Gln. After the mutations were verified by sequencing, the wild-type and mutant cDNAs were expressed in COS-1 cells. The expression level and molecular mass of each of the expressed proteins was assessed by SDS−PAGE, followed by immunoblotting with a polyclonal antibody raised against porcine AP-P (Figure

Table 1: Kinetic Analysis of the Wild Type and His519 Mutants of AP-P[a]

	wild type	H519K	H519L
K_m (μM)	58 ± 4	500 ± 100	391 ± 60
k_{cat} (s^{-1})	1.18 ± 0.05	0.17 ± 0.03	1.82 ± 0.34
k_{cat}/K_m ($\times 10^3$ M^{-1})	20.3 ± 3.3	0.34 ± 0.12	4.65 ± 0.6

[a] Wild-type AP-P and the His519 mutants were incubated with bradykinin (0.01−0.5 mM). Whole-cell lysates (10 μL) were incubated for 20 min at 37 °C before termination. Product and substrate were separated and quantified by HPLC as described under Materials and Methods. The quantity of AP-P present in the lysates was determined by densitometric analysis of Western blots. The results are the mean (± SEM) of triplicate assays from triplicate transfections.

3B). All of the mutants were successfully expressed at the cell surface (Figure 4), although the E568Q mutant was routinely expressed at much lower levels than the other mutants. None of these mutants showed any detectable activity against bradykinin or Gly-Pro-hydroxyPro (<1% of wild type). In contrast, Glu588, which is not conserved across all members of clan MG, when mutated to A or Q retained 66% and 83% the activity of wild type, respectively, with bradykinin as substrate.

DISCUSSION

Prior to the complete sequencing of mammalian AP-P, a limited partial sequence of guinea pig AP-P (*33*) had suggested that mammalian AP-P may be related to members of the so-called "proline peptidase" family. An earlier study (*18*), which involved sequence comparisons of *E. coli* AP-P and methionyl aminopeptidase together with prolidase, creatinase, and agropine synthase, suggested these proteins may share a common structural feature referred to as the pita-bread fold. This structural scaffold has evolved to allow a diverse set of enzyme activities, of which a subset is the metal-dependent peptidases of the clan MG (*16*). Others, such as creatinase and agropine synthase, neither are peptidases nor do they show metal ion dependence. The elucidation of the complete protein sequence (*13*) and cDNA cloning (*14*) of the porcine membrane-bound enzyme, and more recently the human membrane-bound enzyme (*34*), has allowed the unequivocal assignment of mammalian AP-P to clan MG. The sequence similarities between mammalian AP-P and the other members are restricted to the C-terminal half of the protein, where the active site is predicted to be located. The more recent cloning of mammalian cytosolic AP-P (*22*, *35*) also allows its assignment to this peptidase clan.

The structural solution of *E. coli* methionyl aminopeptidase (*17*) revealed a two-domain structure in which two cobalt ions were sandwiched between two β-sheets surrounded by four α-helices, which together comprise the pita-bread fold. The overall structure has an approximate local pseudo-2-fold symmetry. The two metal ions of methionyl aminopeptidase are liganded, in sequence order, by two aspartic acids, a histidine, and two glutamic acid residues. These residues are absolutely conserved in all type I and type II methionyl aminopeptidases from prokaryotes and eukaryotes. The structure of *E. coli* AP-P has been solved at 2.0 Å, revealing an active site in the C-terminal domain containing a dinuclear manganese center in which a bridging water molecule or hydroxide ion acts as the nucleophile attacking the scissile peptide bond (*20*). The residues involved in metal ion binding are two aspartic acids, a histidine, and two glutamic acids, and they correspond to equivalent residues of methionyl aminopeptidase on sequence alignment (see Figure 1).

The model of the active site of porcine membrane-bound AP-P (see Figure 2) conserves all the known features of other members of this peptidase clan and predicts two aspartic acids (Asp449 and Asp460), a histidine (His523), and two glutamates (Glu554 and Glu568) as critical active-site residues. Their requirement for enzyme activity has been confirmed by site-directed mutagenesis, each of the mutants being fully expressed in a glycosylated form but in a catalytically inactive state. AP-P in which a histidine (His519) and a glutamate (Glu588) in the active-site region, which are not conserved among all the other clan members, had been mutated remained catalytically active.

Although the structural data have strongly implicated two metal ions in enzyme activity, cobalt in methionyl aminopeptidase (*17*) and manganese in *E. coli* AP-P (*20*), more recent studies have questioned both the number and the nature of the metal ions involved. *E. coli* methionyl aminopeptidase has been shown to function as an Fe^{2+} enzyme and, indeed, the likely metal ion involved in vivo is Fe^{2+} (*36*). By similar criteria, Zn^{2+}, rather than Co^{2+}, has been implicated as the natural metal ion in yeast methionyl aminopeptidase, type I (*37*). A more detailed study of the metal binding properties of *E. coli* methionyl aminopeptidase has shown that maximal catalytic activity is observed with the addition of only 1 equiv of divalent metal ion, either Co^{2+} or Zn^{2+}, suggesting that it functions as a mononuclear enzyme in vivo (*21*). The position in the active site occupied by the second metal ion is proposed to be occupied by the primary amino group at the N-terminus of the peptide substrate. These data would not be inconsistent with our previous observation that membrane-bound AP-P purified to homogeneity from pig kidney contains 1 equiv of Zn^{2+} and no detectable Co^{2+} (*12*, *27*) and that the cytosolic form of human AP-P functions as a single manganese(II)-dependent enzyme (*22*). The results of this mutagenesis study would also be consistent with a one-metal-containing active site in which residues Asp460, His523, Glu554, and Glu568 are coordinating the single metal ion, and residue Asp449 and the second carboxylate oxygens on Asp460 and Glu568 are involved in binding the amino terminus of the substrate.

Mutation of His429 to leucine and lysine and His532 to lysine in porcine AP-P also led to a complete loss of catalytic activity. This was not due to decreased expression or incomplete trafficking, as the proteins were expressed at the cell surface in comparable amounts to wild-type AP-P. This would be consistent from the sequence alignment (Figure 1) and model (Figure 2) with these residues corresponding to His243 and His361, respectively, of *E. coli* AP-P, which are proposed to play a role in shuttling protons from the metal center to solvent during catalysis (*20*). Fumagillin, an antiangiogenesis agent, forms a covalent bond with a histidine residue in *E. coli* (*38*) and human (*32*) methionyl aminopeptidases, inhibiting activity. The model of porcine AP-P indicates that this residue (His429) is conserved. However, preincubation of porcine AP-P with 10−100 μM fumagillin for 30 min failed to inhibit hydrolysis of bradykinin by the enzyme (data not shown). This difference in sensitivity of methionyl aminopeptidases and AP-P to inhibition by fumagillin probably reflects the different

substrate specificities of the two enzymes and/or differences in other residues surrounding the active site of each protein.

In conclusion, our data for mammalian AP-P confirm the essential nature of three histidine and four acidic residues for enzyme activity as observed in *E. coli* methionyl aminopeptidase. The modeling and mutagenesis data are consistent with the membrane-bound enzyme functioning as a mononuclear enzyme as has recently been proposed for cytosolic AP-P (*22*) and for *E. coli* methionyl aminopeptidase (*21*).

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