Proteolytic Fragmentation Reveals the Oligomeric and Domain Structure of Porcine Aminopeptidase A[†,‡]

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ABSTRACT: Aminopeptidase A (glutamyl aminopeptidase; EC 3.4.11.7) has been cloned from porcine brain and kidney cortex cDNA libraries and the complete primary sequence of the enzyme deduced. This predicts a type II integral membrane protein of 942 amino acids with 14 potential N-linked glycosylation sites and a His-Glu-Xaa-Xaa-His zinc binding motif. Aminopeptidase A was purified from porcine kidney cortex by a combination of anion exchange and hydrophobic interaction chromatographies following its release from the membrane by trypsin. The purified protein migrated as three major polypeptides on SDS−polyacrylamide gel electrophoresis of M_r 147 000, 107 000, and 45 000. N-Terminal sequencing revealed that both the M_r 147 000 and 107 000 polypeptides had the same N-terminal sequence resulting from cleavage of aminopeptidase A by trypsin at the Lys-42−Asp-43 bond just outside the membrane-spanning hydrophobic region. Immunoelectrophoretic blot analysis following electrophoresis under nonreducing conditions revealed that the trypsin-cleaved form of the enzyme no longer migrated as a disulfide-linked dimer, placing the interchain disulfide link N-terminal to Lys-42. N-Terminal sequencing of the M_r 45 000 polypeptide in the purified preparation of aminopeptidase A revealed that it resulted from cleavage at the Asn-602−Gly-603 bond by an endogenous protease. This posttranslational proteolytic cleavage occurred in porcine kidney cortex microvillar membranes but not in porcine intestinal microvillar membranes. Incubation of purified porcine kidney aminopeptidase N (membrane alanyl aminopeptidase; EC 3.4.11.2) with trypsin resulted in a similar fragmentation pattern to that observed in aminopeptidase A, suggesting that these and other members of the type II membrane-spanning zinc aminopeptidase family may have two distinct domains: an N-terminal domain, containing the zinc binding site and residues identified as being involved in catalysis, and a C-terminal domain of unknown function, that are separated by a protease-susceptible region.

Mammalian zinc aminopeptidases have a variety of functions, being involved in protein maturation, the activation, modulation, and degradation of bioactive peptides, and the scavenging of dietary peptides for nutritional purposes. In addition, some aminopeptidases are also involved in cell differentiation, leukemic transformation, cell adhesion, signal transduction, and viral cell entry [reviewed in Taylor (1993), Wilk and Healy (1993), and Wang and Cooper (1996)]. Aminopeptidase A (glutamyl aminopeptidase, AP-A, EC 3.4.11.7)[1] is one such enzyme that catalyzes the removal of acidic residues from the N-terminus of oligopeptide substrates, and as such has been implicated in the *in vivo* metabolism of angiotensin II to angiotensin III (Ahmad & Ward, 1990; Zini *et al*., 1996) and of cholecystokinin-8 (Migaud *et al*., 1996). The enzyme may also be involved in the metabolism of the artificial sweetener aspartame (Hooper *et al*., 1994). AP-A is a widely distributed enzyme being found primarily on the microvillar membrane of kidney and intestinal epithelial cells, as well as on the vascular endothelium of many organs (Li *et al*., 1993b; Wang & Cooper, 1996). AP-A purified from a number of species is an extensively glycosylated protein composed of two M_r 140 000−160 000 disulfide-linked subunits.

Molecular cloning of the murine BP-1/6C3 antigen predicted that it was a type II integral membrane protein containing 965 amino acids and possessing a His-Glu-Xaa-Xaa-His zinc binding motif (Wu *et al*., 1990). Subsequent studies revealed that BP-1 molecules immunoprecipitated from murine pre-B cells exhibited AP-A activity that was inhibited by amastatin (Wu *et al*., 1991), and that recombinant mouse and human BP-1 possessed AP-A activity (Wang & Cooper, 1993; Li *et al*., 1993a). In addition, murine BP-1 has an overall sequence identity of 34% with rat aminopeptidase N (membrane alanyl aminopeptidase; AP-N; EC 3.4.11.2) (Wu *et al*., 1990). It is now clear that AP-A and AP-N are just two members of a larger family of type II membrane-spanning zinc aminopeptidases that share significant sequence homology, especially in regions containing the zinc ligands (Hooper, 1994). Other mammalian members of this family include thyrotropin-releasing hormone degrading enzyme (EC 3.4.19.6) (Schauder *et al*., 1994), the GluT4 vesicle aminopeptidase (Keller *et al*., 1995), and placental leucine aminopeptidase/oxytocinase (Rogi *et al*., 1996). Site-directed mutagenesis and expression have shown that the

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[‡] The nucleotide sequence data reported have been deposited in the GenBank Nucleotide Sequence Database under Accession Number U66371.

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[1] Abbreviations: AP-A, aminopeptidase A; AP-N, aminopeptidase N; DipF, diisopropyl fluorophosphate; E-64, [(*trans*-epoxysuccinyl)-leucylamido]-4-guanidinobutane.

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second His in the zincin motif (Wang & Cooper, 1993) and a downstream Glu (Vazeux *et al*., 1996), that is conserved between the members of the zinc aminopeptidase family (Hooper, 1994), are essential for the catalytic activity of AP-A.

In the present study, we have used oligodeoxynucleotides based on the murine BP-1/6C3 sequence to clone and sequence the corresponding porcine cDNA. For the first time we show from protein sequence information of AP-A purified from porcine kidney cortex that this cDNA encodes AP-A. In addition, proteolytic fragmentation of the purified enzyme by trypsin and an endogenous protease reveals the location of the interchain disulfide bond and the domain structure of AP-A, respectively. Proteolytic fragmentation of porcine kidney AP-N by trypsin indicates that it too has a similar domain structure, a feature that may be conserved in other members of the type II membrane-spanning zinc aminopeptidase family.

EXPERIMENTAL PROCEDURES

Materials. Trypsin, elastase, amastatin, diisopropyl fluorophosphate (DipF), phenylmethanesulfonyl fluoride, [(*trans*-epoxysuccinyl)leucylamido]-4-guanidinobutane (E-64), pepstatin, elastin-Congo red, Ala-*p*-nitroanilide, and αGlu-*p*-nitroanilide were from Sigma Chemical Co. (Poole, Dorset, U.K.). Microvillar membranes were prepared from porcine kidney cortex by the method of Booth and Kenny (1974) and from porcine intestine by the method of Kessler *et al*. (1978). Protein was determined using the bicinchoninic acid method (Smith *et al*., 1985) modified for use in 96-well microtiter plates (Hooper, 1993) with bovine serum albumin as standard. DE-52 (DEAE-cellulose) resin was from Whatman Ltd. (Maidstone, Kent, U.K.). Phenyl-Sepharose resin, alkyl-Superose and phenyl-Superose columns, concanavalin A−Sepharose resin, and T7 sequencing kit were from Pharmacia Biotech (St. Albans, U.K.). Hybond N+ hybridization membrane was from Amersham International plc (Little Chalfont, U.K.). Peptide-*N*-glycosidase F was purchased from Oxford GlycoSystems Ltd. (Abingdon, U.K.). Penicillin and Taq DNA polymerase were purchased from Gibco-BRL (Paisley, U.K.). [α-^{35}S]dATP and [α-^{32}P]-dCTP (1000 Ci/mmol) were from New England Nuclear (Stevenage, U.K.). Restriction enzymes, T4 DNA ligase, and Random Primed DNA labeling kit were from Boehringer Mannheim (Lewes, U.K.). AP-N was purified from porcine kidney cortex following solubilization with Triton X-100 as described previously (Bowes & Kenny, 1987).

Purification of Aminopeptidase A. All procedures were performed at 4 °C unless otherwise stated. Pig kidney cortex (200 g) was homogenized in 50 mM Tris/HCl, 0.33 M sucrose, pH 7.4, to yield a 10% (w/v) homogenate. This was centrifuged at 8000*g* for 15 min, and the supernatant was removed and further centrifuged at 26000*g* for 2 h. The pellet was resuspended in 200 mL of 20 mM Tris/HCl, pH 8.0, to produce a crude microsomal membrane preparation. Trypsin was added to the solution at a ratio of 1 mg of trypsin to 10 mg of protein and incubated for 1 h at 37 °C. Solubilization was stopped by addition of DipF to a final concentration of 0.1 mM. The suspension was centrifuged at 31000*g* for 90 min, and the supernatant was applied to a DE-52 column (2.0 cm × 10 cm), previously equilibrated with 20 mM Tris/HCl, pH 8.0. After the column was washed with 10 volumes of equilibration buffer, a 200 mL linear gradient of 0.0−0.5 M NaCl in 20 mM Tris/HCl, pH 8.0, was applied. Pooled fractions were dialyzed against 20 mM sodium phosphate, pH 7.0. $(NH_4)_2SO_4$ was added to a final concentration of 1.7 M and the sample applied to a phenyl-Sepharose column (2 cm × 12.5 cm). After the column was washed, a 300 mL linear gradient of 1.7 M to 0.0 M $(NH_4)_2SO_4$ in 20 mM sodium phosphate, pH 7.0, was applied. Pooled fractions were dialyzed against 20 mM sodium phosphate, pH 7.0. $(NH_4)_2SO_4$ was added to a final concentration of 1.7 M, and the sample was applied to a phenyl-Superose column (1 mL). After the column was washed, a 30 mL linear gradient of $(NH_4)_2SO_4$ as described for the phenyl-Sepharose column was applied to the column. Fractions containing AP-A activity were pooled and used as the source of purified enzyme.

Enzyme Assays. AP-A and AP-N were assayed with αGlu-*p*-nitroanilide and Ala-*p*-nitroanilide as substrate, respectively, and the released *p*-nitroaniline was quantified spectrophotometrically in 96-well plates (Hooper, 1993). The activity of elastase was checked with elastin-Congo red as substrate.

Protein Sequencing and Enzymic Deglycosylation. N-Terminal sequencing of purified AP-A was carried out by automated solid-phase Edman degradation (Hooper *et al*., 1990) using the microsequence facility at the Protein Sequencing Unit, Department of Biochemistry and Molecular Biology, University of Leeds. AP-A was deglycosylated with peptide-*N*-glycosidase F as described previously (Hooper & Turner, 1987).

cDNA Cloning and Sequencing. The amino acid sequences of human AP-A (Li *et al*., 1993a) and mouse BP-1/6C3 (Wu *et al*., 1990) were aligned using the GAP program (Needleman & Wunsch, 1970) and degenerate primers designed based on regions of high homology. The sequences of the sense primers were as follows: F1, 5′-TGYTTYGA-RTAYAARAARCARGA-3′ (human residues 160−167); F2, 5′-TTYGCITGYAARATGGGIGA-3′ (human residues 751−757). Those of the antisense primers were the following: R1, 5′-CATIGCRAARTAITCYTCRAARTA-3′ (human residues 339−332); R2, 5′-YTGHATCCARTTCCAIGCCAT-3′ (human residues 872−866), where H = A, G, or T; Y = C or T; R = A or G; and I = inosine. F1 and R1 were used in PCR to amplify a region of the porcine cDNA located toward the 5′ end of the coding region, and F2 and R2 were used in PCR to amplify a region of the porcine cDNA located toward the 3′ end of the coding region. Aliquots of a porcine brain cDNA library and of a porcine kidney cortex cDNA library were used as template in the PCR with F1/R1 primers and F2/R2 primers. Conditions for both PCR reactions were the following: initial 3 min at 93 °C followed by 35 cycles of 1 min at 93 °C, 1 min at 45 °C, 1 min at 72 °C, with an additional extension at 72 °C for 5 min in the last cycle. Products of the expected size for the F1/R1 and F2/R2 reactions were gel-purified, subcloned into the pCRII′TA′ cloning vector (Invitrogen), and sequenced by the dideoxynucleotide method with the T7 sequencing kit. Four aliquots, each containing 1×10^5 pfu, were generated by the reamplification of a porcine kidney cortex cDNA library constructed in λZAP (Stratagene). Similarly, another four aliquots, each containing 1×10^5 pfu, were also generated by the reamplification of a porcine brain cDNA library constructed in λgt11 (Clonetech). The four aliquots from

Table 1: Purification of Aminopeptidase A from Porcine Kidney[a]

	protein (mg)	total act. (μmol of pNA/min)	sp act. (μmol of pNA min^{-1} mg^{-1})	recovery (%)	enrichment (x-fold)
homogenate	22720	688.8	0.03	100	1
microsomal membranes	3454	266.0	0.08	39	3
trypsin-solubilized supernatant	699	142.8	0.20	21	7
after DEAE-cellulose	151	75.0	0.49	11	16
after phenyl-Sepharose	21.5	57.1	2.68	8	88
after phenyl-Superose	0.44	9.5	21.64	1	714

[a] AP-A was purified from 200 g of porcine kidney cortex as described under Experimental Procedures. Activity was assayed with αGlu-pNA as substrate.

both libraries were screened using the F1/R1 and F2/R2 primer combinations. A PCR product of the expected size for each reaction was amplified from one of the aliquots from both the brain and kidney libraries. Dilutions of the positive aliquots were made, plated out, and screened by plaque hybridization. The F1/R1 (540 bp) PCR product and F2/R2 (360bp) PCR product were gel-purified, labeled with [α-^{32}P]dCTP using the random primed labeling kit, and used in the plaque hybridization. Hybridization was carried out for 16 h at 55 °C in saline−sodium phosphate (10 mM sodium phosphate, pH 7.4, containing 0.15 M NaCl and 1 mM EDTA), 6% PEG 6000, 0.5% milk powder, 1% SDS, 0.1% $Na_4P_2O_7$, and 250 μg of single-stranded salmon sperm DNA per 40 mL solution. Final wash conditions were 10-fold-diluted saline−sodium phosphate−EDTA containing 0.1% SDS for 30 min at 60 °C. Autoradiography was carried out for 48 h at −70 °C using preflashed film. Positive plaques were selected and subjected to a further screen to check the purity of the chosen plaques. Positive phagemids were rescued as described in the λZAP-cDNA synthesis kit (Stratagene), and both strands of each clone were sequenced. Inserts from positive phagemids isolated from the brain library were released by an *Eco*RI restriction digest and subcloned into pBluescript and both strands of each clone sequenced. The 5′ 1.4 kb clone from the brain library and the 3′ 1.67 kb clone from the kidney library did not overlap by approximately 450 bp. Therefore, two primers were designed to amplify the missing sequence using the proof-reading enzyme *Pfu* (Stratagene), using an aliqout of the pig kidney cortex cDNA library as template. The sense primer, PF1 (5′-AGATTTTGGCACTGGGGCTATG-3′), was designed toward the 3′ end of the 1.4 kb clone. The antisense primer, PR1 (5′-AGTTGAAAAGTCCTTGTGGTTGAG-3′), was designed toward the 5′ end of the 1.67 kb clone. Conditions for the PF1/PR1 PCR reactions were the following: initial 3 min at 93 °C followed by 5 cycles of 1 min at 93 °C, 1 min at 60 °C, 2 min at 72 °C, and 30 cycles of 1 min at 93 °C, 1 min at 58 °C, 2 min at 72 °C, and an additional extension at 72 °C for 10 min in the last cycle. Products of two PF1/PR1 reactions (PFU1 and PFU2) of the expected size were gel-purified and subcloned into pBluescript-SK(Stratagene). Both strands of each clone were sequenced. Sequencing of the 5′ 1.4 kb, 3′ 1.67 kb, PFU1, and PFU2 AP-A clones was performed using Taq Dye Deoxy Terminator cycle sequencing chemistry in conjuction with the ABI373A DNA sequencing system. DNA and protein sequences were assembled and analyzed using the Wisconsin-GCG programs.

Production of Polyclonal Antiserum. A New Zealand White rabbit was immunized with 50 μg of purified AP-A in Freund's complete adjuvant (subcutaneous). The immunization was repeated 3 weeks later in Freund's incomplete adjuvant and again after a further 3 weeks. A final intravenous immunization was performed 3 weeks later, 10 days after which the animal was bled out. An IgG fraction was prepared from the serum by affinity chromatography on a column of protein G−Sepharose. The antibody did not recognize purified porcine kidney AP-N on immunoelectrophoretic blot analysis (result not shown).

SDS/Polyacrylamide Gel Electrophoresis and Immunoelectrophoretic Blot Analysis. SDS/PAGE was performed with a 7−17% (w/v) polyacrylamide gradient as described previously (Relton *et al*., 1983). Reducing and nonreducing conditions were achieved by including or omitting dithiothreitol in the sample loading buffer, respectively. Immunoelectrophoretic blot analysis was carried out with Immobilon P [poly(vinylidene) difluoride] membranes as described previously (Hooper & Turner, 1987). Bound antibody was detected using peroxidase-conjugated secondary antibody in conjunction with the ECL (Amersham) detection method.

Incubations with Trypsin and Elastase. Incubations in the presence of trypsin were carried out at 37 °C in 20 mM Tris/HCl, pH 7.4. The reaction was stopped by the addition of sample loading buffer and boiled for 5 min prior to analysis by SDS/PAGE. Incubations in the presence of elastase were carried out at 37 °C in 20 mM Tris/HCl, pH 8.8, and the reactions stopped as described for the trypsin incubations.

RESULTS

Purification of Aminopeptidase A from Porcine Kidney Cortex. AP-A was released from porcine kidney cortex membranes by trypsin and then purified by a combination of anion exchange and hydrophobic interaction chromatographies as detailed under Experimental Procedures. AP-N coeluted with AP-A from both the DEAE-cellulose anion exchange column and the phenyl-Sepharose hydrophobic interaction column. The two activities were, however, resolved by chromatography on phenyl-Superose. Attempts were made unsuccessfully to remove the contaminating AP-N activity by varying the concentration of trypsin used to cleave the proteins from the membrane, by chromatography on concanavalin A−Sepharose, and by preparative isoelectric focusing (results not shown). The final preparation of AP-A which was essentially free of AP-N activity had a specific activity of 21.64 μmol min^{-1} mg^{-1} with αGlu-pNA as substrate. The enzyme was enriched 714-fold with respect to the starting homogenate, with a recovery of 1.3% (Table 1).

Structural Properties of Aminopeptidase A. On SDS/polyacrylamide gel electrophoresis under reducing conditions, purified AP-A migrated as three major polypeptide

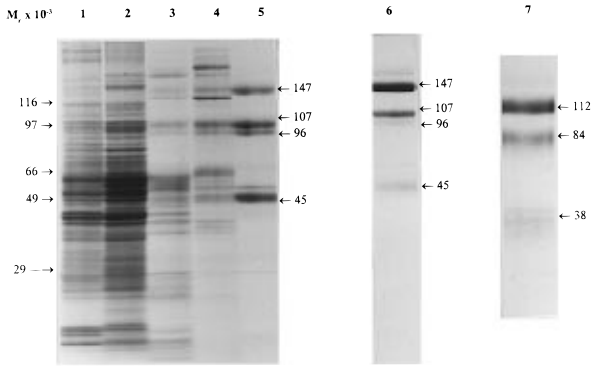

FIGURE 1: Purification and deglycosylation of porcine kidney aminopeptidase A. Samples were prepared and analyzed by SDS/polyacrylamide gel electrophoresis as described under Experimental Procedures. Lane 1, porcine kidney homogenate (0.1 mg of protein); lane 2, microsomal membrane fraction before solubilization with trypsin (0.1 mg of protein); lane 3, supernatant fraction after solubilization of microsomal membranes with trypsin and centrifugation at 31000*g* for 1.5 h (0.1 mg of protein); lane 4, sample from lane 3 after chromatography on DEAE-cellulose (50 μg of protein); lane 5, sample from lane 4 after chromatography on phenyl-Sepharose (25 μg of protein); lane 6, sample from lane 5 after chromatography on phenyl-Superose (10 μg of protein); lane 7, purified AP-A (9 μg of protein) incubated with 0.5 unit of *N*-glycosidase F for 16 h at 37 °C. The gel was stained with Coomassie Brilliant Blue. The molecular weights of the four polypeptides observed in the purified sample of AP-A are indicated on the right of lane 6, and those of the deglycosylated polypeptides on the right of lane 7.

bands with apparent M_r of 147 000, 107 000, and 45 000 (Figure 1, lanes 6 and 7). A minor polypeptide of M_r 96 000, whose intensity varied between preparations (see Figure 3, lanes 1 and 5, for comparison), was also observed. This suggested that it may be derived by limited proteolysis of the M_r 107 000 polypeptide and was therefore not characterized further. Under nonreducing conditions, the purified enzyme migrated as three major polypeptide bands with apparent M_r of 147 000, 107 000, and 45 000 (result not shown). N-Terminal sequencing of the three major polypeptide bands revealed that the M_r 147 000 and 107 000 polypeptides had the same N-terminal sequence of DGGQG, while the M_r 45 000 polypeptide had the sequence GNAFLKINPD. A sample of purified porcine kidney AP-A was enzymically deglycosylated with *N*-glycosidase F and the products analyzed by SDS/polyacrylamide gel electrophoresis (Figure 1, lane 8). The three major polypeptide bands (M_r 147 000, 107 000, and 45 000) were all reduced in size to bands of apparent M_r 112 000, 84 000, and 38 000, respectively.

cDNA Cloning and Sequencing of Porcine Kidney Aminopeptidase A. Degenerate oligonucleotides were designed based on homologous regions of the amino acid sequences of human AP-A (Li *et al*., 1993a; Nanus *et al*., 1993) and mouse BP-1/6C3 (Wu *et al*., 1990). Using these primers, cDNAs corresponding to parts of the 5′ and 3′ regions of the porcine coding region were amplified. Following a PCR-based selection of cDNA sublibraries enriched in AP-A coding sequences, the 5′ 540 bp and 3′ 360 bp PCR products were used as probes in conventional plaque hybridization. Two clones were isolated: a 1.4 kb AP-A clone from the brain library which comprised 86 nucleotides of the 5′ untranslated region and 1320 nucleotides of the coding region, and a 1.67 kb clone from the kidney library which comprised 677 nucleotides of 3′ untranslated region, including a poly$(A)_{20}$ tail, and 1015 nucleotides of the coding region (Figure 2). These two clones did not overlap by 500 nucleotides; therefore, the remainder of the sequence was obtained by PCR using the proofreading enzyme, *Pfu*. Amplified cDNA which overlapped with the 5′ 1.4 kb and 3′ 1.67 kb clones was cloned and sequenced from two separate reactions (Figure 2). The complete nucleotide sequence deduced from the overlapping clones revealed a single open reading frame of 2829 nucleotides which encodes a protein of 942 amino acids with a calculated M_r of 108 283 and predicts 14 potential N-linked glycosylation sites. This is consistent with the observed decrease in the molecular weight of the protein from 147 000 to 112 000 after removal of N-linked sugars (Figure 1, lane 8). The existence of three in-frame stop codons 5′ to the first Met confirmed the initiation codon. A polyadenylation signal (AATAAA) at 3500 nucleotides preceeds the poly(A) tail. A hydropathy plot of the translated sequence revealed a potential transmembrane domain at the N-terminus (data not shown) between residues 15 and 35. A typical zinc binding motif (His-Glu-Xaa-Xaa-His) is found between residues 383 and 387. The third zinc binding ligand, a glutamic acid residue, that has recently been positively identified by site-directed mutagenesis of murine AP-A (Vazeux *et al*., 1996), is also conserved in porcine AP-A (Glu-406).

Proteolytic Fragmentation of Aminopeptidase A and Aminopeptidase N. In an attempt to determine whether the observed proteolytic fragmentation of AP-A (see Figure 1, lanes 6 and 7) was due to the action of exogenous proteases, purified AP-A was incubated with either trypsin or elastase (Figure 3). Neither trypsin nor elastase caused further breakdown of any of the three major polypeptides of AP-A (Figure 3, lanes 2 and 6). In contrast, a purified sample of AP-N, that had been solubilized from the membrane with Triton X-100, was rapidly degraded by trypsin to generate polypeptide fragments (Figure 3, lane 4) of similar sizes to those observed in the purified preparation of AP-A. The inclusion of a cocktail of protease inhibitors in the homogenization buffer failed to prevent the observed fragmentation of porcine kidney AP-A (Figure 4, lane 1). The proteolytic fragmentation of AP-A was also observed in the more purified kidney microvillar membrane preparation (Figure 4, lane 3), but was not observed in microvillar membranes isolated from porcine intestine (Figure 4, lane 4).

Dimeric Structure of Aminopeptidase A. Immunoelectrophoretic blot of the samples from the purification of AP-A from porcine kidney cortex following electrophoresis under reducing conditions clearly indicated the presence of the three major proteolytic fragments in all the samples (Figure 5a). In contrast, when the samples were electrophoresed under nonreducing conditions and then subjected to immunoelectrophoretic blot analysis (Figure 5b), only those samples following incubation of the membranes with trypsin contained the proteolytic fragments, while the homogenate and microsomal membrane samples revealed the presence of only two polypeptide bands, one with an apparent M_r of approximately 220 000−290 000 and the other of M_r 45 000.

DISCUSSION

The cDNA encoding porcine AP-A has been isolated and sequenced (Figure 2). Problems with the integrity of the porcine brain and kidney cDNA libraries meant that two

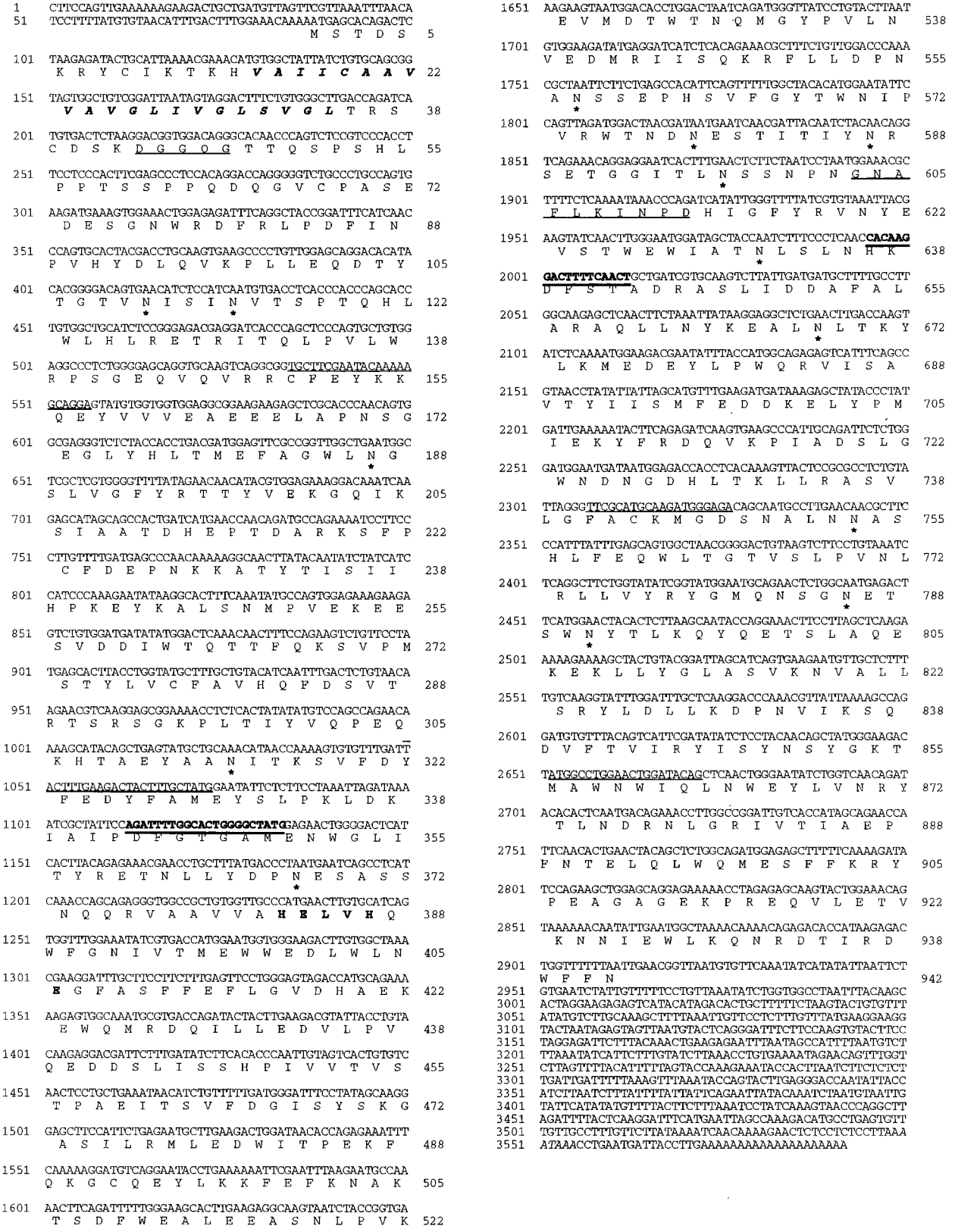

FIGURE 2: cDNA sequence and deduced amino acid sequence of porcine aminopeptidase A. The amino acid sequence is numbered from the initiating Met residue which forms the N-terminus of the mature protein. The N-linked glycosylation sites are indicated by asterisks. The His-Glu-Xaa-Xaa-His zinc binding motif (residues 383−387) and the third zinc binding ligand (Glu-406) are shown in boldface type. The putative transmembrane region is in boldface italics. The underlined cDNA segments show the location of the (+ and −) degenerate PCR primers. The cDNA segments in boldface and underlined show the location of the (+ and −) specific PCR primers for amplification of the *Pfu* clones. Nucleotides 1−1406 are from the 5′ 1.4 kb clone; nucleotides 1407−1899 are from the *Pfu* clones; and nucleotides 1900−3593 are from the 3′ 1.67 kb clone.

cDNA clones encoding the N- and C-termini of the protein were isolated, and that the intervening sequence had to be obtained by PCR using the proofreading enzyme, *Pfu*. The complete nucleotide sequence deduced from the overlapping

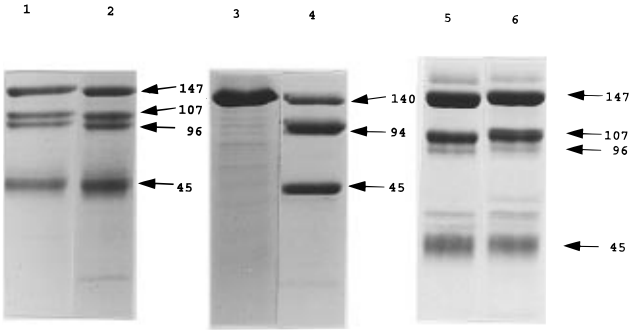

FIGURE 3: SDS/polyacrylamide gel electrophoresis of trypsin- and elastase-treated aminopeptidase A and aminopeptidase N. AP-A and AP-N were purified from porcine kidney cortex following trypsin and Triton X-100 solubilization of the membranes, respectively. Samples were prepared and analyzed under reducing conditions as described under Experimental Procedures. Lane 1, purified porcine kidney AP-A (10 μg of protein) incubated for 2 h at 37 °C in the absence of trypsin; lane 2, AP-A (10 μg of protein) incubated for 2 h at 37 °C in the presence of trypsin (1 μg of protein); lane 3, purified porcine kidney AP-N (10 μg of protein) incubated for 30 min at 37 °C in the absence of trypsin; lane 4, AP-N (10 μg of protein) incubated for 30 min at 37 °C in the presence of trypsin (1 μg of protein); lane 5, AP-A (6 μg of protein) incubated for 1 h at 37 °C in the absence of elastase; lane 6, AP-A (6 μg of protein) incubated for 1 h at 37 °C in the presence of elastase (6 μg of protein). The gel was stained with Coomassie Brilliant Blue. The molecular weights of the various proteolytic fragments are indicated.

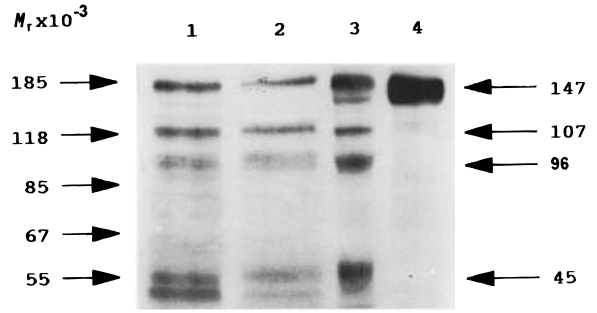

FIGURE 4: Immunoelectrophoretic blot of porcine kidney and intestinal aminopeptidase A. Samples were electrophoresed under reducing conditions and then subjected to immunoelectrophoretic blot analysis with a polyclonal antibody raised to AP-A as described under Experimental Procedures. Lane 1, porcine kidney cortex homogenate (0.1 mg of protein) prepared in the presence of a cocktail of protease inhibitors (EDTA, 1 mM; 1,10-phenanthroline, 1 mM; DipF, 0.1 mM; phenylmethanesulfonyl fluoride, 1 mM; E-64, 10 μM; pepstatin, 1 μM); lane 2, porcine kidney cortex homogenate (0.1 mg of protein) prepared in the absence of inhibitors; lane 3, porcine kidney cortex microvillar membrane preparation (0.1 mg of protein); lane 4, porcine intestinal microvillar membrane preparation (80 μg of protein). The molecular weights of the proteolytic fragments are indicated on the right.

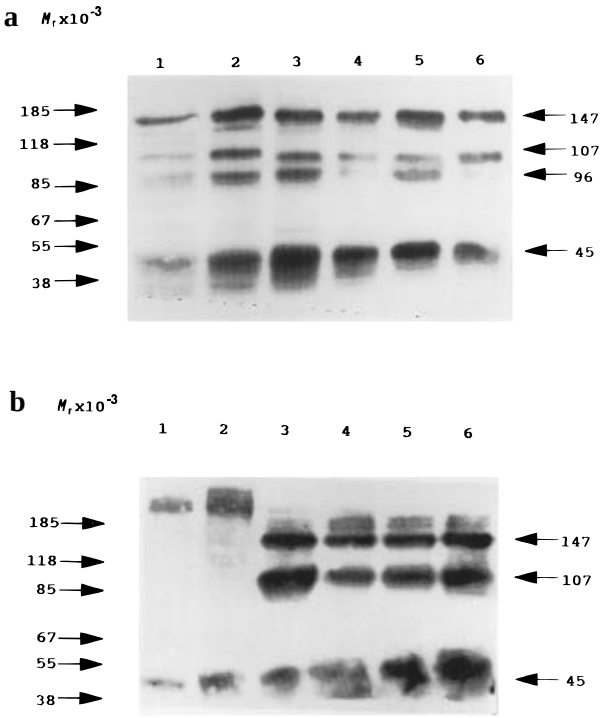

FIGURE 5: Immunoelectrophoretic blot of porcine kidney aminopeptidase A under reducing and nonreducing conditions. Samples from the purification of AP-A from porcine kidney cortex were electrophoresed under (a) reducing and (b) nonreducing conditions, and then subjected to immunoelectrophoretic blot analysis with a polyclonal antibody raised to AP-A as described under Experimental Procedures. Lane 1, porcine kidney cortex homogenate (0.1 mg of protein); lane 2, microsomal membrane fraction before solubilization with trypsin (0.1 mg of protein); lane 3, supernatant fraction after solubilization of microsomal membranes with trypsin and centrifugation at 31000*g* for 1.5 h (0.1 mg of protein); lane 4, sample from lane 3 after chromatography on DEAE-cellulose (25 μg of protein); lane 5, sample from lane 4 after chromatography on phenyl-Sepharose (12 μg of protein); lane 6, sample from lane 5 after chromatography on phenyl-Superose (2 μg of protein). The molecular weights of the proteolytic fragments are indicated on the right.

clones revealed a single open reading frame of 2829 nucleotides that encodes a protein of 942 amino acids. Porcine AP-A has an overall identity of 85% to human AP-A (Li *et al*., 1993a; Nanus *et al*., 1993) and 78% to the murine BP-1/6C3 antigen (Wu *et al*., 1990). As with human and murine AP-A, the porcine cDNA predicts a type II integral membrane protein.

On SDS/polyacrylamide gel electrophoresis, the purified porcine kidney AP-A migrated as three major polypeptide bands of M_r 147 000, 107 000, and 45 000 (Figure 1). The M_r 147 000 and 107 000 polypeptides had the same N-terminal sequence of DGGQG which is found at positions 43−47 of the deduced amino acid sequence (Figure 2). This is consistent with AP-A having been cleaved on release from the membrane by trypsin at the Lys-42−Asp-43 bond, seven residues C-terminal to the putative transmembrane region. The calculated M_r of the N-terminal peptide left in the membrane after trypsin cleavage is 4450. This agrees closely with previous work on porcine intestinal AP-A which used an isotopic dilution method to calculate the M_r of the transmembrane tryptic peptide as 4500 (Benajiba & Maroux, 1980). Two Lys residues are present in an essentially identical position in the stalk region of porcine AP-N (Delmas *et al*., 1994), and N-terminal sequencing of the trypsin-released form of rabbit AP-N revealed that the protein had been cleaved at the Lys-39−Asn-40 bond, five residues from the transmembrane region (Feracci *et al*., 1982; Watt & Yip, 1989).

Among the mammalian cell-surface peptidases, AP-A is one of only four that exist as a disulfide-linked dimer (Wu *et al*., 1991). As the disulfide-linked dimer of M_r 220 000−290 000 was lost upon trypsin treatment of the enzyme (Figure 5), it can be concluded that the Cys residue involved in formation of the dimer lies N-terminal to Lys-42. Of the three Cys residues N-terminal to Lys-42, all of which are conserved in human and mouse AP-A, the most likely candidate is Cys-39 which lies on the extracellular side of

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                                                        T
                                                        ↓
          1                                             61
Pig AP-A  ----MSTDSKRYCIKTKH*VAIICAAVVAVGLIVGLSVGL*TRSCDS-KDGGQGTTQSPSHLP
Human AP-A MNFAEREG.....Q.....*L..V..G.....A*.....SG...P..APA.....
Mouse AP-A MNFAEEEP..K....G.....*GV*.................E-- ----.D..PA..QP.

          567                                   X       635
          *           *       *       *       ↓
Pig AP-A  NSSEPHSVFGYTWNIPVRWTNDNESTITIYNRSETGGITLNSSNPNGNAFLKINPDHIGFYRVNYEVST
Human AP-A .P.Q.P.DL.....K..E..ITSSVLF....KE.....S.....................A.
Mouse AP-A DP.Q.P.EL.....ADNDN.R..V...LDK.....A.LS.D.................GG.
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FIGURE 6: Sequence alignment of porcine, human, and murine aminopeptidase A. The sequences at the N-terminus (residues 1−61) and around the internal cleavage site (residues 567−635) of porcine AP-A are aligned with the corresponding regions of human (Li *et al*., 1993a; Nanus *et al*., 1993) and murine (Wu *et al*., 1990) AP-A. The putative membrane-spanning region is shown in boldface italics. The trypsin cleavage point in porcine AP-A is indicated by T. The internal cleavage of AP-A by the unknown protease is indicated by X. The N-linked glycosylation sites in porcine AP-A are indicated by asterisks. (Periods) indicate on identical residue to porcine AP-A; (hyphens) indicate spaces introduced for optimal alignment. Alignment generated by using the Wisconsin GCG program, PILEUP.

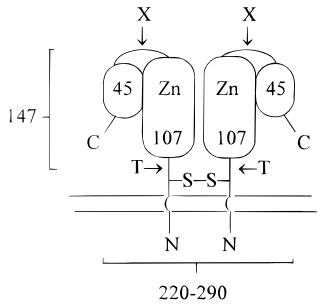

FIGURE 7: Diagrammatic representation of the oligomeric and domain structure of aminopeptidase A showing the sites of proteolytic cleavage. The polypeptide of AP-A is shown interacting with the lipid bilayer through the N-terminal hydrophobic domain with the bulk of the protein on the extracellular surface of the membrane. The position of the proposed interchain disulfide bond (Cys-39) is indicated. The larger (M_r 107 000) catalytic domain (Zn 107) and the smaller (M_r 45 000) domain (45) are shown within the large extracellular region. The site of cleavage between the two domains at the Asn-602−Gly-603 bond by the unknown protease is indicated by X. Cleavage of the protein from the membrane by trypsin (T) at Lys-42 results in the release of the M_r 147 000 fragment. Numbers correspond to molecular weights x 10^{-3}.

the membrane (Figure 6), as the other two cysteines, Cys-9 and Cys-19, are in a reducing environment. This would place the disulfide bond between the two subunits very close to the membrane surface, only four residues away from the end of the hydrophobic membrane-spanning region (Figure 7). This is analogous to membrane dipeptidase (EC 3.4.13.19) where Cys-361 forms the disulfide link which is only seven residues away from the site of attachment of the glycosylphosphatidylinositol anchor (Ser-368) (Keynan *et al*., 1996). In contrast, the single Cys involved in the dimerization of both endothelin converting enzyme-1 and endopeptidase-24.18 (meprin A; EC 3.4.24.18) is found near the middle of the cDNA-derived amino acid sequence (Shimada *et al*., 1996; Chevallier *et al*., 1996). In all four cases, there is only a single interchain disulfide bond.

The M_r 45 000 polypeptide in the purified preparation of porcine kidney AP-A had the N-terminal sequence GNAFLKINPD which corresponds to residues 603−612 of the deduced amino acid sequence (Figure 2). This indicates that AP-A has been posttranslationally cleaved N-terminal to Gly-603, possibly at the Asn-602−Gly-603 bond, within a region that is highly conserved between the porcine, human, and murine sequences (Figure 6). That this proteolytic cleavage is not due to the trypsin used to solubilize AP-A from the membrane is evidenced by the fact that (i) incubation of purified AP-A with trypsin did not result in further breakdown of the M_r 147 000 polypeptide (Figure 3) and (ii) the pattern of proteolytic fragmentation was observed in the homogenate and microsomal membrane samples before addition of the trypsin (Figure 5a). The presence of Gly with its small side chain in the P_1' position suggested the possibility of an elastase-like protease being involved in this posttranslational proteolytic event. However, purified elastase failed to cleave the M_r 147 000 polypeptide in the purified sample of AP-A (Figure 3). That this observed proteolytic fragmentation of AP-A is not due to nonspecific proteolysis during the tissue homogenization and subsequent purification is evidenced by the fact that the inclusion of a cocktail of protease inhibitors directed against serine, metallo, thiol, and aspartic acid proteases, in the homogenization buffers, had no effect on the observed proteolytic processing (Figure 4), and that the proteolytic fragmentation of AP-A was consistently observed in numerous preparations as well as in the more purified kidney microvillar membranes (Figure 4). Previously it has been shown that AP-A purified from porcine kidney following either detergent or trypsin solubilization from the membrane appeared as four polypeptide bands on SDS/polyacrylamide gel electrophoresis with almost identical M_r to those of the present study, although at the time their identity was not established and the possibility of contaminants could not be ruled out (Danielsen *et al*., 1980).

The observation that 3 of the 14 potential N-linked glycosylation sites lie within 24 residues of the N-terminal side of Gly-603 (Figure 6) suggests that this may be a region of the polypeptide that is at the surface of the protein, possibly forming a loop that is susceptible to proteolysis (see Figure 7). The observation that both purified AP-A and AP-A detected in the homogenate and membrane samples by immunoelectrophoretic blot analysis appeared as the three polypeptides of M_r 147 000, 107 000, and 45 000 suggests that not all AP-A molecules are cleaved at the Asn-602−Gly-603 bond. This raises the possibility that AP-A may exist *in vivo* as a "heterodimer" with one uncleaved and one cleaved polypeptide chain (Figure 7). Whether this posttranslational proteolytic cleavage alters the activity of AP-A could not be determined as every preparation contained a mixture of the cleaved and uncleaved forms. Although AP-A in porcine kidney was found to be proteolytically processed, immunoelectrophoretic blot analysis of porcine intestinal membranes revealed only a single polypeptide band of M_r 147 000 (Figure 4). Earlier work also showed that porcine intestinal AP-A, whether solubilized from the membrane by detergent or trypsin, migrated as a single polypeptide band on SDS/polyacrylamide gel electrophoresis (Benajiba & Maroux, 1980). This result reinforces the fact that the processing in the kidney is not due to nonspecific proteolysis as the risk of proteolysis would be expected to be greater in the intestine than in the kidney due to the presence of the pancreatic digestive proteases. Purified rat kidney AP-A also appears as three major polypeptide bands on SDS/polyacrylamide gel electrophoresis (Song *et al*., 1994) of comparable M_r to those observed in porcine kidney AP-A. Thus, it would appear that the proteolytic cleavage of AP-A is a tissue/cell-specific event occurring in the kidney but not in the intestine, presumably due to the absence of the processing protease in the latter tissue.

In contrast to AP-A, incubation of purified porcine kidney AP-N with trypsin resulted in the rapid cleavage of the M_r 140 000 polypeptide to products of M_r 94 000 and 45 000 (Figure 3). N-Terminal sequencing of the M_r 45 000 polypeptide revealed the sequence SSAFDYLWIVPIS-SIKNGVM, which corresponds to residues 573−592 of porcine AP-N (Delmas *et al*., 1994). Immediately preceding Ser-573 is an Arg residue that is presumably the site of action of trypsin. This cleavage site corresponds to that previously identified as occurring *in vivo* in porcine intestinal AP-N (Olsen *et al*., 1988).

Alignment of the porcine AP-A and AP-N sequences reveals that the Asn-602−Gly-603 bond in AP-A and the Arg-572−Ser-573 bond in AP-N lie within close proximity of each other, and that in both enzymes the cleaved bond is preceded by two or three putative N-linked glycosylation sites. Thus, it would appear that both enzymes consist of a larger N-terminal domain that contains the residues identified as being involved in zinc binding and catalysis and a smaller C-terminal domain that is separated from the catalytic domain by a glycosylated, protease-susceptible region. Comparison of the amino acid sequences of other members of the type II membrane-spanning zinc aminopeptidase family [thyrotropin-releasing hormone degrading enzyme (EC 3.4.19.6) (Schauder *et al*., 1994), the GluT4 vesicle aminopeptidase (Keller *et al*., 1995), and placental leucine aminopeptidase/oxytocinase (Rogi *et al*., 1996)] with the sequences of AP-A and AP-N reveals that they are all of a similar overall length with the highest sequence similarity within the N-terminal domain around the zinc binding motif. Other shorter regions of sequence similarity are present in the C-terminal domain. In all of these enzymes, the region corresponding to the protease-susceptible region in AP-A and AP-N also contains several putative N-linked glycosylation sites, suggesting that they may all consist of two distinct domains separated by a glycosylated, protease-susceptible region. That such a protease-susceptible region is present in other members of this family is evidenced by the observation that trypsin treatment of thyrotropin-releasing hormone degrading enzyme (subunit M_r 116 000) resulted in its limited fragmentation to two major products of M_r approximately 70 000 and 45 000 (Bauer, 1994) which would be consistent with the generation of N- and C-terminal domains, respectively. The biological role (if any) of the C-terminal domain in the type II membrane-spanning zinc aminopeptidases awaits to be determined, although interestingly, the region of AP-N to which the porcine coronavirus transmissible gastroenteritis virus binds has been mapped to a region between residues 717 and 813 which lies within the C-terminal domain (Delmas *et al*., 1994).

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