Prodomal Processing of Asp1 (BACE2) Is Autocatalytic*

Ishrut Hussain‡, Gary Christie‡, Klaus Schneider§, Stephen Moore‡, and Colin Dingwall‡

From the §Neurology Centre of Excellence for Drug Discovery and the ¶Department of Computational and Structural Sciences, GlaxoSmithKline, New Frontiers Science Park, Third Avenue, Harlow, Essex CM19 5AW, United Kingdom

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Alzheimer's disease is a neurodegenerative disorder characterized by the deposition of the amyloid β-peptide in the brain as amyloid plaques (1, 2). The amyloid β-peptide is generated through sequential proteolytic processing of the amyloid precursor protein (APP)β by β- and γ-secretases is central to the etiology of Alzheimer's disease. The highly elusive β-secretase was recently identified as a transmembrane aspartic proteinase, Asp2 (BACE). The Asp2 homolog Asp1 (BACE2/DRAP) has also been reported to exhibit β-secretase cleavage of amyloid precursor protein. Most aspartic proteinases are generated as inactive proenzymes, requiring removal of the prodomain to generate active proteinase. Here we show that prodomain processing of Asp1 occurs between Leu62 and Ala63 and is autocatalytic. Asp1 cleaved a maltose-binding protein-Asp1 prodomain fusion protein and a synthetic peptide at this site. Mutation of one of the conserved catalytic aspartic acid residues in the active site of Asp1 to asparagine (D110N) abolished this cleavage. Mutation of P1’ and P2’ residues in the substrate to phenylalanine reduced cleavage at this site. Asp1 expressed in cells was the mature form, and prodomain processing occurred intramolecularly within the endoplasmic reticulum/early Golgi. Interestingly, a proportion of mature Asp1 was expressed on the cell surface. When full-length Asp1(D110N) was expressed in COS-7 cells, it was not processed, suggesting that no other proteinase can activate Asp1 in these cells.

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¶ To whom correspondence should be addressed. Tel.: 44-1279-622651; Fax: 44-1279-622555; E-mail: Colin_Dingwall@sbphrd.com.

The abbreviations used are: APP, amyloid precursor protein; MBP, maltose-binding protein; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometric/spectrometry; Ahx, aminohexanoic acid; dnp, 2,4-dinitrophenyl; HPLC, high performance liquid chromatography; ER, endoplasmic reticulum.

EXPERIMENTAL PROCEDURES

cDNA Constructs—Asp1 (base pairs 61–276) and Asp2 (base pairs 64–225) fragments spanning the prodomain cleavage site (Ala21–Tyr22) were amplified by polymerase chain reaction, in which the reverse oligonucleotide encoded a His6 tag followed by a termination codon at the 3′-end. The fragments were cloned in frame to the maltose-binding protein (MBP) sequence at the BamHI/HindIII sites in pMalc2 (New England Biolabs, Inc.) to generate MBP-Asp1pro-His6 and MBP-Asp2pro-His6. Sequences were confirmed by dideoxy nucleotide sequencing. Generation of the Asp1-Fc/Signal plgPlus construct has been described previously (13). The Asp2-Fc construct was generated by subcloning the Asp2 cDNA (base pairs 61–1359) into the HindIII/XhoI sites in the Signal plgPlus vector (R&D Systems). Mutant constructs and the MBP-Asp1 prodomain construct terminating at the prodomain cleavage site were generated by QuikChange™ site-directed mutagenesis (Stratagene) and sequenced.

Expression and Purification of Fusion Proteins—Asp1-Fc and Asp2-Fc were expressed in COS-7 cells and purified from the culture medium as described previously (13). The MBP fusion proteins were expressed in Escherichia coli and purified. Briefly, protein expression was induced in 800 ml of LB medium with 0.3 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37 °C. The cells were harvested by centrifugation at 6000 × g for 10 min and lysed by sonication in 50 ml of 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10 mg of lysozyme, and 0.1 mM phenylmethylsulfonyl fluoride at 4 °C. The cell lysate was centrifuged at 10,000 × g for 20 min, and the supernatant was incubated with amylose resin (5 ml) at 4 °C for 1 h. The amylose resin was packed into a disposable column and washed twice with 10 ml of 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride. The protein was eluted with 10 ml maltose, 20 ml Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride. Fractions (1 ml) containing protein were pooled and dialyzed against 10 ml Tris-HCl (pH 6.8), 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride.

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Cleavage Assays—MBP fusion proteins (2.5 μg) were incubated with Asp1-Fc or Asp2-Fc (25 nm) in 100 mM sodium acetate (pH 4.5–6.5) in a final volume of 60 μl at 37 °C, and an aliquot (12 μl) was removed at the indicated times. An equal volume of gel sample buffer was added to terminate the reaction, and samples were analyzed on 8% Tris/glycine gels (Novex). Proteins were visualized by staining with Coomassie Brilliant Blue.

Mass Spectrometric Analysis of MBP-Asp1 Fusion Proteins Incubated with Asp1-Fc—Cleavage assays were set up as described above. 10–30 μl of the incubation solutions were purified using 10-μl pipette tips loaded with a C18 resin (ZipTip, Millipore Corp.) employing the protocol supplied by the manufacturer. For MALDI-MS analysis, peptides were directly eluted from the ZipTip onto the MALDI plate with matrix solution (a-cyano-4-hydroxycinnamic acid in water/acetonitrile (2:1) containing 0.1% trifluoroacetic acid). For Nanospray MS analysis, peptides were eluted from the ZipTip using water/acetonitrile (1:1) containing 5% formic acid. The eluate was immediately loaded into a Nanospray microcapillary (type N, Protana, Odense, Denmark). MALDI-time-of-flight MS was performed on a ToFSpec SE instrument (Micromass, Manchester, United Kingdom). Nanospray MS and MS/MS experiments were performed on an orthogonal acceleration quadrupole time-of-flight mass spectrometer (Q-ToF, Micromass) equipped with a Z-spray ion source for Nanospray analysis.

Peptide Cleavage Studies—Peptide ADGLALALEPALAK(Ahx-dnp)G (50 μM) was incubated with Asp1-Fc or Asp2-Fc (50 nm) in 50 mM sodium acetate and 20 mM NaCl (pH 4.5) for 2 h in a final assay volume of 30 μl. The reaction was terminated by the addition of 4 volumes of 5% trifluoroacetic acid. The assay components were loaded onto a Poros R1 column (Applied Biosystems) in 0.08% trifluoroacetic acid and eluted with a linear gradient of 0.08% trifluoroacetic acid in acetonitrile (linear gradient of 5–50% acetonitrile over 15 min). The chromatographic dnp group on the full-length peptide and carboxyl terminus-derived cleavage products was followed by monitoring at 360 nm. Amino terminus-derived products are not detected at 360 nm due to loss of the dnp group. To determine the Km, reactions contained 10 mM enzyme and increasing substrate concentrations. Reactions were incubated at 37 °C for 30 min and terminated by the addition of trifluoroacetic acid, and the amount of peptide cleaved was determined by HPLC as described above. Data were fitted to the Michaelis-Menten equation using GraFit Version 4.0.9. Incubations with inhibitory peptides were carried out as described above following preincubation of Asp1-Fc with 10 μM MBP-tagged Asp1 prodomain peptide or 1 μM APP β-secretase inhibitor H-Lys-Thr-Glu-Ile-Ser-Glu-Val-Asn-Stat-Val-Ala-Glu-Phe-OH (Calbiochem) at 37 °C for 30 min.

Cell Transfections and Immunodetection—COS-7 cells overexpressing APP751 carrying the Swedish mutation were transiently transfected with Asp1mycHis6/pcDNA3.1. 48 h post-transfection, cells were washed extensively with phosphate-buffered saline prior to incubation with EZ-link™ sulfo-NHS-Lc-biotin (Pierce) at 10 μg/ml in phosphate-buffered saline for 1 h at room temperature. Cells were washed extensively with phosphate-buffered saline containing 20 mM glycine and harvested for lysis as described previously (4). Cell-surface biotinylated proteins were purified using streptavidin-agarose and eluted in gel sample buffer. Samples were resolved on Tris/glycine-SDS-polyacrylamide gels, followed by Western blot analysis with an anti-His6 antibody (Stressgen Biotech Corp.).

RESULTS

Asp1 Cleaves MBP-Asp1pro-His6 at the Prodomain Cleavage Site—To investigate prodomain processing of Asp1, we generated a recombinant MBP-Asp1 prodomain fusion protein that encodes MBP fused to Asp1 (amino acids 21–91) encompassing the prodomain cleavage site, with a His6 tag at the C terminus (MBP-Asp1pro-His6) (Fig. 1a). Modeling studies using the published sequence of Asp1 (BACE2) and sequence alignments were used to define the limits of the prodomain. The MBP-Asp1pro-His6 protein was expressed in E. coli and purified by amylose resin. Purified MBP-Asp1pro-His6 migrated as a doublet of ~51 kDa on gels (Fig. 1b) and was immunoreactive with both an anti-MBP and an anti-His6 antibody (Fig. 1c). We have shown by N-terminal sequencing that both bands have the same MBP N terminus; hence, the difference in the two bands is most likely to be in the number of histidine residues at the C terminus.

To confirm this, in-gel digestion of these two bands followed by peptide mass fingerprinting employing MALDI-time-of-flight-MS was performed. The results indicated that both bands correspond to the MBP-Asp1pro-His6 fusion protein. However, the N-terminal peptides as well as the His6-containing C-terminal peptides were not observed directly in the MALDI mass spectra. The failure to detect these peptides may have been caused by suppression effects known to occur in the MALDI analysis of digest mixtures (23). A minor band of lower molecular mass (~40 kDa) was also observed that was immunoreactive with the anti-MBP antibody, but not with the anti-MBP antibody.
Fig. 2. Asp1-Fc cleaves MBP-Asp1pro-His6 at the prodomain cleavage site. a and b, MBP-Asp1pro-His6 and MBP-Asp2pro-His6, respectively, were incubated with either Asp1-Fc (25 nM) or Asp2-Fc (25 nM) in 100 mM sodium acetate (pH 4.5) at 37 °C for the indicated times. Reactions were resolved on 8% Tris/glycine-SDS-polyacrylamide gels for staining with Coomassie Brilliant Blue. Cleavage of MBP-Asp1pro-His6 at the predicted site resulted in the generation of a 47-kDa cleavage product. To determine whether the Asp2-Fc or Asp1-Fc fusion protein could process the Asp2 prodomain, we generated an MBP-Asp2pro-His6, MBP-Asp1pro-His6 was incubated with Asp1-Fc at 37 °C for the indicated times, followed by Western blot analysis with the anti-MBP or anti-His6 antibody. c, shown is the sequence of Asp1 around the prodomain cleavage site, with the cleavages observed highlighted. Cleavage at the predicted site is indicated with a solid arrow, and the other cleavages are indicated with broken arrows.

H1Se antibody, suggesting that it represents a truncated form of the substrate or free MBP.

Cleavage of MBP-Asp1pro-His6 at the prodomain cleavage site (Leu1→Ala) is expected to generate two cleavage products, a large 47-kDa amino-terminal fragment and a small 4-kDa carboxyl-terminal fragment. To determine if Asp1 or Asp2 can mediate cleavage at this site, MBP-Asp1pro-His6 was incubated with pure catalytically active Asp1 or Asp2, both expressed as soluble Fc fusion proteins (Fig. 1d). Incubation of MBP-Asp1pro-His6 with Asp1-Fc resulted in a clear shift in the molecular mass of the prodomain substrate from ~51 to ~47 kDa (Fig. 2a), consistent with cleavage at the predicted site and loss of the 4-kDa carboxyl-terminal fragment, which was not detected. Western blot analysis showed that whereas MBP-Asp1pro-His6 was immunoreactive with both anti-MBP and anti-His6 antibodies, the 47-kDa cleavage product was immunoreactive only with the anti-MBP antibody due to loss of the C-terminal His6 tag following cleavage (Fig. 2c). MS analysis of MBP-Asp1pro-His6 incubated with Asp1-Fc was undertaken to detect the 4-kDa carboxyl-terminal fragment. MALDI-MS revealed the presence of one major and two minor carboxyl-terminal peptides in the samples incubated Asp1-Fc that were absent from control samples. To define the cleavage sites, Nanospray MS/MS analysis of the three cleavage peptides was performed. MS/MS sequence data showed clearly that the bulk of the processing of MBP-Asp1pro-His6 occurred at the expected site, yielding the carboxyl-terminal peptide starting with ALEP (Fig. 2d). In addition, MS/MS sequencing of the two minor peptides revealed cleavage of MBP-Asp1pro-His6 to yield carboxyl-terminal peptides beginning with LAMVD and AMVD (Fig. 2d). The ability of Asp1 to mediate prodomain cleavage at the correct site in the context of a recombinant fusion protein suggests that in vivo, this protease may autoactivate, removing its prodomain to generate the mature enzyme. In contrast, cleavage of MBP-Asp1pro-His6 by Asp2 is very inefficient (Fig. 2a), suggesting that it is unlikely to activate Asp1 in vivo.

To determine whether the Asp2-Fc or Asp1-Fc fusion protein can process the Asp2 prodomain, we generated an MBP-Asp2pro-His6 fusion protein in which amino acids 22–66 of Asp2 were fused to MBP with a C-terminal His6 tag. This protein was also expressed in E. coli and purified using amylose resin. Incubation of this protein (~49 kDa) with Asp1-Fc or Asp2-Fc did not result in a detectable shift in molecular mass (Fig. 2b). Hence, in contrast to Asp1, prodomain cleavage of Asp2 is unlikely to be autocatalytic, and it does not appear to be mediated by Asp1. This result is consistent with reports suggesting that prodomain processing of Asp2 is mediated by another protease (20–22).

Asp1 Prodomain Processing Is Optimal at pH 4.5—To determine the optimum pH profile for cleavage, MBP-Asp1pro-His6 was incubated with Asp1-Fc at pH 4.5, 5.5, or 6.5. Of the pH values tested, Asp1-Fc cleaved MBP-Asp1pro-His6 efficiently at pH 4.5, with 80% of the substrate converted into product within 30 min (Fig. 3b). At pH 5.5, cleavage was still evident, although only 24% of the substrate was converted to product within 30 min. At pH 6.5, cleavage was less efficient, with only 22% turnover of substrate within 30 min.

The Asp1 Active-site Mutant D110N Cannot Autoactivate or Cleave MBP-Asp1pro-His6—The catalytic activity of aspartic proteases is dependent on the presence of two aspartic acid residues in the active site. Hence, processing of MBP-Asp1pro-His6 should be abolished if either of the catalytic aspartic acid residues in Asp1 is mutated. The first catalytic aspartic acid residue in Asp1-Fc was mutated to asparagine (D110N), and the protein was expressed in COS-7 cells and purified from the medium using protein A-Sepharose. In the cleavage assays, Asp1(D110N)-Fc was unable to cleave MBP-Asp1pro-His6 at the prodomain cleavage site since no shift in molecular mass was apparent (Fig. 3a). N-terminal sequencing of mutant Asp1-Fc revealed that 100% of the protein still possessed its prodomain, whereas wild-type Asp1-Fc expressed in COS-7 cells was fully processed and had the amino-terminal sequence ALEP (13). Thus, it appears that the mutant Asp1(D110N)-Fc is not processed in COS-7 cells due to its inability to autoactivate.

Mutations in the Prodomain Cleavage Site Affect Cleavage Site Selection—Phenylalanine scanning mutagenesis was conducted through the Asp1 prodomain cleavage site to determine the effect on prodomain processing. Residues P5–P9 (ALAL) in MBP-Asp1pro-His6 were mutated individually to phenylalanine, and the purified mutant proteins were incubated with Asp1-Fc. Cleavage of the mutant MBP-Asp1pro-His6 substrates appeared to be as efficient as that of the wild-type substrate when expressed as the proportion of substrate cleaved (Fig. 3c). However, MALDI-MS analysis of the 4-kDa cleavage product revealed that mutation of the P5' and P9' residues has different effects on cleavage than mutation of the P5–P9 residues.
residues reduced the level of the peptide detected with the N-terminal sequence ALEP and increased the level of peptide detected with the N-terminal sequence LAMVD. In contrast, mutations at the P₁ and P₂ positions had no effect on cleavage. This indicates that the active site of Asp1 is unable to accommodate large bulky residues at either the P₁ or P₂ position.

**Asp1 Cleaves Synthetic Peptides at the Prodomain Cleavage Site**—We went on to determine whether Asp1 can cleave a synthetic peptide spanning the prodomain cleavage site of Asp1. Incubation of Asp1-Fc with peptide ADGLALEPALAKH/dnp/G resulted in cleavage to give a single dnp-labeled product (Fig. 4, a and b). MS analysis showed that this cleavage occurred at the predicted prodomain cleavage site, between Leu⁵⁷ and Ala⁶³. Asp2-Fc also cleaved the Asp1 prodomain peptide at this site, albeit poorly in comparison with Asp1-Fc (data not shown). An initial velocity plot of Asp1-Fc-mediated cleavage of the synthetic peptide with respect to substrate concentration is shown in Fig. 4c, giving a $K_m$ at pH 4.5 of $97 \pm 30$ μM. To determine if the Asp1 prodomain has any inhibitory activity, assays were carried out in the presence of an MBP-tagged Asp1 prodomain peptide (residues 21–62). Preincubation of 50 nM active Asp1-Fc (50 nM) with a 10 μM concentration of this peptide resulted in $16\%$ inhibition of cleavage of synthetic Asp1 proenzyme peptide under standard conditions (see “Experimental Procedures”). This contrast indicates a $>50\%$ inhibition of cleavage by 1 μM APP β-secretase inhibitor, indicating that the prodomain is a very weak inhibitor of Asp1.

**Prodomain Processing of Asp1 in Vivo**—To determine if Asp1 autophosphorylates in vivo and to identify the intracellular compartment in which prodomain processing occurs, COS-7 cells expressing APP751 carrying the Swedish mutation (K651M/ N652N) were transiently transfected with Asp1MycHis₆ or mutant Asp1(D110N)-MycHis₆. To detect Asp1 proenzyme, cell lysates with untagged wild-type Asp1. Western blot analysis of the cell lysates with the anti-Asp1 C terminus antibody showed that the prodomain of Asp1(D110N)MycHis₆ intermolecularly, the mature Asp1(Fc) protein corresponds to the prodomain-containing form of Asp1, whereas the 50-kDa protein is the mature protease that lacks the prodomain. Hence, at low temperature, prodomain processing of Asp1 is reduced, resulting in the accumulation of the proenzyme. In contrast to wild-type Asp1, when Asp1(D110N)-MycHis₆ was deglycosylated, it migrated exclusively as the proenzyme of ~54 kDa following growth at 37 and 15 °C (Fig. 5a, fourth and fifth lanes). Thus, processing is abolished in the mutant enzyme. A further implication of this result (see below) is that, in these cells, there is no other enzyme that is capable of processing Asp1.

The detection of mature as well as pro-Asp1 following growth at 15 °C (Fig. 5, a and b) suggests that processing can occur early in the secretory pathway. When cells were grown at 20 °C, which inhibits the transit of protein from the trans-Golgi network to the cell surface (25), very little proenzyme accumulated (Fig. 5b), implying that very little prodomain processing occurs in post-Golgi compartments. To further confirm the identity of the intracellular site where prodomain processing of Asp1 occurs, cells were grown in the presence of drugs known to interfere with the trafficking of proteins (Fig. 5b). Growth in the presence of the fungal metabolite brefeldin A, which causes a redistribution of proteins that normally reside in the Golgi into the ER (26), revealed the accumulation of both pro and mature forms of Asp1. In contrast, growth in the presence of the monovalent ionophore monensin, which disrupts late Golgi and endosomal functions (27), caused the accumulation of mature Asp1, but little proenzyme. Hence, the majority of prodomain processing of Asp1 does not occur in late Golgi/endosomal compartments, but rather in the ER/early Golgi compartments.

The results described above for the processing of the active site mutant enzyme indicate that no other intracellular protease is capable of cleaving the prodomain of Asp1 and that prodomain cleavage is an exclusively intramolecular event. To confirm this finding, Asp1(D110N)-MycHis₆ was coexpressed with untagged wild-type Asp1. Western blot analysis of the cell lysates with the anti-Asp1 C terminus antibody showed that expression of both Asp1 proteins was successful (Fig. 5c, upper panel). If the cotransfected untagged Asp1 is able to cleave the prodomain of Asp1(D110N)-MycHis₆, intermolecularly, the mature form of this protein should now be detected with the anti-myɛ or anti-His$_{96}$ antibody. However, Western blot analysis with the anti-His$_{96}$ antibody revealed that Asp1(D110N)-MycββHis$_{96}$ still migrated as the proenzyme in these cells.
Hence, prodomain processing of Asp1 occurs exclusively through an intramolecular reaction. Mature Asp1 Is Present at the Cell Surface—Proportions of cellular APP and Asp2 have been shown to be present on the cell surface (28–30). To determine whether Asp1 is also expressed at the cell surface, we performed biotinylation experiments on COS-7 cells overexpressing APP751 carrying the Swedish mutation and transiently transfected with Asp1.

**Fig. 4. Asp1-Fc cleaves a synthetic peptide at the prodomain cleavage site.** a, peptide incubated in the absence of Asp1-Fc. b, hydrolysis of the Asp1 prodomain peptide by Asp1-Fc. A reverse-phase HPLC profile shows the dnp-labeled substrate and product peaks at 360 nm. The amino acid sequences of the peptides as determined by MS are shown. c, kinetic plot of the cleavage of the synthetic peptide by Asp1-Fc (10 nM). Std., standard.

**Fig. 5. Prodomain processing of Asp1 in vivo.** COS-7 cells expressing APP751 carrying the Swedish mutation were transiently transfected with the following. a, Asp1MycHis6 or Asp1(D110N)MycHis6, followed by growth at the indicated temperatures. Cell lysates were generated, and proteins were deglycosylated by treatment with N-glycosidase F prior to Western blot analysis with the anti-His6 antibody. b, Asp1MycHis6, followed by growth at reduced temperature or in the presence of brefeldin A (BFA) or monensin (MON). Cell lysates were generated, and proteins were deglycosylated by treatment with N-glycosidase F prior to Western blot analysis with the anti-His6 antibody. c, untagged wild-type Asp1, Asp1(D110N)MycHis6, or both. Cell lysates were generated, and proteins were deglycosylated by treatment with N-glycosidase F prior to Western blot analysis with the anti-Asp1 C terminus antibody (upper panel) or the anti-His6 antibody (lower panel). d, Asp1MycHis6, followed by cell-surface biotinylation as described under “Experimental Procedures.” Cell-surface (lane 1) and intracellular (lane 2) proteins were subjected to Western blot analysis for Asp1 (upper panel), APP (middle panel), or chaperone proteins Grp94 and Grp78 (lower panel). e, Asp1MycHis6, followed by Western blot analysis of biotinylated cell-surface proteins with the anti-His6 antibody, showing that Asp1 at the cell surface (lane 1) aligns with the mature form of the proteinase (lane 2).
Transfected cells were incubated with sulfosuccinimidobiotin to biotinylate the cell-surface proteins. Cell lysates were generated, and the biotinylated cell-surface proteins were immunoprecipitated with streptavidin-agarose. A significant level of total Asp1 was detected at the cell surface (Fig. 5d, upper panel, lane 1). As a control, the cell-surface and intracellular cell extracts were also probed for APP, which is known to reside on the cell surface (28, 29), and for the endoplasmic reticulum-localized chaperones Grp94 and Grp78 (31). Consistent with published data, we found that APP was present at the cell surface and intracellularly (Fig. 5d, middle panel, lanes 1 and 2), whereas the endoplasmic reticulum-resident proteins were detected only within the cell (lower panel, lane 2). To determine if Asp1 residing at the cell surface is the mature protease lacking the prodomain, the biotinylated cell extracts were deglycosylated with N-glycosidase F prior to incubation with streptavidin-agarose. Following deglycosylation, the biotinylated cell-surface Asp1 migrated at a molecular mass consistent with the mature form of the protein (Fig. 5e, lane 1). Thus, prodomain processing of Asp1 occurs during its transit through the secretory pathway en route to the cell surface, and the protein expressed on the cell surface is the processed, potentially active form of the enzyme. However, prodomain processing is not a prerequisite for transit to the cell surface, as Asp1(D110N)mytchHis6, which still possesses its prodomain, was also expressed at the cell surface (data not shown).

**DISCUSSION**

All known aspartic proteases are generated as proenzymes, requiring removal of the prodomain to generate mature protease (32). Although most aspartic proteases such as pepsin and cathepsin E autoactivate upon acidification, others such as renin require a separate proteinase activity for prodomain cleavage. It has recently been reported that Asp2 does not autoactivate and that removal of its prodomain is mediated by furin or a furin-like enzyme (20–22). We report that in contrast to Asp2, Asp1 is capable of autoactivating cleavage, cleaving its prodomain to generate mature protease. In *vitro*, Asp1 cleaved both an MBP-Asp1 prodomain fusion protein and a synthetic peptide at the predicted prodomain cleavage site (Leu62Ala63 in full-length Asp1). This cleavage was abolished when one of the conserved aspartic acid residues in the active site of Asp1-Fc was mutated to asparagine. In addition, mutation of either the P1 (Ala) or P3 (Leu) residue in the MBP-Asp1 prodomain substrate to phenylalanine reduced cleavage at this site. The other cleavages observed when MBP-Asp1pro-His6 was incubated with Asp1-Fc are unlikely to be physiologically relevant, as Asp1-Fc secreted from cells had only one N-terminal stringent regulation of its activity compared with Asp2. However, it is feasible that other intracellular factors exist that influence the activity of Asp1.

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