The C-terminal domain, but not the interchain disulphide, is required for the activity and intracellular trafficking of aminopeptidase A

Lisa D. OFNER and Nigel M. HOOPER¹

Proteolysis Research Group, School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, U.K.

Mammalian aminopeptidase A (APA; glutamyl aminopeptidase; EC 3.4.11.7) is a type II membrane-spanning protein consisting of a short N-terminal cytosolic domain, a single transmembrane domain and a large extracellular C-terminal domain containing the active site. The extracellular domain consists of a 107 kDa domain, containing the zinc-binding motif and all the residues involved in catalysis, separated by a protease-susceptible hinge region from the 45 kDa C-terminal domain of unknown function. To investigate the role of the 45 kDa domain, a construct of murine APA (G594Δ) lacking this C-terminal domain was expressed in COS-1 cells. This truncated form of APA, although expressed, lacked enzymic activity and failed to reach the cell surface. Confocal immunofluorescence microscopy revealed that

G594Δ co-localized with the lectin concanavalin A and had a similar staining pattern as protein disulphide-isomerase, indicating that it was retained in the endoplasmic reticulum. Thus the C-terminal 45 kDa domain appears to be acting like a pro-domain and seems to be required for the correct folding and trafficking of APA. In contrast, mutation of cysteine-43 to serine, which is involved in the disulphide-linkage of the APA homodimer, did not affect the enzymic activity, cellular location or rate of trafficking through the secretory pathway of APA.

Key words: aminopeptidase N, COS cells, ectoenzyme, pro-domain, zinc metallopeptidase.

INTRODUCTION

Aminopeptidase A (APA; glutamyl aminopeptidase, EC 3.4.11.7) is a widely expressed mammalian ectoenzyme that specifically cleaves acidic residues (aspartic acid or glutamic acid) from the N-terminus of peptide substrates [1]. APA is involved *in vivo* in the conversion of brain angiotensin II into angiotensin III [2] and in the metabolism of cholecystokinin-8 [3]. The enzyme is also the major intestinal zinc metallopeptidase responsible for the metabolism of the artificial sweetener aspartame [4]. On the basis of its role in the brain renin–angiotensin system, inhibitors of APA have been reported as potential central antihypertensive agents [5]. APA is identical with the murine B-lymphocyte differentiation antigen BP-1/6C3 and is likely to be involved in early B-cell development [6–8]. Plasma APA activity is significantly and consistently lower in Alzheimer's-disease patients than in age-matched controls, and thus the enzyme may have potential use as a diagnostic/predictive marker for this disease [9].

APA is a member of the M1 family of zinc metallopeptidases [10]. A subgroup of this family comprises the mammalian type II membrane-spanning aminopeptidases, consisting of APA, aminopeptidase N (APN; membrane alanyl aminopeptidase, EC 3.4.11.2), placental leucine aminopeptidase (PLAP; cystinyl aminopeptidase, EC 3.4.11.3) and thyrotropin-releasing hormone-degrading ectoenzyme (TRH-DE; pyroglutamyl peptidase II, EC 3.4.19.6). These mammalian aminopeptidases consist of a short (approx. 20 amino acids) N-terminal cytosolic domain, a single transmembrane domain and a large (approx. 900 amino acid) C-terminal extracellular domain. On the basis of proteolytic-fragmentation studies we previously subdivided the C-terminal extracellular domain of APA into two parts: a 107 kDa domain containing the HEXXH zinc-binding motif and a 45 kDa C-terminal domain, separated by a protease-sensitive hinge region [11]. Sequence alignments and data from proteolytic

digestion of APN [12] indicate that the ectodomains of the other, type II, membrane-spanning aminopeptidases have a similar two-domain structure.

Although both APA and APN are subject to proteolytic cleavage between the two extracellular domains *in vivo*, the resulting fragments appear to remain tightly associated with one another, and this proteolytic fragmentation does not alter the enzymic activity of the proteins [11,13]. Interestingly all the residues involved in metal binding and catalysis that have been identified through site-directed mutagenesis and/or sequence comparisons with other zinc metalloproteinases, such as thermolysin [14–18], are all located in the 107 kDa domain of APA. Thus it is unclear whether the 45 kDa domain is required for enzymic activity or whether it serves some other function.

All of the mammalian type II membrane-spanning aminopeptidases exist as homodimers. APN is non-covalently associated [19], while APA and TRH-DE both have a single disulphide bond between the two subunits [11,20]. During the aforementioned proteolytic-fragmentation study, we mapped the single cysteine residue involved in the interchain disulphide to Cys³⁹ of porcine APA [11].

In the present study we used site-directed mutagenesis to investigate the role of the interchain disulphide and the C-terminal 45 kDa domain in the enzymic activity, folding and trafficking of murine APA. Cys⁴³, which is equivalent to Cys³⁹ in porcine APA, was mutated to serine and the mutant protein expressed in COS-1 cells. This C43S mutant was expressed at the cell surface in an enzymically active, glycosylated form, but was no longer disulphide-linked. In contrast, removal of the C-terminal 45 kDa domain by mutating the codon for Gly⁵⁹⁴ in the hinge region to a stop codon resulted in the expression of a truncated protein (G594Δ) that was enzymically inactive and which did not reach the cell surface. Confocal immunofluorescence microscopy revealed that, without this C-terminal domain, APA

Abbreviations used: APA, aminopeptidase A; APN, aminopeptidase N; ER, endoplasmic reticulum; TBS, Tris-buffered saline; TRH-DE, thyrotropin-releasing hormone-degrading ectoenzyme; C43S, Cys⁴³ → Ser mutant; G594Δ, G⁵⁹⁴ → stop codon mutant; wt, wild-type; ADAM, a disintegrin and metalloproteinase-like.

¹ To whom correspondence should be addressed (e-mail n.m.hooper@leeds.ac.uk).

was retained in the endoplasmic reticulum (ER), indicating that this region of the protein is required for correct folding and trafficking of the protein.

EXPERIMENTAL

cDNA cloning and site-directed mutagenesis

cDNAs encoding the C43S and G594Δ mutants were constructed by oligonucleotide-directed mutagenesis of the cDNA encoding murine APA in the vector SRαBP-1 (a gift from Professor M. D. Cooper, Department of Medicine, University of Alabama at Birmingham, Birmingham, AL, U.S.A.) [6,14] using the Stratagene QuikChange Mutagenesis Kit. The mutants generated were confirmed by DNA sequencing.

Cell culture and membrane preparation

COS-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal-bovine serum and 50 units/ml penicillin/streptomycin. Cells were transiently transfected with wild-type (wt) or mutant APA cDNA constructs using LIPOFECTAMINE™ (Gibco BRL, Paisley, Renfrewshire, Scotland, U.K.) according to the manufacturer's protocol. At 48 h after transfection, cells were washed with PBS and then scraped into PBS. The cell suspension was centrifuged at 100 *g* for 5 min and the cell pellet resuspended in 0.1 M Tris/HCl, pH 7.4, before being disrupted using a nitrogen bomb at 5.934 MPa (850 lbf/in²) for 10 min. The resulting suspension was centrifuged at 55000 *g* for 1 h and the crude particulate pellet fraction resuspended in 0.1 M Tris/HCl, pH 7.4, containing 1% (v/v) Triton X-100 and incubated on ice for 1 h to solubilize the proteins.

Enzymic assay

APA activity was assayed using 5 mM α -glutamic acid *p*-nitroanilide as substrate with 0.1 M Tris/HCl (pH 7.4)/1 mM CaCl₂ in 96-well microtitre plates. Following incubation at 37 °C, the released *p*-nitroaniline was detected by measuring absorbance at 405 nm and calibrating against a standard curve [11]. Protein concentration was determined using the bicinchoninic acid ('BCA') method with BSA as standard [21].

Temperature-block and cell-surface-activity assay

COS-1 cells grown in six-well plates and transiently expressing either wtAPA or the C43S mutant were incubated at 15 °C for 4 h. Growth medium was then removed and the cells were washed in 0.1 M Tris/HCl, pH 7.4, before adding 2 ml of 0.1 M Tris/HCl (pH 7.4)/1 mM CaCl₂ containing 2.5 mM α -glutamic acid *p*-nitroanilide and incubated at 37 °C. At regular intervals the substrate containing medium was removed and its absorbance measured at 405 nm.

SDS/PAGE and Western-blot analysis

Samples were mixed with an equal volume of either reducing or non-reducing electrophoresis sample buffer and boiled for 5 min. Proteins were resolved by SDS/PAGE using a 7–17%-(w/v)-acrylamide gradient gel and transferred to Immobilon P PVDF membrane, as described previously [22]. The membranes were blocked by incubation in PBS containing 0.1% (v/v) Tween 20, 5% (w/v) dried milk powder and 2% (w/v) BSA for 1 h at room temperature. The polyclonal antibody against murine APA has been described elsewhere [23] and was a gift from Professor S. Wilk (Mount Sinai Medical Center, New York, NY, U.S.A.). All primary- and secondary-antibody incubations were performed in

PBS containing 2% (w/v) BSA and 0.1% Tween 20. Bound antibody was detected using peroxidase-conjugated secondary antibodies in conjunction with the enhanced chemiluminescence (ECL®; Amersham Life Sciences, Little Chalfont, Bucks., U.K.) detection method.

Immunofluorescence microscopy

Cells were grown on coverslips in six-well plates, washed with Tris-buffered saline (TBS; 25 mM Tris/HCl/137 mM NaCl/2.68 mM KCl, pH 7.5) and then fixed in methanol/acetone (1:1, v/v) for 10 min. After further washes in TBS the cells were incubated in blocking buffer [0.2% (w/v) gelatin and 1% (v/v) goat serum in TBS] for 30 min at room temperature. The cells were incubated with either the polyclonal anti-APA antibody or a polyclonal anti-(protein disulphide-isomerase) antibody diluted in blocking buffer for 2 h at room temperature, and then incubated with an FITC-conjugated anti-rabbit secondary antibody diluted in blocking buffer for 30 min. Alternatively, the cells were incubated for 1 h at room temperature with Rhodamine Red-conjugated concanavalin A (Sigma Chemical Company, Poole, Dorset, U.K.). The cells were finally washed with TBS, prior to mounting on slides and then viewed under a Leica confocal microscope.

Enzymic deglycosylation

For deglycosylation with N-glycosidase F, samples were made 20 mM with respect to sodium phosphate, pH 7.6, 50 mM with respect to EDTA, 5% (w/v) with respect to SDS and 5% (v/v) with respect to β -mercaptoethanol. Samples were boiled for 5 min, diluted 5-fold with 1.25% (v/v) Triton X-100 and incubated for 16 h at 37 °C with 1 unit of N-glycosidase F. For deglycosylation with endoglycosidase H, samples were boiled for 5 min in 100 mM sodium citrate, pH 5.5 containing 0.2% (w/v) SDS. After cooling, 0.1 unit of endoglycosidase H was added and the sample incubated for 24 h at 37 °C.

RESULTS

Expression and enzymic activity of the APA mutants

The cDNAs encoding wtAPA, C43S or G594Δ (Figure 1) were transiently expressed in COS-1 cells and membranes prepared

Figure 1 Schematic diagram showing the domain structure of murine APA and the constructs used in the present study

Murine wtAPA is depicted with its uncleaved N-terminal signal sequence that doubles as a transmembrane anchor (black box). The extracellular 107 kDa domain containing the zinc-binding motif (denoted by Zn) and the smaller C-terminal 45 kDa domain are indicated, separated by the protease-susceptible hinge region [11]. In the C43S mutant, Cys⁴³ is mutated to serine, while the G594Δ construct is truncated at the hinge region and lacks the 45 kDa C-terminal domain.

Figure 2 Immunoelectrophoretic blot analysis of the APA mutants

COS-1 cells were transfected with the cDNA encoding either wtAPA (wt), C43S or G594 Δ . A crude particulate fraction was prepared from the cells as described in the Experimental section and subjected to electrophoresis under (A and C) reducing or (B) non-reducing conditions on an SDS/7–17% polyacrylamide gel. Protein was then transferred to a PVDF membrane and probed with a polyclonal antibody against murine APA. Un, untransfected COS-1 cells. Molecular-mass markers (in kDa) are indicated on the right of each panel.

Figure 3 Confocal immunofluorescence microscopy of cells expressing APA

COS-1 cells were grown on coverslips and transfected with wt or mutated APA cDNA as described in the Experimental section. The cells were then incubated with the primary APA antibody, followed by an FITC-conjugated secondary antibody and viewed in a Leica CLSM microscope. (A) Untransfected cells; (B) cells transfected with wtAPA; (C) cells transfected with C43S; (D) cells transfected with G594 Δ . The images shown are representative of three separate experiments. The scale bar represents 20 μ m.

from the cells subjected to immunoelectrophoretic blot analysis using a polyclonal antibody raised against murine APA [23]. In the crude particulate fraction from cells transfected with wtAPA,

the antibody detected a polypeptide of 147 kDa under reducing conditions (Figure 2A, lane 2) and a band of 280 kDa under non-reducing conditions (Figure 2B, lane 2), consistent with murine

Figure 4 Double labelling of the aminopeptidase A expressing cells

COS-1 cells were grown on coverslips and transfected with either wtAPA or G594 Δ cDNA as described in the Experimental section. Cells were subjected to immunocytochemistry using (A) FITC-conjugated secondary antibody with the anti-APA primary antibody, (B) Rhodamine Red-conjugated concanavalin A, (C) shows dual staining for both APA and concanavalin A (overlapping distribution is represented by the orange staining), and (D) FITC-conjugated secondary antibody with an anti-(protein disulphide-isomerase) primary antibody with a Leica TCS ST microscope. The images are representative of three separate images. The scale bar represents 20 μ m.

APA existing as a disulphide-linked homodimer [7]. In contrast there was no difference in electrophoretic mobility of the C43S mutant under reducing or non-reducing conditions, with the antibody detecting a protein of 147 kDa under both conditions (Figures 2A and 2B, lane 3). This clearly indicates that Cys⁴³ is the cysteine residue involved in the interchain disulphide bond in murine APA and is equivalent to Cys³⁹ in porcine APA [11]. As the C43S mutant had a monomeric molecular mass identical with that of the recombinant wt enzyme, it would suggest that it was similarly glycosylated. Immunoelectrophoretic blot analysis of membranes from cells expressing the G594 Δ mutant revealed a polypeptide of 107 kDa (Figure 2C, lane 3), which is consistent with the size of the N-terminal proteolytic fragment of porcine APA [11]. With that particular batch of antibody the non-specific band at approx. 120 kDa also appeared in the wtAPA and untransfected cells (Figure 2C, lanes 1 and 2). Analysis of the crude particulate fraction prepared from the COS-1 cells expressing wtAPA, C43S or G594 Δ indicated that the C43S mutant had a specific activity similar to that of the wt protein (4.3 ± 0.7 and 5.3 ± 0.1 nmol of *p*-nitroaniline/min per mg of protein respectively). In the untransfected cells there was negligible hydrolysis of the α -glutamic acid *p*-nitroanilide substrate under the same conditions (0.2 ± 0.1 nmol of *p*-nitroaniline/min per mg of protein), whereas no enzymic activity (0 nmol of *p*-nitroaniline/min per mg) was detected in the cells expressing the

G594 Δ mutant. Thus the interchain disulphide bond does not appear to be required for enzymic activity, but removal of the C-terminal 45 kDa domain does have an adverse effect on activity.

Immunocytochemical localization of the APA mutants

COS-1 cells transfected with wtAPA, C43S or G594 Δ were subjected to confocal immunofluorescence microscopy in order to determine the cellular location of the constructs. Cell-surface fluorescent staining was clearly seen for the cells expressing wtAPA (Figure 3B) or C43S (Figure 3C), indicating that removal of the interchain disulphide does not drastically affect the subcellular location of the enzyme. However, the cells expressing the G594 Δ mutant showed no cell-surface staining (Figure 3D). Instead there was a pronounced intracellular staining, particularly around the nucleus (Figure 3D). Identical results for all three constructs of APA were obtained using Chinese-hamster ovary cells instead of COS-1 cells (results not shown).

In order to determine the precise intracellular location of the G594 Δ mutant, cells expressing G594 Δ or wtAPA were subjected to immunocytochemistry with concanavalin A conjugated to Rhodamine Red. This lectin specifically binds to mannose-rich carbohydrate cores in glycoproteins and labels the ER [24,25]. Immunocytochemistry with concanavalin A revealed the net-like meshwork of the ER around the nucleus of the cells (Figure 4B),

Figure 5 Rate of trafficking of APA through the secretory pathway

COS-1 cells transfected with either wtAPA or C43S mutant cDNA were grown in six-well plates. Following transfection, the cells were incubated at 15 °C for 4 h before returning them to 37 °C. The cells were then assayed for surface APA enzymic activity at the indicated time intervals using α -glutamic acid p -nitroanilide as substrate. The results are the means $\pm$ S.E.M. of triplicate determinations. Un, untransfected cells; pNA, p -nitroaniline.

similar to the staining observed with the G594 Δ mutant (Figure 4A, lower panel). Merging of the images obtained with the Rhodamine Red-conjugated concanavalin A and the FITC-labelled anti-APA antibody, revealed an almost exact overlap of the localization of the G594 Δ mutant with this ER marker (Figure 4C, lower panel). In contrast, there was little overlap of wtAPA with the concanavalin A (Figure 4C, middle panel), consistent with most of this form of the protein being located at the cell surface. To confirm that the net-like meshwork staining observed with the G594 Δ mutant represented the ER, the cells were also subjected to immunocytochemistry with an antibody against protein disulphide-isomerase, the ER marker protein. Protein disulphide-isomerase also displayed the characteristic net-like meshwork staining representative of the ER (Figure 4D). The ER localization of the G594 Δ mutant was confirmed by assessing its deglycosylation with endoglycosidase H. A reduction in its molecular mass on incubation with endoglycosidase H (results not shown) revealed that the N-glycans were in their high-mannose form, confirming that this construct had not travelled beyond the *cis*-Golgi compartment. In contrast, wtAPA was only susceptible to deglycosylation with N-glycosidase F (results not shown), consistent with it having acquired complex N-glycans.

Rate of trafficking of APA through the secretory pathway

As the C43S mutant was expressed at the cell surface, covalent dimerization was obviously not a prerequisite for transport to the cell surface. However, dimerization is required for the efficient trafficking of APN through the secretory pathway [26,27]. We therefore investigated whether the lack of the interchain disulphide bond affected the rate of trafficking of APA. Cells expressing either wtAPA or C43S were first incubated at 15 °C for 4 h to block export of newly synthesized protein from the ER. The cells were then returned to 37 °C and the presence of APA at the cell surface was determined by activity assays (Figure 5). At time points up to 20 min after return to 37 °C, no APA enzymic activity was detected at the surface of the cells expressing either wtAPA or C43S, indicating that any APA already at the cell

surface at the beginning of the experiment had been internalized and/or degraded. However, after 30 min at 37 °C, significant enzymic activity was detected at the surface of the cells expressing wtAPA, and this level of activity remained relatively constant for up to 120 min. A remarkably similar level of cell-surface enzymic activity was observed for the C43S mutant after 30 min at 37 °C, as seen for the wt protein. These data indicate that the lack of the interchain disulphide bond has no significant effect on the rate of transport of APA from the ER to the cell surface.

DISCUSSION

The aims of the present study were to evaluate the roles of the interchain disulphide and the C-terminal 45 kDa domain in the activity, folding and intracellular trafficking of APA. For this purpose, the cysteine residue involved in the sole interchain disulphide was mutated to serine, and the C-terminal 45 kDa domain was deleted by introducing a stop codon in the protease-sensitive hinge region that lies just upstream of this domain. The resulting recombinant proteins were expressed in COS-1 cells and analysed by Western blotting, activity assays and confocal immunofluorescence microscopy.

Cys⁴³ in murine APA, equivalent to Cys³⁹ in porcine APA [11], was identified as the single cysteine residue involved in the disulphide linkage of the two monomers in the homodimer. Among the mammalian cell-surface peptidases, only five exist as disulphide-linked dimers. In addition to APA, these are the related type II membrane-spanning aminopeptidase TRH-DE [20], endothelin-converting enzyme-1 [28], membrane dipeptidase [29] and meprin [30,31]. In the case of APA, TRH-DE and membrane dipeptidase the cysteine residue involved in the disulphide link lies close to the membrane surface. In all three of these peptidases, mutation of the cysteine residue did not significantly affect the specific activity of the mutant enzymes. As seen here for APA, and reported recently for TRH-DE [20], the intermolecular disulphide link was not essential for trafficking and transport of the protein to the cell surface. Thus it would appear that the interchain disulphide is not essential for the correct folding and function of either APA or TRH-DE.

The role of the C-terminal 45 kDa domain was investigated by generating a mutant with a stop codon in the protease-sensitive hinge region which thus lacked the last 351 residues of the protein. On expression in COS-1 cells the resulting mutant protein, G594 Δ , was readily detected with a polyclonal antibody raised against murine APA [23]. Despite the fact that all the residues currently identified as critical for metal binding and catalysis in APA are located in the 107 kDa domain [14–18], and thus are present in the G594 Δ mutant, no enzymic activity was associated with the recombinant protein. This would suggest that either there is one or more critical catalytic residues within the C-terminal 45 kDa domain or that the C-terminal domain is required for correct folding of the remainder of the protein. The observation that the G594 Δ mutant failed to reach the cell surface and was retained within the ER would be consistent with the latter interpretation.

Interestingly, during the course of the present study, a short variant of APA was cloned from a rat hippocampus cDNA library that may result from alternative mRNA splicing [32]. This short variant of APA encodes a protein that contains the N-terminal cytosolic domain, the transmembrane region and the zinc-binding catalytic ectodomain, but lacks the C-terminal 45 kDa domain. In fact the protein is just 20 amino acids shorter than the G594 Δ mutant used in the present study. On expression of this short variant of APA in COS-1 cells, a polypeptide of 80 kDa was detected in total cell lysates, although the subcellular

location of the protein was not examined. Unlike the full-length protein, no APA enzymic activity was detected in the cells transfected with the short variant of APA, leading Lee et al. [32] to conclude that the structure of the C-terminal region of APA is essential for activity.

To date, no biological function has been ascribed to the C-terminal 45 kDa domain of any of the type II membrane-spanning aminopeptidases, other than the finding that porcine coronavirus-transmissible gastroenteritis virus binds to the C-terminal domain of APN between residues 717 and 813 [12]. However, our data clearly indicate that this domain is required for the correct folding and intracellular trafficking of APA and is likely to fulfil a similar function in the other members of this subfamily.

Several mammalian zinc metalloproteinases are synthesized with N-terminal pro-domains that precede the catalytic zinc-binding domain in the polypeptide chain, e.g. in the matrix-metalloproteinase ('MMP') [33] and the ADAMs (a disintegrin and metalloproteinase-like) families [34]. In these proteinases the pro-domain contains a free cysteine residue that co-ordinates to the zinc ion in the active site, thus keeping the proenzyme in an inactive form – the so-called 'cysteine-switch' mechanism. There is no evidence that the C-terminal 45 kDa domain plays a similar role in APA as, like the other type II membrane-spanning aminopeptidases, this peptidase is constitutively active. However, insect cells failed to secrete a deletion mutant of tumour necrosis factor- α convertase (ADAM17) encoding the catalytic domain, but lacking the pro-domain [35], and the pro-domain region of ADAM 12 was required for exit of the proteinase from the ER when expressed in COS-7 cells [36]. Thus the pro-domain is required for translocation from the ER to the Golgi apparatus, possibly by assisting the catalytic domain in folding to a secretion-competent conformation. From our data the C-terminal 45 kDa domain of APA would appear to fulfil a similar role, but, unlike the situation in these other metalloproteinases, it is not removed from the mature form of the protein and is located C-terminal to the catalytic zinc-binding domain.

In conclusion, we have shown that the interchain disulphide link is not required for either transport through the secretory pathway or the catalytic activity of APA. However the C-terminal 45 kDa domain appears to have a role in the folding and trafficking of the protein out of the ER.

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