Sequential proteolysis of the amyloid precursor protein (APP) by β- and γ-secretase activities yields the amyloid β peptide that is widely deposited in the brains of individuals with Alzheimer’s disease. The membrane-anchored aspartyl protease β-site APP-cleaving enzyme (BACE) exhibits all of the characteristics of a β-secretase and has been shown to cleave APP at its β-site in vitro and in vivo. We found that BACE undergoes cleavage on a surface-exposed α-helix between amino acid residues Leu-228 and Ala-229, generating stable N- and C-terminal fragments that remain covalently associated via a disulfide bond. The efficiency of BACE endoproteolysis was observed to depend heavily on cell and tissue type. In contrast to brain where holoprotein was predominant, BACE was found primarily as endoproteolyzed fragments in pancreas, liver, and muscle. In addition, we observed a marked up-regulation of BACE endoproteolysis in C2 myoblasts upon differentiation into multinucleated myotubes, a well established model system of muscle tissue specification. As in liver, BACE exists as endoproteolyzed fragments in the hepatic cell line, HepG2. We found that HepG2 cells are capable of generating amyloid β peptide, suggesting that endoproteolyzed BACE retains measurable β-secretase activity. We also found that BACE endoproteolysis occurs only after export from the endoplasmic reticulum, is enhanced in the trans-Golgi network, and is sensitive to inhibitors of vesicular acidification. The membrane- bound proteases tumor necrosis factor α-converting enzyme and furin were not found to be responsible for this cleavage nor was BACE observed to mediate its own endoproteolysis by an autocatalytic mechanism. Thus, we characterize a specific processing event that may serve to regulate the enzymatic activity of BACE on a post-translational level.

Alzheimer’s disease (AD)1 is characterized pathologically by the florid accumulation of insoluble amyloid β peptide (Aβ) in the central nervous system (1). A causative role for Aβ in AD progression has been accepted by many for some time (2, 3), and recent evidence from mouse models has underscored the importance of this peptide in the process of cognitive decline (4–6). Aβ is produced by the sequential processing of the amyloid precursor protein (APP) by two proteolytic activities that have historically been referred to as β- and γ-secretase. Alternatively, cleavage of APP by a so-called α-secretase activity within the Aβ region of the protein precludes the generation of the peptide (7). Numerous studies have identified the membrane-anchored aspartyl protease BACE as the β-secretase (8–12), whereas evidence continues to mount that γ-secretase activity is mediated at least in part by the presenilins proteins (PS1 and PS2) (13–17). A group of metalloprotease disintegrins, including tumor necrosis factor-α converting enzyme (TACE), appears to be responsible for at least a significant fraction of cellular α-secretase activity (18, 19).

The central role of Aβ in AD pathogenesis requires careful study of the proteins involved in APP/Aβ processing and metabolism. The α-secretase activity of TACE, for instance, may depend on C-terminal phosphorylation of the enzyme by protein kinase C (18, 19). The presenilins have also been demonstrated to undergo phosphorylation (20–22), although the functional impact of these modifications remains unclear. More thoroughly characterized is the endoproteolysis of PS1 and PS2, an event that generates distinct presenilin N- and C-terminal fragments that are thought to represent the biologically active forms of the proteins. At endogenous levels the cleavage fragments of PS1 and PS2 predominate, and under conditions of overexpression, the vast majority of the presenilins exist as holoproteins, presumably due to the saturation of the endoproteolytic pathway (23–27).

Despite its fairly recent discovery, BACE has already been the subject of numerous investigations designed to elucidate its basic cell biology. During maturation in the secretory pathway, BACE undergoes glycosylation at 3–4 N-linked sites and is separated from its propeptide domain (28–30). This latter proc-

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1 The abbreviations used are: AD, Alzheimer’s disease; Aβ, amyloid β; APP, amyloid precursor protein; BACE, β-site APP-cleaving enzyme; BACE-Ct, BACE C-terminal fragment; BACE-Nt, BACE N-terminal fragment; TACE, tumor necrosis factor-α converting enzyme; PS1, presenilin 1; PS2, presenilin 2; ER, endoplasmic reticulum; TGN, trans-Golgi network; HA, influenza hemagglutinin; PBS, fetal bovine serum; RIPA, radiolmmunoprecipitation assay; BHK, baby hamster kidney; DMEM, Dulbecco’s modified Eagle’s medium; SFV, Semliki Forest virus; mAb, monoclonal antibody.
Endoproteolysis of BACE within Its Catalytic Domain

Easing step is mediated by a member of the protease convertase family of proteases (31, 32). In addition, BACE is palmitoylated at cysteine residues within its cytoplasmic domain and C-terminally phosphorylated (33, 34). Mature BACE has a t½ of 12–16 h and cycles between the cell surface, the endosomal system, and the TGN multiple times through the course of its life span (29, 30, 34). The cleavage site specificity of BACE for its APP substrate has been shown to depend heavily on the intracellular localization of the protease (35).

In this study, we describe a specific endoproteolytic event that may have a significant effect on BACE activity. Whereas the existence of BACE breakdown products has been documented (10), the protein fragments themselves have not been well characterized, and the process by which they are generated remains unclear. We demonstrate BACE endoproteolysis occurs between amino acid residues Leu-228 and Ala-229, generating stable N- and C-terminal fragments that remain associated in a heterodimeric complex stabilized in part by a disulfide bond. This endoproteolytic process is regulated in a cell type- and tissue type-dependent fashion. We show that BACE endoproteolysis does not appear to take place in neurons. However, the processing event occurs robustly in a variety of native tissues and may play an important role in the differentiation of muscle cells. We also demonstrate that endoproteolysed BACE appears to retain at least some β-secretase activity.

Finally, we investigate the cell biological determinants and molecular mechanisms underlying the cleavage of BACE. Our results indicate that BACE, like the presenilins, undergoes an endoproteolytic cleavage event with intriguing functional implications.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Expression—The generation of BACE, BACE-KK, and BACE-TGN constructs has been described (30, 35). TACE and m-furin-HA were expressed in pcDNA3.1 (19, 36). Expression of DNA constructs in cultured cells was obtained using Geneporter transfection reagent (Gene Therapy Systems, San Diego) with a DNA/Geneporter ratio of 1 μg/10 μl. For some experiments BACE and APP were expressed using a replication-defective Semliki Forest virus (SFV) vector expressing wild-type human BACE and wild-type APP vectors (37). In these experiments the cells were incubated with virus using a multiplicity of infection of 2–10 for 1 h in DMEM without serum. Cells were rinsed and incubated in normal growth medium for 3–4 h prior to metabolic [3H]Thiouracil labeling. For experiments expressing APPwt in HepG2 cells, SFV-infected cells were metabolically labeled for 6 h following a 4-h incubation period.

N-terminal Radiosequencing—Custom DMEM deficient in either methionine or isoleucine was prepared using a minimum Eagle’s medium–α-Selectamite kit (Invitrogen) and supplemented with 10% dialyzed FBS. 293 cells expressing HA-tagged BACE were preincubated in either the methionine-free or isoleucine-free medium for 30 min followed by an 18-h incubation in the same media supplemented with either [35S]methionine or [3H]isoleucine, respectively. The cells were then washed in PBS, lysed in RIPA, and immunoprecipitated with mAb HA11. Immunoprecipitates were eluted in 1× Laemmli sample buffer plus 0.34 M β-mercaptoethanol, electrophoresed on 8% polyacrylamide gels, and transferred to Immobilon-P polyvinylidene fluoride membrane (Millipore). Bands corresponding to BACE-Ct were then cut from the membrane and sequenced by automated Edman degradation collecting each amino acid separately. The amino acids were assayed for radioactivity in a Beckman LS 6500 scintillation counter.

Fluorescence Microscopy—Antibody staining of HeLa cells was performed as described previously (30). Briefly, cells were fixed and permeabilized in ice-cold methanol followed by incubation in primary antibody diluted in PBS supplemented with 2% FBS and 0.02% saponin. BACE constructs were recognized using rb-HA11, and a monoclonal antibody directed against GM-130 (Transduction Laboratories) was used to detect Golgi. Cells were then incubated in fluorescent-conjugated secondary antibodies diluted in the same solution as the primaries. All micrographs were taken with a Nikon E800 microscope utilizing UV illumination.

RESULTS

BACE Is Endoproteolytically Processed to Generate Stable N- and C-terminal Fragments—While characterizing the biochemical properties of BACE under conditions of overexpression in cell culture, we repeatedly noticed the presence of an ~37-kDa band by SDS-PAGE whose immunoreactivity closely paralleled that of the BACE holoprotein. This species could be readily demonstrated in cell lysates by Western blot using a variety of antibodies directed against the C terminus of BACE (Fig. 1, B and C). Furthermore, peptide competition experiments confirmed that the band was specific and not a background artifact (Fig. 1C). We interpreted these findings to suggest that a portion of the BACE holoprotein undergoes endoproteolysis generating a distinct ~37-kDa C-terminal fragment (BACE-Ct), a mechanism by which the activity of the protein could be regulated (Fig. 1A). We observed significant levels of BACE endoproteolysis in a variety of cell types including human embryonic kidney 293 cells (Fig. 1B), baby hamster kidney (BHK) cells (Fig. 1C), Chinese hamster ovary cells, HeLa cells, and N2a cells (data not shown).
By using metabolic pulse-chase analysis, we investigated the kinetics of BACE endoproteolysis. 293 cells expressing BACE were radiolabeled with \( ^{35} \text{S} \)methionine/cysteine for 30 min and chased in non-radioactive media for various times up to 24 h, after which the cells were lysed and subjected to immunoprecipitation. In these experiments, BACE-Ct first emerged 4 h into the chase after which the fragment accumulated slightly, reaching a maximum at the 12-h time point, before being slowly turned over (Fig. 2A). The initial appearance of BACE-Ct several hours after synthesis is characteristic of a physiological breakdown product and is not consistent with BACE-Ct being a post-lysis artifact. In addition, our data indicate that endoproteolyzed BACE is stable and that the cleavage event itself does not simply promote BACE degradation. Pulse-chase experiments were also performed using an antibody directed against the N terminus of BACE (Fig. 2B). These studies demonstrated the accumulation of a 37-kDa N-terminal fragment (BACE-Nt) with kinetics parallel to those of BACE-Ct. Thus, BACE endoproteolysis results in the production of stable N- and C-terminal fragments.

BACE Endoproteolysis Occurs between Leu-228 and Ala-229, and the Resulting Fragments Remain Associated after Cleavage—The exact site of BACE endoproteolysis was determined by N-terminal radiosequencing of BACE-Ct after metabolic labeling with either \( ^{35} \text{S} \)methionine or \( ^{3} \text{H} \)isoleucine (Fig. 3A), which demonstrated that cleavage occurs between amino acid residues Leu-228 and Ala-229. Analysis of the position of these residues in the published BACE crystal structure (40) revealed that both lie in a solvent-exposed \(-\alpha\)-helix connecting the two extracellular lobes of the protein (Fig. 3, B and C). Cleavage at this site would split apart the BACE catalytic domains and could potentially inactivate the enzyme. However, structural studies have indicated the existence of a disulfide bond between residues Cys-216 and Cys-420 (Fig. 3C) (29, 40). Such a connection could serve to maintain the association of BACE-Ct and BACE-Nt even after endoproteolysis.

In light of this possibility, we asked whether a distinct BACE C-terminal fragment could be visualized under non-reducing conditions that should preserve the integrity of disulfide bonds. Triton X-100 lysates from 293 cells expressing BACE were analyzed by Western blot after incubation at either 37 or 55 °C.

**Fig. 1.** A, schematic showing topological models of BACE constructs used. Luminal and cytoplasmic domains are indicated. White circles denote the positions of the catalytic aspartic acid residues. The cytoplasmic domain of murine furin used to design BACE-TGN is shown in blue. HA epitope tag is shown in red. Endoproteolysis of BACE at Ala-229 is also demonstrated, a process that generates the BACE cleavage products BACE-Nt (green) and BACE-Ct (gray). B, lysates from 293 cells expressing a C-terminally HA-tagged BACE construct (lane B) or mock-transfected (lane M) were subjected to Western blot with mAb HA11. Arrows denote BACE-Ct. IP, immunoprecipitation. C, lysates from BHK cells expressing untagged wild-type BACE were analyzed by Western blot with 88.6 (rabbit polyclonal antisera recognizing BACE C terminus). Blots were probed with antibody preadsorbed with specific (S), nonspecific (NS), or no preadsorption (−) (see “Experimental Procedures”).
in the presence or the absence of β-mercaptoethanol. Under non-reducing conditions, the BACE holoprotein migrated at a lower apparent molecular weight than under reducing conditions, suggestive of intramolecular disulfide bonding and consistent with previous results (Fig. 3D) (30). Whereas BACE-Ct was readily apparent in the presence of β-mercaptoethanol, it could not be detected in the absence of reducing agent, consistent with the notion that the BACE N- and C-terminal fragments remain covalently associated by a disulfide bond even after endoproteolysis (Fig. 3D).

**BACE Endoproteolysis Occurs Readily in Several Different Non-neuronal Tissues and May Play a Role in Cellular Differentiation**—To determine whether BACE endoproteolysis takes place at significant levels in vivo, we assayed for the presence of BACE-Ct in a variety of native tissue samples derived from rat. In lysates from pancreas, liver, and muscle, Western blot demonstrated high levels of a C-terminal BACE fragment that co-migrated with BACE-Ct derived from BHK cells (Fig. 4A). BACE holoprotein was not readily apparent in these tissues. By contrast, samples from cerebral cortex, cerebellum, diencephalon, and brain stem exhibited negligible amounts of BACE-Ct, whereas the holoprotein was clearly evident (Fig. 4A). Analysis of human tissue yielded similar results. Samples from cerebral cortex and cerebellum revealed only BACE holoprotein was not readily apparent in these tissues. By contrast, samples from cerebral cortex, cerebellum, diencephalon, and brain stem exhibited negligible amounts of BACE-Ct, whereas the holoprotein was clearly evident (Fig. 4A). Analysis of human tissue yielded similar results. Samples from cerebral cortex and cerebellum revealed only BACE holoprotein, whereas lysates from muscle tissue exhibited a large band co-migrating with BACE-Ct. The specificity of the antibody was confirmed by peptide blocking (Fig. 4B). To determine whether BACE-Nt is also present at significant levels in native
tissue, we performed Western blots on rat muscle lysates using a BACE N-terminal antibody (Fig. 4C). Although the N-terminal BACE antibody (left panel) was less sensitive than the C-terminal BACE antibody (right panel), the N-terminal antibody still detected an ~35–37 kDa band co-migrating with BACE-Ct. The similar size of the Nt- and Ct-BACE fragments in muscle is consistent with data showing that immunoprecipitations using both Nt- and Ct-BACE antibodies detected apparently identical, closely spaced doublet bands at ~37 kDa in size (Fig. 2B). This would be expected under immunoprecipitating conditions because BACE-Nt and -Ct fragments remain attached via a common disulfide bond. These findings are also in keeping with the expected size of the fragments based upon the position of the endoproteolytic cleavage. Thus these results indicate that N- and C-terminal cleavage products of BACE endoproteolysis selectively accumulate in cell lines and in vivo. The existence of highly stable, BACE cleavage fragments suggests that this form of BACE may have a specific physiological function in certain tissue and cell types.

We further investigated the endoproteolytic processing of BACE in muscle tissue using C2 murine myoblasts that can be induced to differentiate into multinucleated myotubes (39). We found that differentiation dramatically altered the processing of endogenously expressed BACE. Undifferentiated C2 myoblasts exhibited relatively low levels of BACE holoprotein but not BACE-Ct (Fig. 4D). However, upon differentiation into myotubes, this expression pattern was reversed. Whereas BACE holoprotein was down-regulated (note that four times the number of cells were loaded in the C2 lane compared with the C2(Diff) lane in order to visualize the holoprotein), BACE-Ct expression was markedly up-regulated (Fig. 4D). The specificity of the BACE antibody was confirmed by BACE-Ct peptide antibody preadsorption (Fig. 4D). Pulse-chase analysis of BACE metabolism in differentiated C2 myotube cultures (Fig. 4E) confirmed the existence of a precursor-product relationship between BACE and BACE-Ct in muscle tissue with kinetics similar to those observed in other non-neuronal cell types (Fig. 2, A and B). These findings demonstrate that endoproteolytic conversion of BACE is a marked feature attending muscle differentiation, suggesting a physiological role in the specification of muscle cells and perhaps, more broadly, in tissue development.

Cell Types Containing Endoproteolysed BACE Retain β-Secretase Activity—We next asked if β-secretase activity and Aβ production can occur in cells where endogenous BACE is expressed in the endoproteolyzed form. Because BACE-Ct fragments are highly expressed in liver, we examined BACE expression in the human hepatic cell line, HepG2. Western blot analysis of HepG2 cells revealed high levels of BACE-Ct with virtually no detectable BACE holoprotein (Fig. 5A). The specificity of the antibody for BACE was confirmed by peptide blocking (Fig. 5A). These findings indicate that stable levels of endogenous BACE exist almost exclusively as endoproteolysed fragments in HepG2 cells. APP processing was examined by metabolically labeling HepG2 cells expressing SVF-induced wild-type human APP<sup>695</sup>. Cells expressing SVF-β-galactosidase (lacZ) served as controls. Immunoprecipitation with antibodies directed against the C-terminal domain of APP revealed the presence of APP C-terminal fragments consistent with α- and β-secretase-mediated APP cleavage (Fig. 5B). Immunoprecipitation of the conditioned medium with 6E10, a monoclonal antibody recognizing amino acid residues 1–10 of Aβ, readily detected Aβ (Fig. 5B). The same experimental approach also detected the production of Aβ in differentiated C2 myotube cultures (data not shown). These results strongly suggest that endoproteolysed BACE retains at least some APP β-site cleaving enzyme activity.

Cell Biological Characteristics of BACE Endoproteolysis—To determine more accurately the cellular compartment(s) where the BACE endoproteolytic event takes place, we used BACE constructs targeted to specific organelles. Placing a di lysine motif on the C terminus of BACE (BACE-KK) effectively retains the protein in the ER (30), whereas replacing the cytoplasmic tail of BACE with the intracellular domain of the proprotein convertase furin targets the protease to the TGN (BACE-TGN) (Fig. 1A) (35). The cellular distributions of BACE-KK and BACE-TGN were confirmed by immunofluorescence (Fig. 6A). When expressed in HeLa cells, BACE-KK exhibited a fine reticular staining pattern, consistent with ER localization and identical to previous results (30), whereas BACE-TGN displayed positive immunoreactivity in a perinuclear distribution that colocalized well with the Golgi marker GM-130. The BACE targeting mutants were expressed in 293 cells, and immunoprecipitations of radiolabeled protein were performed to assay for endoproteolysis. Whereas BACE-Ct was undetectable in cells expressing BACE-KK, the fragment accumulated at roughly twice the wild-type level in cells expressing BACE-TGN (Fig. 6B). These findings indicated that endoproteolysis occurs only after BACE is exported from the ER and that the efficiency of the cleavage event itself is enhanced in the TGN. The larger size of BACE-Ct in cells expressing BACE-TGN simply reflects the increased length of the furin cytoplasmic tail relative to its wild-type BACE counterpart.

To define further the cell biology of BACE endoproteolysis, we asked whether the generation of BACE-Ct requires an acidic microenvironment. A variety of proteases, including BACE, are optimally active at acidic pH values (10, 12). We addressed this issue using two pharmacological reagents. Chloroquine, a weak base, accumulates in acidic vesicles where it counteracts the established pH gradient (41, 42), whereas bafilomycin A1 selectively blocks vacuolar-type H<sup>+</sup>-ATPases (43), protein complexes that pump protons into a variety of cellular compartments including endosomes and the TGN (44). 293 cells expressing BACE were labeled with <sup>35</sup>S)cysteine/methionine for 16 h in the presence of either chloroquine or bafilomycin A1. This treatment eliminated the production of BACE-Ct, as visualized by immunoprecipitation of radiola-
Endoproteolysis of BACE within Its Catalytic Domain

We next sought to determine what enzyme(s) mediate the endoproteolytic processing of BACE. In light of our results implicating the TGN as a likely site for BACE endoproteolysis, we considered two proteases as good candidates. The previously mentioned α-secretase TACE was originally found to be involved with the cleavage of cell surface proteins (45, 46). However, more recent evidence has implicated TACE in protein kinase C-regulated α-secretase activity within the TGN, where the protein competes with β-secretase for APP substrate (19). In addition to TACE, furin is known to be active primarily in the TGN where it mediates the processing of several different precursor proteins (47). Furin or a furin-like protease has also been demonstrated to cleave BACE at its propeptide site (31, 32), demonstrating that these two proteins interact in cells. Arguing against a role for furin, however, is the fact that the BACE endoproteolysis site is not marked by the consensus sequence, Arg-Xaa-(Lys/Arg)-Arg, typically associated with furin cleavage (47).

The impact of TACE and furin on the endoproteolysis of BACE was determined by co-expression studies. 293 cells were transfected with either BACE alone, BACE and TACE, or BACE and mfurin-HA (an HA-tagged murine furin construct). The cells were then labeled with [35S]cysteine/methionine for 8 h, lysed, and immunoprecipitated with mAb HA11. Analogous experiments using BACE-TGN were also performed. We found that co-expression of TACE or mfurin-HA with either BACE-HA or BACE-TGN did not lead to an appreciable increase in the accumulation of BACE-Ct (Fig. 7, A and C). We also attempted to up-regulate TACE activity by treating the cells with the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) 4 h into the [35S]cysteine/methionine labeling period. No effect on BACE-Ct levels was observed (Fig. 7A). TACE activity was confirmed by co-transfection with APP in 293 cells. A 30-min [35S]cysteine/methionine label followed by PMA stimulation led to an increase in secreted APPβ derived from both endogenous APPβ751 and overexpressed APPβ695 (Fig. 7B), consistent with reported results (19). We confirmed the expression of furin-HA by Western blot (Fig. 7D). Thus, neither TACE nor furin was found to mediate the cleavage of BACE.

We also considered whether an autocatalytic mechanism might be responsible for BACE endoproteolysis. Several enzymes, including furin and possibly the presenilins, undergo autocatalytic processing events that significantly impact the activity of the proteases themselves (13, 15, 47). In addition, the dependence of BACE endoproteolysis on an acidic microenvironment correlates well with the documented acidic pH optimum for BACE. To address this possibility, we generated a BACE construct whose second active site aspartate residue (Asp-289) mutated to asparagine (BACE-D289N). This mutation has been demonstrated to eliminate completely the protease activity of BACE (8), and in our hands, co-expression of BACE-D289N with APP in 293 cells did not lead to the typically observed increase in Aβ secretion (data not shown). If BACE does autocatalyze its own endoproteolysis, one would expect BACE-Ct not to be produced in cells expressing BACE-D289N or to be produced only at very low levels due to the presence of endogenous BACE. We transfected BACE-D289N and BACE into 293 cells and assayed for endoproteolysis by immunoprecipitation of radiolabeled protein. No significant differences were found between the BACE-Ct levels of cells expressing BACE-D289N and BACE-TGN and those expressing BACE-HA.

**Fig. 6.** A, immunofluorescence micrographs of HeLa cells expressing BACE, BACE-KK, or BACE-TGN. All cells were fixed and permeabilized with methanol prior to BACE staining (see “Experimental Procedures”). The lower row of panels indicates the extent of co-localization (yellow) between BACE-TGN (green) and the Golgi resident protein GM-130 (red). B, 293 cells expressing either BACE, BACE-KK, or BACE-TGN were radiolabeled with [35S]cysteine/methionine for 18 h, lysed, and immunoprecipitated. Arrows denote BACE-Ct derived from either BACE-HA or BACE-TGN. C, 293 cells expressing BACE or BACE-TGN were pretreated with either normal growth medium (−), growth medium containing 100 µM chloroquine (Chl), or growth medium containing 1 µM bafilomycin A1 (Baf) for 3 h. The cells were then radiolabeled with [35S]cysteine/methionine for 18 h with chloroquine or bafilomycin A1 included as before, lysed, and immunoprecipitated. Arrows indicate BACE-Ct. D, bafilomycin (Baf) blocked the endoproteolysis of BACE in differentiated C2 cells expressing SVF-mediated wt BACE. NI, non-infected controls. Cells were metabolically labeled and then chased in cold medium for the indicated time (hours). Lysates were immunoprecipitated with BACE-Ct antibodies. Lower panel is darker exposure of a portion of the upper panel. Arrows indicate BACE-Ct.
expressing either BACE-D289N or BACE (Fig. 7E). Our findings, therefore, are not consistent with BACE directly mediating its own endoproteolysis. Nevertheless, an autocatalytic mechanism cannot be completely excluded (see “Discussion”).

**DISCUSSION**

The proteolysis of APP by BACE is a crucial step in the generation of Aβ in brain and represents an important potential target of therapeutic strategies aimed at slowing or even halting the progression of AD. Consequently, a better understanding of the cellular mechanisms regulating β-secretase activity is of obvious importance. We and others (29, 30) have found that BACE is a stable protein with a t½ of 12–16 h. This long life span strongly implies that, in order for cells to effectively exert tight control over β-secretase activity, regulatory mechanisms must exist at a post-translational level. We have described an endoproteolytic processing pathway that cleaves BACE into distinct N- and C-terminal fragments, each ~37-kDa in size. BACE endoproteolysis appears to be a normal cellular metabolic process resulting in the generation of a highly stable form of the protein. In this regard BACE may be similar to a wide variety of proteins that are known to be regulated by endoproteolysis including zymogens, clotting factors, complement proteins, and hormones (48, 49). In addition, several membrane-bound proteases such as the A Disintegrin And Metalloprotease (ADAM) and proprotein convertase families require specific cleavage events during the maturation process to obtain biological activity (47, 50, 51).

Structural analysis of BACE endoproteolysis revealed that cleavage occurs on a solvent-exposed α-helix bridging the two extracellular lobes of the protein. Analogous helices are absent from soluble aspartyl proteases like pepsin (40, 52), consistent with the fact that these enzymes have not been shown to undergo endoproteolysis at similar positions in their amino acid sequences (53, 54). Typically, cleavage at this site would be expected to inactivate BACE by dissociating the two catalytic aspartic acid residues. However, we found that the BACE-Nt- and -Ct remain covalently associated after proteolysis via a common disulfide bridge. This finding suggests the possibility that such post-translational regulation may alter and/or attenuate, rather than abolish, the enzymatic activity of BACE.

Although BACE endoproteolysis occurs readily in a variety of cultured cell types, only a relatively small proportion of the holoprotein appears to undergo cleavage when overexpressed. By contrast, endoproteolyzed BACE accumulates at high levels endogenously in muscle, liver, and pancreas, coupled with undetectable or very low levels of holoprotein. This dichotomy is reminiscent of the regulated endoproteolysis of the presenilins, which exist primarily as holoproteins when overexpressed, and yet are found almost entirely as N- and C-terminal cleavage fragments endogenously (23, 24, 55, 56). Whereas a specific physiological purpose cannot be assigned to BACE endoproteolysis at this time, this post-translational event appears to be a normal, regulated metabolic process yielding stable cleavage products that accumulate in certain native tissues. Moreover, the pattern of endoproteolyzed BACE expression is generally consistent with BACE mRNA levels in different tissue types. For instance, the pancreas exhibits robust BACE mRNA expression (10) and is among the tissues harboring the highest levels of BACE cleavage fragments. In addition, we have shown that an up-regulation of BACE endoproteolysis is associated with myogenic differentiation in C2 cells, a well established model of muscle development. Together, these findings strongly suggest that endoproteolyzed BACE has a biologically significant, but as yet undefined, function(s) in a number of different cell types, particularly in cells of non-neuronal lineage.

We attempted to localize BACE endoproteolysis by targeting the protein to different sites within the secretory pathway. These studies revealed that the cleavage event takes place only after the protein is exported from the ER and appears to be enhanced in the TGN. We also found BACE endoproteolysis to be sensitive to inhibitors of vesicular acidification, a result consistent with a TGN and/or endosomal localization for BACE cleavage. Multiple lines of evidence implicate the TGN in BACE endoproteolysis. Thus, we considered two membrane-bound proteases, TACE and furin, both of which are active in the TGN. The potential impact of BACE endoproteolysis on APP processing is intriguing because β-secretase activity is known to be quite low in non-neuronal tissues, such as the pancreas, that

![Diagram](https://example.com/diagram.png)
express high levels of BACE mRNA (9, 10). Some have argued that a predominance of alternatively spliced BACE variants with reduced APP processing activity may account for this discrepancy (57, 58). However, more recent data indicate that the most abundant BACE mRNA species in pancreas correspond to full-length protein, implying that down-regulation of β-secretase activity most likely occurs at a post-translational level. We have shown that BACE exists primarily as an endoproteolytic fragment in pancreas, muscle, and liver, whereas holoprotein predominates in neuronal tissue. This would imply that BACE endoproteolysis significantly reduces β-secretase activity in these tissue types. We observed definitive β-secretase activity and subsequent Aβ production in HEK293 cells, where virtually all detectable BACE protein exists as endoproteolytic fragments. Although we cannot completely exclude the possibility that trace amounts of BACE were generated by the highly abundant endoproteolyzed BACE species. Therefore, these findings in conjunction with previous work (9, 10) suggest that endoproteolysis may significantly attenuate, but most likely does not abolish, the β-secretase activity of BACE in certain non-neuronal cell types. An improved understanding of this post-translational event could aid considerably in the crafting of methods to reduce cerebral amyloid deposition by specifically activating BACE endoproteolysis in neural tissue.

Finally, the effects of BACE endoproteolysis may extend beyond APP cleavage. For instance, BACE processing could modulate the ability of the protease to act on other substrates, a possibility made all the more feasible by recent findings implicating the protease in the cleavage of the sialyltransferase, ST6Gal-1 (60). Much work remains to be done to better modulate the ability of the protease to act on other substrates, beyond APP cleavage. For instance, BACE processing could considerably in the crafting of methods to reduce cerebral amyloid deposition by specifically activating BACE endoproteolysis in neural tissue.

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