Research article

Pro-domain removal in ASP-2 and the cleavage of the amyloid precursor are influenced by pH

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Abstract

Background: One of the signatures of Alzheimer's disease is the accumulation of aggregated amyloid protein, Aβ, in the brain. Aβ arises from cleavage of the Amyloid Precursor protein by β and γ secretases, which present attractive candidates for therapeutic targeting. Two β-secretase candidates, ASP-1 and ASP-2, were identified as aspartic proteases, both of which cleave the amyloid precursor at the β-site. These are produced as immature transmembrane proteins containing a pro-segment.

Results: ASP-2 expressed in HEK293-cells cleaved the Swedish mutant amyloid precursor at different β-sites at different pHs *in vitro*. Recent reports show that furin cleaves the pro-peptide of ASP-2, whereas ASP-1 undergoes auto-catalysis. We show that purified recombinant ASP-2 cleaves its own pro-peptide at ph 5 but not pH 8.5 as seen by mass spectrometry, electrophoresis and N-terminal sequencing.

Conclusion: We suggest that ASP-2 processing as well as activity are influenced by pH, and hence the cellular localisation of the protein may have profound effects on the production of Aβ. These factors should be taken into consideration in the design of potential inhibitors for these enzymes.

Background

Alzheimer's disease is a common age-related dementia which is characterised pathologically by the appearance of brain senile plaques [1,2] composed primarily of aggregated forms of Aβ. These are 39–43 residue peptides released following proteolytic processing of the transmembrane precursor glycoprotein, APP. The amyloidogenic pathway requires the APP to be sequentially cleaved by β and γ secretases [3,4]. β-Secretase cleaves APP close to the membrane to produce βAPPs (secreted), and the 12-kDa, C100 transmembrane stub, subsequently cleaved by γ-secretase to produce the Aβ peptide and a cytoplasmic fragment with very short half life. α-Secretase

cleaves APP within the Aβ sequence thus preventing its formation producing the N-terminal αAPPs domain and the 10-kDa membrane-localised C-terminal stub, C83. As aggregated Aβ is thought to promote neuronal death [5–7], the secretases represent potential drug targets for the treatment and/or prevention of AD. Presenilin-1 was suggested to be the ideal candidate for γ-secretase [8] whereas α-secretase has been characterised as ADAM10 disintegrin and metalloprotease [9]. Recently, several groups used expression cloning, genomic search, or purification and proteomic analysis [10–13] to clone and identify β-secretase as an aspartic endopeptidase (EC 3.4.23) named BACE (β-site APP clevage enzyme), ASP-2 (aspartic protease 2), or

memapsin 1. An additional candidate, ASP-1, BACE-2 or memapsin-2, has also been cloned [14–16]. The ASP-2 gene codes for a signal peptide, a pro-peptide (22-TQH-GIRLPLRSGLGGAPLGLRLPR-46), followed by the catalytic domain, a transmembrane segment and a cytoplasmic C-terminal tail. Several cysteine residues are present, six of which are in the lumenal domain which may form intramolecular disulphide bridges contributing to the folding of the active site [17]. Both ASP-1 and -2 are extensively glycosylated [18] and phosphorylated [19], and contain S-palmitoyl groups which may aid membrane anchorage[20].

Nearly all aspartic proteinases (EC 3.4.23.X) are synthesized as zymogens which are converted to active enzymes at acidic pH by proteolytic cleavage of the pro-segment [21,22]. This process is autocatalytic for some pro-enzymes such as pepsinogen [23–25] and cathepsins D [26] and E [27]. Furin is thought to cleave the ASP-2 pro-domain [20,28,29], though other pro-protein convertases were effective as well [20,30]. In contrast, ASP-1 has been demonstrated to cleave its own pro-peptide [31]. Although the pro-sequences of ASP-1 and ASP-2 are dissimilar we investigated whether ASP-2 also cleaves its pro-peptide. The activity of ASP-2 was assessed using the Swedish mutant form of APP [32] which is more readily cleaved by β-secretase than the wild type [33]. We investigated the effects of pH on ASP-2 processing and activity. We report that pH affects the pro-domain removal of ASP-2 *in vitro* as well as its site of β-secretase cleavage in APP to produce Aβ.

Results

Expression and Ni^{2+}-affinity purification of recombinant ASP-1 and ASP-2 in HEK293 cells

HEK293 cells were transfected with the β-secretase candidates ASP-1 or ASP-2 pcDNA3.1*myc*His clones and selected for stable transfection with Geneticin (800 μg/ml). HEK293 cell lines have been used by others to overexpress these enzymes [34,35] and have also been used to study the effects of β-secretase cleavage on recombinant APP [33,36]. Cell lysates from positive clones were analysed by SDS-PAGE and western blotting with an anti-*myc* antibody to detect the recombinant ASP-1 and ASP-2 proteins. The *myc* epitope is encoded in frame downstream of the C-terminal of the proteins followed by a 6-Histidine tag for affinity purification. Both recombinant ASP-1 and ASP-2 were expressed and detected by western blotting as 55–70-k and 65–80-k bands, respectively, higher than the estimated molecular weights of the pro-apoproteins (53.4 and 53.7-kDa including the pro-peptide domains), mainly due to glycosylation as predicted by their amino acid sequences and shown in recent reports by Charlwood *et al.*, 2001. Recombinant ASP-2 was purified by Ni^{2+}-affinity chromatography. Silver-stained SDS-PAGE confirmed the

purity of ASP-2 from other contaminants as seen in figure 1A; lower bands (dotted arrows) seen in the purified preparation are due to artefactual materials also present in the negative control sample (note the presence of higher molecular weight forms of ASP-2). Immunoblotting of the same preparation of ASP-2 detected with the anti-myc antibody, (figure 1, 1B) confirms purification of ASP-2. The most prevalent of these ASP-2 proteins was a 75-k band. We used an affinity-purified antibody raised against the C-terminal peptide of ASP-2 (anti-ASP-2) to characterise the recombinant proteins. This antibody was specific for ASP-2 and did not cross-react with ASP-1 or other cellular proteins as characterised by ELISA and western blotting.

Removal of N-linked oligosacharides increased the relative mobility of ASP-2 from 65–80-k to 50–60-k, a size resembling the estimated molecular weights of the core protein confirming glycosylation as reported by others either by suppression of N-glycosylation or deglycosylation [18,34]. When increased amounts of protein were loaded (as in figure 2, 2A) the anti-ASP-2 IgG detected ASP-2 bands of higher Mw, larger than 200-k or at Mr∼140-k, shown with triple and double arrows, respectively; the 140 k band was also visible by silver staining as indicated by the double arrow in figure 1A. These correspond in size to three or two ASP-2 molecules, respectively, implying ASP-2 oligomerisation into a trimer and dimer. The sensitivity of the putative dimer (140-k) to PNGase resulted in a reduction of size by ∼30-k, to yield a band migrating as 100-k, equivalent to the behaviour of 2 linked monomers. Reduction with DTT and β-mercaptoethanol (figure 2, 2B) almost completely removed the ∼200-k protein and increased the intensity of the 75-k band, but failed to affect the appearance of the putative dimer (140-k) suggesting strong intramolecular interactions. The oligomers were visible in freshly produced and purified ASP-2, though the 200-k protein was more prominent (data not shown) upon longer storage and at higher protein concentrations indicating that this was partly due to non-specific aggregation. An additional observation was the delayed mobility of ASP-2 in reducing conditions (figure 2, 2B) which is possibly due to loss of globular structure during reduction of intramolecular disulphide links described previously by Haniu *et al.*, 2000.

ASP-2 undergoes a two-step pro–peptide cleavage at acidic pH

We investigated the processing of ASP-2 at different pH values, since self-activation of other aspartic proteases such as pepsinogen [23] has been shown to be sensitive to pH. Pure ASP-2 was dialysed into a buffer of ph 5 or pH 8.5, immediately following purification. Incubation of the protein at room temperature was carried out prior to PAGE analysis. A sample before dialysis was also analysed alongside the ASP-2 at pH 8.5 and ph 5. A western blot

Figure 1
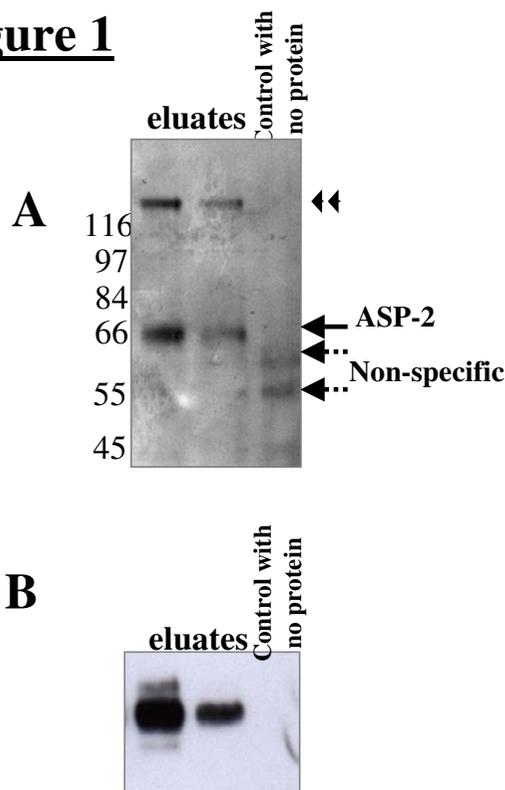
Figure 1
Ni^{2+}-affinity purification of ASP-2. A detergent extract was prepared from a total of 50 × 10^{6}ASP-2-transfected cells as described in the methods, in the presence of protease inhibitors and loaded on an equilibrated Ni^{2+}-charged agarose column, following dilution of the lysate to 0.5% (wt/vol) detergent and pure protein was eluted in fractions of 1 ml with 300 mM imidazole solution with 0.1% detergent. Eluates were loaded on a SDS-PAGE and proteins were detected by silver staining, (panel A) and anti-*myc* antibody developed by ECL (Panel B). Molecular weight standards are indicated in the first lane whereas the last lane shows a sample of elution buffer in loading buffer without protein. Typically, 20% yield was recovered following purification, amounting to approximately 40–60 μg of protein for a preparation from 50 × 10^6 cells. Solid arrows indicate the positions of the ASP-2 protein; the double solid arrow designates a pitative ASP-2 dimer.

analysis of these samples, depicted in figure 3, 3A, reveals a small shift in molecular weight of 3 k for the monomeric band and ~8 k for the dimer at ph 5. This was the first indication that cleavage of the pro-peptide (2.56 k) was occurring, which was also mirrored, by increased mobility in the putative dimer. PNGase treatment of the ph 5 and pH

8.5 ASP-2 samples revealed the deglycosylated proteins to be resolved in 3 and 2 bands (figure 3, 3B), respectively. The bands of the ph 5 sample were smaller (~3-k) than the ASP-2 bands in the pH 8.5 sample. The fact that PNGase did not reduce the ASP-2 protein to a single band of the unmodified protein is probably due to other modifications mentioned earlier, such as palmitoylation [20] and phosphorylation [18,19]. The triplet at ph 5 is consistent with deglycosylated proteins with the pro-segments removed (processed), with and without additional modifications. The highest band is possibly non-processed deglycosylated protein, because its intensity decreases upon incubation at 37°C.

Pure ASP-2 that had been incubated at ph 5 after adjustment of the pH with acetic acid was analysed by mass spectrometry to further detect the cleaved pro-peptide (figure 4). The sample dialysed at ph 5 could not be used since the small, cleaved fragments would have been dialysed out of the 4 k-exclusion membrane. Compared to pH 8.5 (profile B), the sample at ph 5 (profile A) displays two distinct peaks of size 1094 and 1352 Da. The peak at 1094 corresponds closely to the estimated m/z of the N-terminal fragment of the pro-sequence namely, QHGIRL-PLR (1089Da) (the N-terminal fragment from the Pro-sequence from which the N-terminal T had been removed-as found by N-terminal sequencing-see below, P1) and the peak at 1352 corresponds to a dehydrated form (-18 mass) of the penultimate N-terminal fragment of the Pro-sequence, P2 SGLGGAPLGLRLPR (1364 Da) respectively.

The mass spectra indicated firstly that pro-peptide cleavage occurs readily at ph 5 at two sites within the pro-domain. N-terminal sequencing of these protein preparations revealed the presence of two sequences in the pH 8.5 preparation, namely ETDEEPEE (mature protein starting at E46), and QHGIRL (immature protein with an intact pro-domain starting at Q23). The presence of protein without its pro-domain, within the pH 8.5 sample, is not surprising as this protein was purified from the cells which themselves are expected to process nascent ASP-2. In contrast, Edman degradation of the preparation at pH 5 gave the sequence ETDEEPEE, predominantly matured protein, which was produced by processing *in vitro* following purification and dialysis -at low pH, suggesting that pro-peptide cleavage of ASP-2 can occur at two sites by autocatalysis at ph 5 *in vitro*. To further ascertain autocatalytic cleavage we added EDTA to inhibit endogenous cleavage by furin-like proteases as well as the BACE inhibitor (H-Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-Sta-Val-Ala-Glu-Phe-OH, Bachem, UK) to the incubations at ph 5. Mass spectrometric analysis of those samples (figure 5) showed that EDTA had no effect on the pro-peptide cleavage of ASP-2 as the previously identified peaks of 1094 and 1352 Da are still present in this sample as they are in

Figure 2

A

B
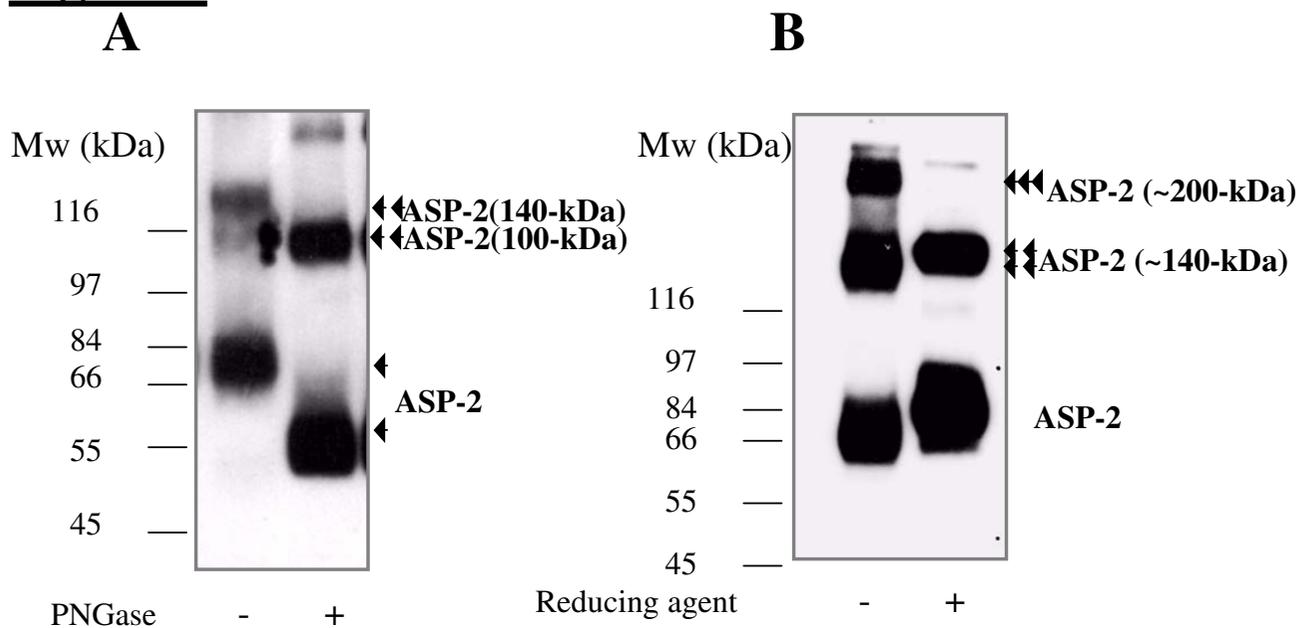
Figure 2
ASP-2 post-translational modifications; glycosylation and disulphide linkagesPanel A: ASP-2 samples were denatured by boiling in the presence of SDS and prior to treatment with PNGase, according to the manufacturer's protocol. Glycosylated and deglycosylated samples are depicted. A reduction in size of ~15-k is seen in the presence of PNGase due to the removal of N-linked sugars. Note the presence of higher ASP-2 bands, indicated with double arrows, which are similarly, deglycosylated. **Panel B**: ASP-2 samples were denatured in the presence or absence of a mixture of 10 mM DTT and 2% (vol/vol) β-mercaptoethanol designated as reducing agent. A reduction in the amount of the largest ASP-2 protein (marked with 3 arrows) is noted in the presence of reducing agent as well as a reduction in the mobility of ASP-2 proteins. Ni^{2+}-affinity purified ASP-2 was used at a concentration of 40 nM. Western blots were detected with anti-ASP-2. Bold arrows designate larger putative, dimer and multimer.

the control which did not contain any inhibitor. Most importantly incubation of ASP-2 with the BACE inhibitor resulted in the disappearance of these peaks suggesting that pro-peptide cleavage is a result of autocatalysis.

Diverse cleavage of APPswe by ASP-2 at pH 5 and pH 8.5
The activity of recombinant ASP-2 was assessed using the Swedish mutation APP (APPswe) purified using the anti-CT15 IgG from stably transfected HEK293 cells. Initially [^{35}S]-labelled C-terminal APPswe polypeptides were iso-

lated by immunoprecipitation with anti-CT15 IgG and protein-A-agarose and resuspended in reaction buffer (pH 8.5) prior to the addition of ASP-2. Figure 6, 6A, depicts an autoradiogram of a 16.5% Tris-Tricine gel where APPswe was incubated with or without ASP-2. In the absence of ASP-2 we noted the presence of a small band of ~9.25 (± 0.6, n = 3)-k corresponding to the C-83 APPswe fragment produced by endogenous α-secretase cleavage. The addition of ASP-2 resulted in an additional band at ~12.4-k consistent with the estimated size of the C-termi-

Figure 3.
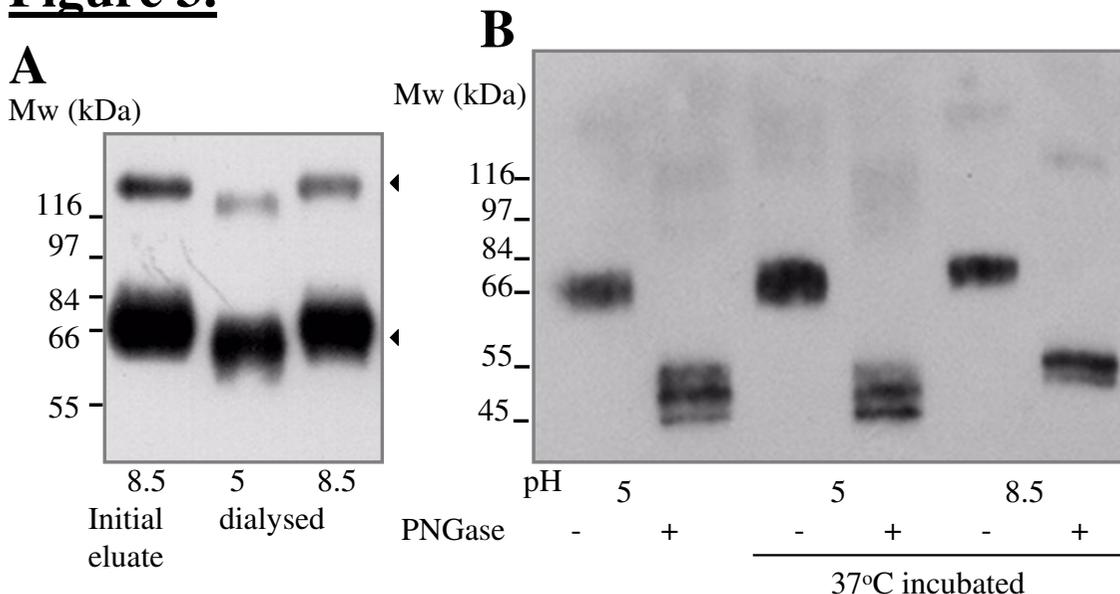
Figure 3
Reduction in size of ASP-2 upon incubation at acidic pH proposes autocatalytic pro-peptide cleavagePanel A:
Pure ASP-2 samples were dialysed at ph 5 and pH 8.5 immediately following purification and subsequently incubated for 2 hrs at room temperature as described in the methods. The initial non-dialysed sample was also incubated before all the samples were all analysed by SDS-PAGE and western blotting. A reduction of the size of ASP-2, ph 5 was observed compared to the initial eluate and the pH 8.5 samples. Note the presence of higher Mw bands ~140-k which also displayed increased mobility at ph 5. **Panel B:** The same ASP-2 samples dialysed at different pH were treated with PNGase for better resolution of the protein bands. The samples dialysed at pH 5 were either not incubated (first two lanes) or pre-incubated before PNGAse treatment. These samples needed to be titrated to pH 8.5 before deglycosylation. Similarly samples at pH 8.5 were treated or not with PNGase as indicated on the figure. Compared to the respective sample at pH 8.5, the ph 5 deglycosylated ASP-2 reveals the lowest ASP-2 band visible on this blot at ~50-k, possibly mature protein without the pro-peptide and sugars, suggesting maturation of ASP-2. Pre-incubation of the sample at 37°C increased the intensity of this band compared to the samples that were not pre-incubated implying that its appearance is time-dependent on incubation of the protein at a certain pH. For both panels sizes of molecular weight standard proteins are indicated on the left. Both panels were probed with anti-ASP-2 IgG and developed with ECL.

nal stub after cleavage of β-secretase at the Aβ (1–40/42) site. To confirm the identity of this band the same reactions were carried out with non-radiolabelled substrate and the proteolytic fragments were detected by western blotting using either the anti-CT15 antibody (figure 6, 6B top panels) or the 6E10 (figure 6, 6B bottom panels) antibody, specific for the N-terminal of Aβ. These reactions were carried out both at optimum pH for aspartic protease activity, pH 5, as well as at pH 8.5 for comparison and the whole sample was analysed. ASP-2 was also pre-incubated at the respective pH. The arrows indicate the α-secretase cleaved fraction to be present in both pH 8.5 and ph 5. Two fragments (12.39 (± 0.24, n = 3) and 10.43 (± 0.45,

n = 3)-k, designated β_1-C100 and β_2-C90, respectively) appear from ASP-2 cleavage at ph 5, consistent with fragments occurring by β-secretase cleavage at the Aβ (1–40/42)-producing β_1 site starting with D1, and the E11, β_2-site resulting in Aβ (11–40/42). Incubation of the purified recombinant ASP-2 with the APPswe at ph 8.5 also produced cleavage at the β_1-site as only the 12.39 k band is visible. The 12.4-k band was also labelled by the 6E10 antibody further confirming that it is the result of APPswe cleavage at the β_1-site. These results reveal that the activity of ASP-2 is influenced by pH which apparently governs the site at which ASP-2 cleaves the APPswe protein.

Figure 4.
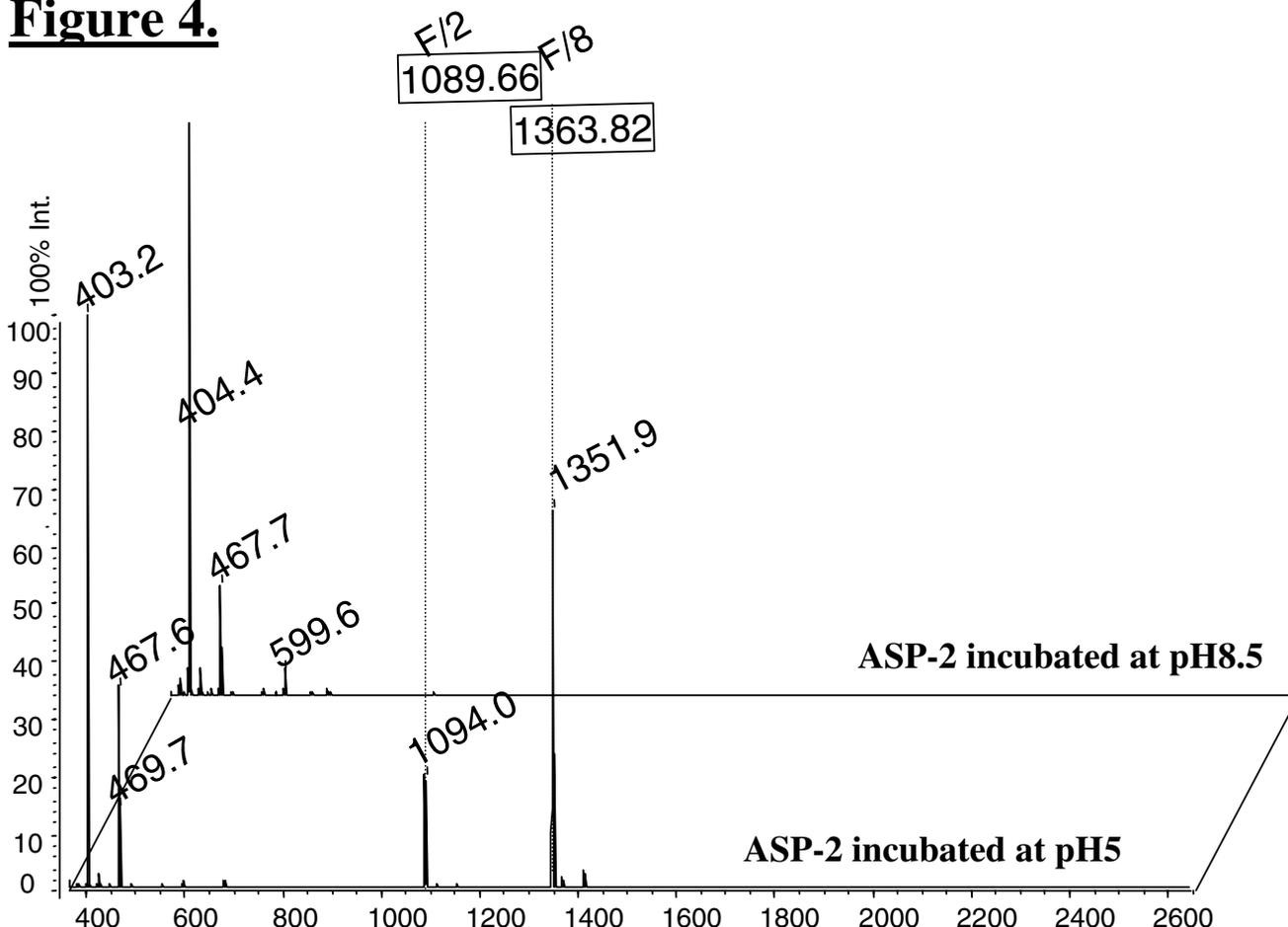
Figure 4
Mass spectrometry of ASP-2 samples at ph 5 and 8.5 Pure ASP-2 samples that were prepared as described were titrated to pH 5 and pH 8.5 and incubated at room temperature. The first panel shows a profile following incubation at pH 8.5 and the second panel, at pH 5. The pro-peptide has an estimated Mw = 2535-Da. the ph 5 contains two peaks of 1094.4 and 1352.3 that are not present at pH 8.5. These correspond closely to the estimated mw of two fragments of the pro-sequence namely, QHGIRLPLR (1089) and SGLGGAPLGLRLPR (1364). Solid lines display the detected peaks in each sample. Dotted lines indicate the estimated positions corresponding to the pro-peptide fragments as calculated above; these estimated values of these peaks are boxed. The profiles seen indicate percentage of intensity against mass/charge ratio.

Discussion
Understanding what governs the activity of ASP-2 toward the APP substrate at a cellular and molecular level could facilitate the discovery of compounds that could inhibit the development of AD. It was therefore important to study the maturation, processing and activity of ASP-2 and relate that to the proteolytic events that lead to Aβ production. In agreement with several other reports [15,17,18,37] glycosylated proteins were successfully produced in HEK293 cells displaying mobility of ~55–70 and 65–80-k, for ASP-1 and ASP-2, respectively. The broadness of these bands suggested a heterogeneous protein population, as a result of variable translational modification which was mainly due to glycosylation and possibly pro-processing and as also detected by others in the fully glycosylated, endoglycosidase H-resistant ASP-2 forms [17,18,31,34]. Using a new anti-ASP-2 IgG, we detected the presence of higher molecular weight proteins in the purified preparations, by analysis under denaturing SDS-PAGE, which corresponded in size to ASP-2 homo-multimers. Oligomerisation has also been identified [38] for pepsin [39] and cathepsin E [27,40]. A putative dimer was clearly noted at ~150-k, corresponding to (ASP-2)$_2$, which was also sensitive to deglycosylation giving rise to a

Figure 5.
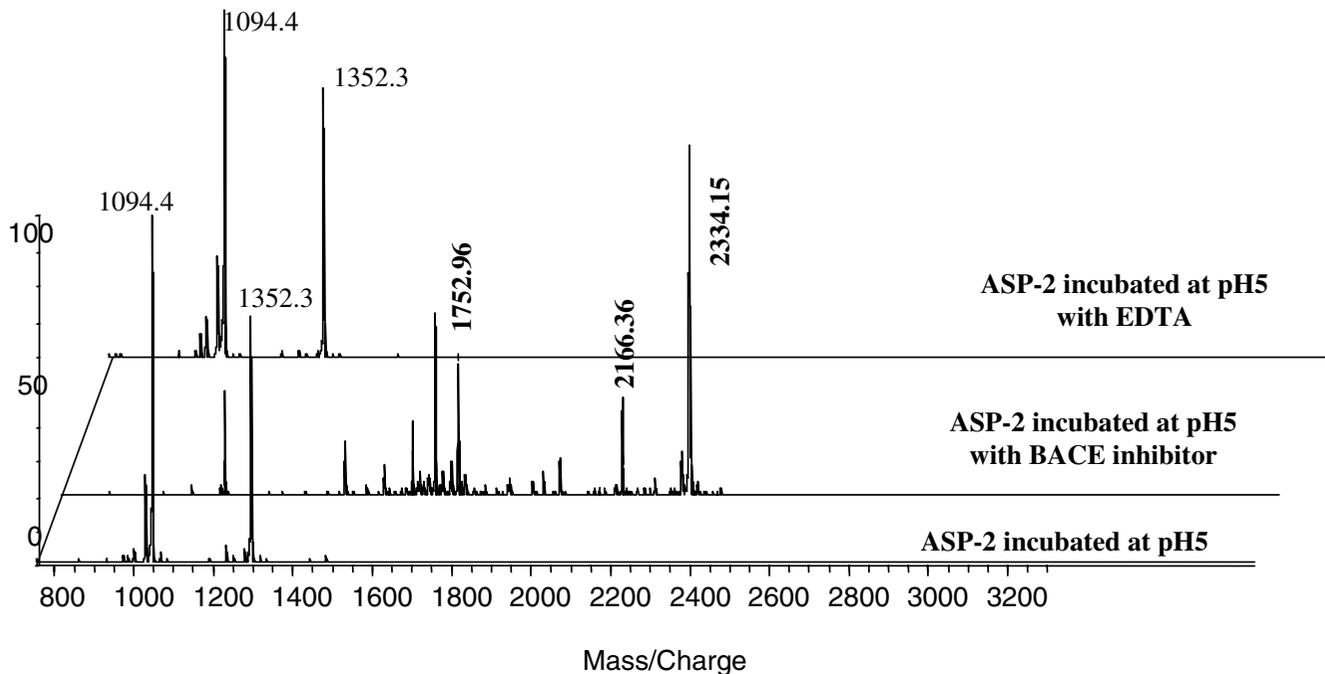
Figure 5
BACE inhibitor reduces pro-peptide cleavage of ASP-2 at pH 5 Pro-peptide cleavage of ASP-2 results in two peaks of 1094.4 and 1352.3 not present in the sample incubated with the BACE inhibitor (Mw = 1651 g/mol). These peaks are present when ASP-2 was incubated with EDTA. Samples of pure ASP-2 were prepared as described. Titration to ph 5 was carried out at 4°C and immediately EDTA (5 mM) or BACE inhibitor (20 μM, according to the protocol in Hussain *et al.* 2001) were added. Control ASP-2 at ph 5 contained no inhibitor. The peak of 1750 seen in the BACE inhibitor sample is also present in the inhibitor profile (not shown) and in agreement with its molecular weight is the inhibitor itself; additional larger bands are also present the inhibitor control sample. Following incubation samples were analysed by mass spectrometry as described in the methods.

change in mobility which corresponded to two monomers. The appearance of the largest ASP-2 forms was partly dependent on concentration and length of storage suggesting this is partly due to non-specific aggregation. In contrast, the putative dimers (~140 kDa) were evident even in fresh samples and were mainly insensitive to reducing agent providing evidence for the existence of strong non-disulphide protein-protein interactions not broken by high salt and non-ionic detergents used during purification or the strong denaturing conditions of SDS-boiling used during SDS-PAGE. Recently a laboratory in Germany reported the isolation of native active ASP-2 dimers from human brain homogenates, (Multhaup, G. and colleagues from Germany, unpublished data[41]).

These native dimers were similar to those observed in our preparations as they were SDS-resistant.

Activation of acidic proteases by removal of a pro-peptide occurs either by autocatalysis as in the case of pepsinogen [23,25] and other aspartic proteases, or the action of other proteases, like Cathepsins S and L which activate Cathepsin C [38]. Several reports by other laboratories, showed furin to successfully cleave the ASP-2 pro-segment [20,29] also demonstrating that recombinant ASP-2 does not cleave its own synthetic or recombinant fusion pro-segments. However, Hussain *et al.* (2001), have recently shown that minimal cleavage of ASP-1 pro-domain is achieved by ASP-2 whereas ASP-1 displayed a unimolecu-

Figure 6.
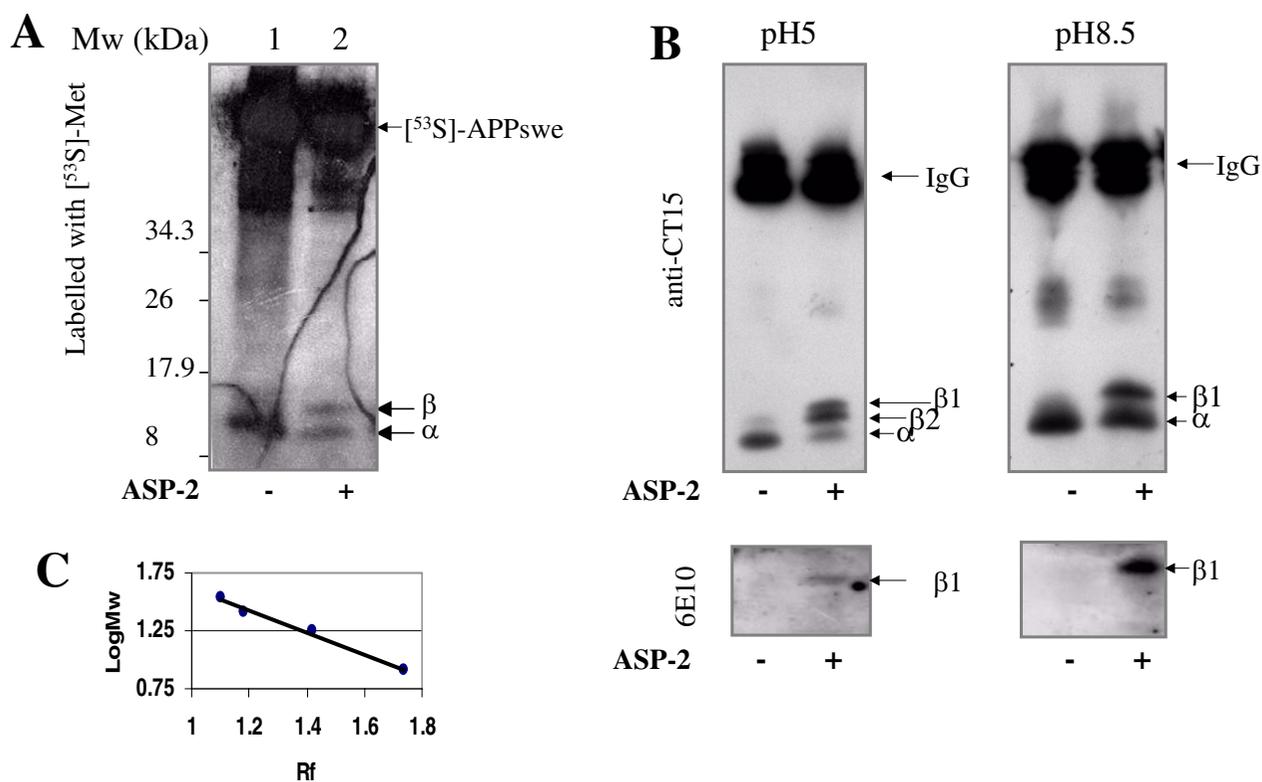
Figure 6
***In vitro* cleavage of APPswe by pure recombinant ASP-2; differential cleavage occurs at acidic or alkaline pHPanel A:** anti-CT15 IgG immunoprecipitated (^{35}S)-labelled APPswe (2 μM APPswe) was incubated with either just buffer (lane 1) or with pure ASP-2 at 6 nM (lane 2). The total reaction was analysed on a16.5% Tris-Tricine gel, stained and fixed, and amplified as described in the methods. The gel was then dried and exposed on a Kodak-X-Omat film. **Panel B:** Immunoprecipitated APPswe samples as above were incubated with or without ASP-2 as described above at pH 5 or ph 8.5 as indicated in the figure. The blots were developed with anti-CT15 IgG that is specific for the C-terminal of APP. The bottom panels are the same reaction probed with the 6E10 clone antibody, which was raised specifically against the first residues of Aβ and thus only recognises β-secretase cleaved fragments that produce Aβ. **Panel C:** A typical gel calibration standard curve for the low molecular weight standards (carbonic anhydrase, 34.3 kDa, soybean trypsin inhibitor, 26 kDa, Lysozyme, 17.9, kDa, Aprotinin, 8 kDa, insulin, 4 kDa) plotted as the Log$_{10}$ of molecular weight against Rf, for size estimation of peptides. For all panels α- and β-secretase cleavage are designated by arrows and the letters α or β for the respective secretase cleavage. β$_1$ and β$_2$ indicate cleavage at the Aspartate (1) and the Glutamate (11) sites of Aβ, producing the C-100 and a C-90 fragment respectively.

lar pro-peptide auto-removal. Here we demonstrated *in vitro* cleavage of the ASP-2 pro-peptide in full-length, pure, recombinant proteins instigated by incubation of ASP-2 at acidic pH, implying autocatalysis. We propose that cleavage occurs at two sites RLPLR↓S$_{32}$ and RLPR↓E$_{46}$ within the pro-domain, suggesting a two-step cleavage resembling the mechanism of maturation of other aspartic pro-

teinases by autocatalysis (for a review see Richter *et al.*, 1998). We have not investigated to see if the pro-sequence removal from ASP-2 is a unimolecular reaction, or due to the attack of a second ASP-2 molecule. APP cleavage at both the Asp1 (β1-site) as well as the Glu11 (β2-site) positions shows that ASP-2 has a preference to cleave N-terminally to an acidic residue as is the case for the pro-

peptide cleavage. However, although the RXXR↓ found in the ASP-2 pro-sequence is thought to be a minimal sequence required for a pro-peptide convertase [43] furin prefers to cleave C-terminal to two adjacent basic residues. Recent results [30] showed that ASP-2 processing takes place in furin-deficient cell lines and suggested that other pro-peptide convertases are involved whereas Bennett *et al.*, 2000 observed that a proportion of recombinant ASP-2 is processed in cells untransfected with furin. Further to those findings we suggest that ASP-2 can remove its pro-domain by autocatalysis *in vitro*, as the BACE inhibitor inhibits this process whereas EDTA does not, so that an additional pro-peptide convertase may not be required. Hence, it is possible that autocatalysis could be preferred under conditions of acidic pH, or in cells or cellular sub-compartments where pro-peptide convertases are not expressed. Although other reports have shown that ASP-2 does not cleave its pro-domain *in vitro*[20,31] the difference between those and our observations could be due to differences in the protein preparations. We used full-length pure proteins for enzyme and substrate whereas, synthetic peptides as well as truncated fusion proteins were employed in those studies. These factors in addition to differences in expression systems (and modifications) could influence the results observed herein as encountered for pepsinogen, which displays different activation kinetics as a fusion than as a whole protein [23]. To conclude we suggest that ASP-2 may cleave its pro-peptide in a two-step mechanism in the absence of any other protease. Multiple-step removal of pro-sequences has been seen with other aspartic proteinases, where complete activation sometimes requires cleavage at more than one site [42].

We showed *in vitro* β-secretase activity of ASP-2, producing a C-terminal APP fragment, Mw 12-kDa, consistent with APPswe cleavage at the β1-site. This cleavage occurs efficiently at ph 8.5 although the optimum pH of ASP-2 activity is acidic. We must mention though that a proportion of ASP-2 in the ph 8.5 preparation is processed (as purified from the cells) and could contribute to the β-cleavage of APP we observe at ph 8.5. Cleavage of APPswe at ph 5, however was different to pH 8.5 as it resulted in two C-terminal APP fragments each corresponding to cleavage at the Aβ-producing site (β_1), and the β_2-site described by Creemers *et al.* (2000), to be the most favourable producing N-terminally truncated, highly cytotoxic [44], Aβ peptides (11–42) found in patients with the Flemish mutation. Creemers et al. (2000) suggested that cleavage at these sites is governed by the expression levels of ASP-2, *in vivo*, rather than its maturation. In addition to that we report here that our results show that pH can also determine the site of APPswe β-cleavage by ASP-2. Results from our laboratory [45] have localised β-cleavage of APP in the TGN and early endosomes, and recently Huse *et al.*

(2000) showed TGN localisation for ASP-2 indicating that these compartments are loci for β_2-site cleavage of APP as their pH is acidic. On the other hand, localisation in the endoplasmic reticulum and early Golgi imply processing at neutral to alkaline pH that according to our results gives cleavage at the β_1-site and production of Aβ (1–40/42).

Conclusions
Taken together, our results of ASP-2 pro-peptide processing and diverse APP proteolysis, both happening at ph 5, imply an additional mode of cellular control of APP β-cleavage whereby subcellular localisation of APP and ASP-2 in different compartments where the pH is either acidic or alkaline influences the proteolytic processing of both ASP-2 as well as of APP by ASP-2 itself and consequently the production of Aβ. Thus APP cleavage at ph 5 is relevant to Alzheimer's in patients with the Flemish and Dutch mutations who demonstrate increased deposition of N-terminally truncated Aβ, resulting from APP processing at the β_2-site.

Methods
Materials
Culture media and antibiotics were from Life Technologies, Paisley, UK. All culture plasticware, were from Nalge Nunc International, Loughborough, UK. Amino Acids for peptide synthesis were from Nova Biochem, Nottingham, UK. Freunds Adjuvant, standard chemicals, 9E10 clone ascites fluid (anti-*myc*), Triton X-100, Nonidet P40, and Kodak X-Omat film were purchased from Sigma-Aldrich Company Ltd, Dorset, UK, 6E10 (anti-Aβ) from ID labs, P.O. Box 3556, Glasgow, Scotland, United Kingdom. C-18 ZIP tips, Centricon-30 concentrators, PVDF membrane were purchased from Millipore. Automated N-terminal peptide sequencing was carried out by AltaBiocscience, University of Birmingham, Edgbaston, UK. All secondary horseradish peroxidase-linked anti-species IgG, Enhanced chemiluminescence reagents, ECL Hyper film, Superdex 200HR, Sephadex G-10, were from Amersham Pharmacia Biotech, UK Ltd, Buckinghamshire, UK, whereas the Ni^{2+}-affinity resin NiNTA was from Novagen, Nottingham, UK. Pfx2 Lipids were initially purchased from Invitrogen Life Technologies, Paisley, UK. Ready-made Tris-Tricine gels and low molecular weight Kaleidoscope Polypeptide Standards were from BIORAD, Hemel Hempstead, UK. Prestained protein molecular weight markers and Acrylamide mixture (30% acrylamide: 0.8% (wt/vol) bisacrylamide) from National Diagnostics, East Riding of Yorkshire, UK. Protease Inhibitors and N-glycosidase-F were purchased from Roche Molecular Biochemicals, East Sussex, UK.

cDNA constructs and transfections
pCEP4APP695 Hygromycin resistant, APPswe Neomycin resistant. pcDNA3.1*Myc*His (A) ASP-1 and 2 were kindly

donated by the GlaxoSmithKline laboratories, Harlow, UK. The clones were engineered in frame with the Histidine tag and the Myc epitope, which in that order are downstream of the coding sequence of the Aspartic proteases (ASP).

Cell manipulations, expression protein purification

For the production of stable colonies HEK293 cells were transfected using pFX2 lipids as described by the manufacturers for 2.5×10^4 cells per transfection reaction. Positive transfectants were identified and selected using Geneticin. Colonies were screened by SDS-PAGE and Western Blotting analysis of cell lysates prepared by direct suspension of the cells in SDS, 0.1% (wt/vol) followed by sonication and suspension in gel loading buffer. HEK293 cells were cultured and propagated as described in Frears *et al.*, 1999. Cell lysates for affinity- or immuno-purification were prepared by lysis of the cells with frequent agitation for 2 hrs at 4°C in phosphate buffered saline (PBS: 150 mM NaCl 2.7 mM KCl, 10 mM Na2HPO4 and 1.75 mM KH_2PO_4, at pH7) supplemented with protease inhibitors, 0.5 mM EDTA, 1 μM Leupeptin, 1 μM Pepstatin and 1 mM PMSF, and Triton X-100, 1% (wt/vol) and NP40, 1% (wt/vol). For affinity purification of ASP-2 EDTA and pepstatin were omitted. Prior to purification the lysate was diluted to 0.5% (wt/vol) detergent with the above buffer/inhibitor solution. Ni^{2+} – affinity purification was carried out as recommended by the manufacturers using a pre-charged Ni^{2+} column, 1 ml of settled bed resin for 50×10^6 cells with minor alterations; all binding and washes (with increasing imidazole concentrations, pH 7.4) were carried out in PBS buffer supplemented with 0.2% (wt/vol) NP40 to reduce non-specific binding. Elution was achieved with 300 mM imidazole/0.2% (wt/vol/) detergent.

Protein incubations

Freshly purified ASP-2 was dialysed immediately into 50 mM CH_3COONa, 20 mM NaCl, 0.2% (wt/vol) NP-40, at either pH 8.5 or 5, at 4°C overnight, in the presence of 1 μM Leupeptin and 1 mM PMSF. The protein solution was then incubated at room temperature for two hours before SDS-PAGE, *in vitro* cleavage reactions, or N-terminal sequencing. For Mass Spectrometry the proteins were first dialysed in the above buffer at pH 8.5, and then the pH was adjusted with dilute acetic acid to ph 5. For N-terminal peptide sequencing the samples were immobilised on a PVDF membrane.

Immunoprecipitations and in vitro cleavage assays

Anti-CT15 (2 μg/ml) was used to immunoprecipitate (Stephens and Austen 1996) APPswe from HEK293 cell lysates/extracts prepared as above. The pellet was resuspended in reaction buffer (20 mM CH_3COONa, ph 5, 50 mM NaCl, 0.2% NP40, 1 mM PMSF and 1 μM Leupeptin) prior to the addition of ASP-2 (purified and pre-incubated in reaction buffer at designated pH). Reactions were carried out for 2 hours at 37°C. Samples were analysed by SDS-PAGE on a 16.5% Tris-Tricine gel as described by the manufacturers followed by western blotting and detection with anti-CT15 IgG (0.5 μg/ml) or 6E10 (4 μg/ml).

Mass spectrometry

Purified ASP-2 which had been dialysed at pH 8.5 as described above was pre-incubated in the reaction buffer titrated at the designated pH without detergent prior to absorption to a C-18 ZIP tip (Millipore) equilibrated with 0.1% (vol/vol) trifluorocetic acid, (TFA). Peptides were eluted with 50% (vol/vol) acetonitrile and analysed on an Axima-CFR KRATOS Mass Spectrometer after addition of an equal volume of 10 mg/ml α-cyano-4-hydroxycinnamic acid (Sigma) in 50% (vol/vol) acetonitrile. Angiotensin 1, the dimeric form of α-cyano-4-hydroxycinnamic acid, and neuropeptide Y were used as external calibrants spotted on an adjacent spot on the chip.

Peptide synthesis and IgG production

The C-terminal peptide of ASP-2 (CLRQQHDDFADDIS-LLK residues 482–501) was synthesised on a Milligen 9050 synthesizer using Fmoc N-terminal protection and, after deprotection and release from resin was purified by HPLC on a column of Vydac C4 with gradients of acetonitrile in 0.1% TFA. In brief, bovine thyroglobulin was activated with succinimidyl 4-(N-maleimido-methyl) cyclohexane-1-carboxylate, desalted on a Sephadex G-10 column and coupled to the HPLC-purified synthetic peptide utilising its free-cysteine as described in [45]. The coupled peptide was used to immunise rabbits and its immunoreactivity was screened by ELISA using immobilised antigenic peptide. Reactive serum was purified on a peptide-conjugated Sepharose column, aliquoted and stored at -20°C. Pure anti-ASP-2 IgG was tested for its specificity towards ASP-2 by western blotting of extracts of recombinant clones as well as mock-transfected cells using purified IgG pre-incubated or not with excess antigenic peptide.

Metabolic labelling and detection of recombinant proteins

80% Confluent flasks of HEK293 APPswe-trasfected cells ($\sim5 \times 10^6$ cells) were depleted of Methionine by incubation in Met-free media supplemented with FCS and Glutamax and pyruvate for 1 hr. The same media was then supplemented with $[^{35}S]$-Met at 50 μCi/ml in 5 mls, and incubated for 3 hrs. Cells were first rinsed with PBS and then lysed and extracted as described earlier. The $[^{35}S]$-labelled protein was immunoprecipitated as described earlier and reactions with ASP-2 were carried out. These were analysed by 7% SDS-PAGE, soaked in Amplify^{TR} for 15 minutes and dried before exposure to Kodak X-Omat film.

SDS-PAGE and Immunoblotting

Proteins were first denatured by boiling in SDS sample buffer (0.25 M Tris-HCl, pH 8.8, 2.2% (wt/vol) SDS, 10% (v /v) Glycerol, 0.05% (wt/vol) bromophenol blue) with or without reducing agent (1% β-mercaptoethanol and/or 10 mM DTT) and electrophoresed on SDS-PAGE gels prepared as described by the manufacturers of the acrylamide solution (National Diagnostics) and run using a BioRad Mini-Gel system at 20 mA per gel. The gels were subsequently transferred onto PVDF membrane using a Biorad semi-dry blotter in 25 mM Tris, 192 mM Glycine, and 20% (vol/vol) methanol. Antibody detection was carried out by immunoblotting as described in Stephens and Austen (1996). For silver staining, gels were fixed with methanol, 40% (vol/vol) and acetic acid 10% (vol/vol), for one hour, before they were soaked for 30 min in DTT (0.5 μg/ml). The gels were rinsed twice with water and soaked for one hour in $AgNO_3$, 0.1% (wt/vol) prior to development with a solution of Na_2CO_3, 3% (wt/vol) and formaldehyde, 0.0185% (vol/vol).

PNGase treatment

Cellular extracts or purified ASP-2 were first heat-denatured in the presence of 0.5% (wt/vol) SDS in PBS, pH8 and the rest of the procedure was carried out as described by the manufacturers.

Abbreviations

Aβ,β amyloid peptide; APPswe, Mutations in APP in which KM is replaced by NL in Swedish family with familial Alzheimer's dementia; DMEM, Dulbecco's modified eagle medium; ECL, Enhanced chemiluminescence; HRP, Horseradish peroxidase; NP40, Nonident P 40; Octylglucoside, Octyl-β-D-glucopyranoside; PMSF, Phenylmethylsulfoxide; PNGase F, N-Glycosidase-F; PVDF, Polyvinylidene Fluoride; SDS-PAGE, Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Authors contributions

CS carried out the molecular and cell biology as well as the design, biochemical analyses of this study and drafted the manuscript. CL carried out protein purification and cell manipulations. BMA conceived the study, participated in its design and coordination and also carried out the mass spectrometry analysis.

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