BACE IsDegraded via theLysosomal Pathway*

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Amphiphilic peptides are formed by aggregates of amyloid-β-peptide, a 37–43-amino acid fragment (primarily Aβ40 and Aβ42) generated by proteolytic processing of the amyloid precursor protein (APP) by β- and γ-secretases. A type I transmembrane aspartyl protease, BACE (β-site APP cleaving enzyme), has been identified to be the β-secretase. BACE has been shown to undergo rapid maturation into a ~70-kDa form in the Golgi apparatus, involving the addition of complex carbohydrates and the removal of the propeptide domain by furin proteolysis (5). The ectodomain contains four glycosylation sites and two signature sequences typically associated with aspartyl proteases (DT/SYT) (6). Furthermore, the BACE ectodomain has been shown to undergo shedding following cleavage by ADAM 10 (5, 7). BACE is targeted through the secretory pathway to the plasma membrane where it can be internalized to endosomes. The carboxyl terminus of BACE contains a di-leucine-based signal for sorting of transmembrane proteins to endosomes and lysosomes. In this study, we set out to determine whether BACE is degraded by the lysosomal pathway and whether the di-leucine motif is necessary for targeting BACE to the lysosomes. Here we show that lysosomal inhibitors, chloroquine and NH4Cl, lead to accumulation of endogenous and ectopically expressed BACE in a variety of cell types, including primary neurons. Furthermore, the inhibition of lysosomal hydrolases results in redistribution and accumulation of BACE in the late endosomal/lysosomal compartments (lysosome-associated membrane protein 2 (LAMP2)-positive). In contrast, the BACE-L/LA mutant, in which Leu499 and Leu500 in the COOH-terminal sequence (DDISLLK) were replaced by alamines, partially co-localized with LAMP2-positive compartments following inhibition of lysosomal hydrolases. Collectively, our data indicate that BACE is transported to the late endosomal/lysosomal compartments where it is degraded via the lysosomal pathway and that the di-leucine motif plays a role in sorting BACE to lysosomes.

The amyloid hypothesis of Alzheimer disease (AD)2 maintains that the accumulation of the amyloid-β-peptide (Aβ) is a critical event in disease pathogenesis. Amyloid plaques are formed by aggregates of Aβ, a 37–43-amino acid peptide (primarily Aβ40 and Aβ42) generated by proteolytic processing of the amyloid precursor protein (APP), a large type I integral transmembrane protein. APP can be cleaved by three proteolytic processing of the amyloid precursor protein (APP), a large type I integral transmembrane protein. APP can be cleaved by three proteolytic enzymes: the γ-secretase, β-secretase, and α-secretase (2, 4). BACE is initially synthesized in the endoplasmic reticulum as an immature precursor protein (pro-BACE) with a molecular mass of 60 kDa, which then undergoes rapid maturation into a ~70-kDa form in the Golgi apparatus, involving the removal of the propeptide domain by furin proteolysis (5). The ectodomain contains four glycosylation sites and two signature sequences typically associated with aspartyl proteases (DT/SYT) (6). Furthermore, the BACE ectodomain has been shown to undergo shedding following cleavage by ADAM 10 (5, 7). BACE is targeted through the secretory pathway to the plasma membrane where it can be internalized to endosomes. The carboxyl terminus of BACE contains a di-leucine-based signal for sorting of transmembrane proteins to endosomes and lysosomes. In this study, we set out to determine whether BACE is degraded by the lysosomal pathway and whether the di-leucine motif is necessary for targeting BACE to the lysosomes.

EXPERIMENTAL PROCEDURES

Materials—Lactacystin, chloroquine, peptide aldehyde MG132, ALLN, and NH4Cl were obtained from either Sigma or Calbiochem. Monoclonal and polycyonal antibodies against Myc tag (9B11) were obtained from Cell Signaling. The rabbit anti-BACE COOH-terminal antibody was purchased from Affinity Bioreagents. The rabbit antibodies recognize the proregion of immature BACE (residues 26–45) and the NH2-terminal end of the mature BACE (residues 46–65) were purchased from Calbiochem. The anti-β-tubulin antibody was purchased from Sigma. Texas Red dextran was purchased from Molecular Probes. The anti-β-catenin antibody was purchased from Transduction Laboratories.

cDNA Vectors—A human BACE wild type cDNA was cloned into pcDNA4 (Invitrogen) with Myc-His tag fused at the COOH terminus of BACE (BACE WT-Myc). BACE wild type and mutant (BACE-L/A) with V5 tag at the COOH terminus have been already described (16).
Lysoosomal Degradation of BACE

mutant BACE-L/A, Leu499 and Leu500 in the COOH-terminal sequence (DDISLLK) were replaced by alanines.

Cell Culture, Transfection, and Western Blotting—H4 human neuroglioma cells, SH-SY5Y human neuroblastoma cells, and CHO cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin. Primary cortical cultures were established from E16 mouse embryos, as described previously (17). H4 human neuroglioma cells were transfected with BACE cDNAs using Lipofectamine 2000 reagent (Invitrogen) according to the supplier’s instructions. H4 cells stably expressing BACE-Myc were selected by culturing them in Dulbecco’s modified Eagle’s medium with supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 200 µg/ml G418. The cells were then washed and lysed in radioimmunoprecipitation assay buffer. For Western blot analysis, total proteins (50–100 µg/lane) were separated by NuPage 4–12% BisTris-polyacrylamide gel electrophoresis (Invitrogen) using MES running buffer (Invitrogen) and then blotted on Immuno-Blot™ polyvinylidene difluoride membranes (Bio-Rad). Proteins were visualized using the Lumi-GLO™ protein detection kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD) as described by the manufacturer.

Immunocytochemistry—H4 cells expressing the BACE WT-Myc (H4-B) or H4 cells transiently expressing BACE WT-V5 or BACE-LL/AA mutant-V5 cDNA on coverslips were fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton X-100. They were then washed with PBS at room temperature and processed with the appropriate primary antibody diluted in PBS supplemented with 2% goat IgG. BACE WT-Myc was detected with anti-Myc monoclonal or polyclonal antibodies. BACE-LL/AA mutant-V5 was detected with anti-V5 monoclonal or polyclonal antibodies. Coverslips were washed twice with PBS followed by incubation for 1 h with the indicated combinations of secondary antibodies diluted in PBS supplemented with 2% goat IgG. Following incubation, coverslips were washed twice with PBS, mounted, and subjected to fluorescent microscopic analysis with a Nikon Eclipse TE-300 microscope. Primary antibodies and dilutions were as follows: anti-Myc monoclonal or polyclonal antibodies (Cell signaling), 1:400; anti-EA1 (BD Transduction Laboratories), 1:500; anti-syntaxin 6 (BD Transduction Laboratories), 1:200; LAMP2 (monoclonal antibody H4B4) (BD Transduction Laboratories), 1:50; anti-V5 polyclonal antibody (Bethyl Laboratories, Montgomery, TX), 1:200. Alexa 488 (green) and Alexa 594 (red) secondary antibodies (Molecular Probes, Eugene, OR) were used at 1:200 dilutions. For dextran internalization, cells cultured on coverslips were incubated with Texas Red-labeled dextran (0.1 mg/ml) in serum-free Dulbecco’s modified Eagle’s medium with 1 mg/ml of bovine serum albumin for 8 h at 37 °C and then washed with PBS and fixed in 4% paraformaldehyde.

RESULTS

Inhibition of Lysoosomal Hydrolases Leads to Increased Levels of Both Ectopically Expressed and Endogenous BACE—Readily available proteasome and lysosomal inhibitors allow a rapid analysis in intact cells of the possible contributions of these two major degradatory pathways (18). Chloroquine and NH4Cl are weak bases known to inhibit lysosomal hydrolases by reducing the acidification of the endosomal/lysosomal compartments (19). Inhibitors of the proteasome include: lactacystin, a Streptomyces metabolite, which inhibits the proteasomal degradation of proteins specifically targeting the 20 S proteasome (20); MG132, which is a less specific proteasome inhibitor that has also been shown to inhibit calpain, cathepsin, and γ-secretase (21); and ALLN, which inhibits both the proteasome and calpain, as well as acidic cathepsins found in the lysosomes (22). We first determined protein levels of BACE following inhibition of proteasomal or lysosomal degradation in H4 cells stably expressing BACE tagged at the COOH terminus with Myc (H4-B cells). H4-B cells were treated with lactacystin (10 µM), ALLN (50 µM), and lysosomal inhibitors, chloroquine (100 µM) and NH4Cl (50 mM). DMSO, dimethyl sulfoxide. B, dose dependence of chloroquine on BACE up-regulation. Cells were treated with various concentrations (10, 50, 100, and 200 µM) of chloroquine for 24 h, C, time dependence for chloroquine (100 µM). Equivalent amounts of protein from each sample were immunoblotted with monoclonal anti-Myc antibody for BACE or anti-β-tubulin antibody. This is a representative experiment of three experiments.

We next set out to test whether treatment with lysosomal inhibitors also induces an increase in endogenous BACE. Naïve H4, SH-SY5Y, CHO cells, and primary cortical neurons were treated with lactacystin (10 µM) or MG132 (1 µM), which is a more specific proteasome inhibitor than ALLN (18), chloroquine (50 µM), or NH4Cl (50 mM) for 24 h. Endogenous BACE was detected by a rabbit polyclonal antibody raised against BACE COOH terminus (Affinity Bioreagents). As expected, endogenous BACE protein levels were markedly increased following treatment with either chloroquine or NH4Cl in H4, SH-SY5Y, CHO cells, and primary cortical neurons (Fig. 2A, B, C, and D, respectively). In contrast, treatment with proteasome inhibitors, lactacystin or MG132, failed to increase endogenous BACE protein levels in cells. Finally, as a positive control for proteasomal degradation, we assessed protein levels of β-catenin, a protein known to be degraded by the proteasome (23), following treatment with proteasomal inhibitors. As expected, treatment with lactacystin or MG132 increased protease levels in primary cortical cultures (Fig. 2D). Collectively, these findings indicate that endogenous BACE degradation requires the activity of lysosomal hydrolases but not the proteasome in a variety of cell lines including primary neurons.

The Inhibition of Lysoosomal Hydrolases Leads to Accumulation of Both Mature and Immature BACE—Since chloroquine and NH4Cl treatment resulted in the accumulation of various isoforms of BACE in H4 cells, we next addressed whether they represent mature or immature BACE. H4-B cells were treated with lactacystin, MG132, or NH4Cl for 24 h. Western blot analysis performed with antibodies that specifically detected immature/pro-BACE or mature BACE (anti-BACE26–45 and anti-BACE46–69, respectively), or with a pan-BACECOOH-terminal antibody, revealed that NH4Cl treatment inhibited the degradation of both
mature and immature BACE (Fig. 3, A and B). These data suggest that some molecules of pro-BACE likely escape the furin-mediated proteolysis in the Golgi and reach the endosomal/lysosomal apparatus.

Inhibition of Lysosomal Hydrolases Does Not Affect Levels of Secreted BACE—It has been previously reported that the extracellular domain of BACE is cleaved and released from the cells in a soluble form (5). Thus, we next tested whether the accumulation of BACE induced by treatment with lysosomal inhibitors was due to inhibition of BACE shedding and/or secretion. Levels of secreted BACE were determined in conditioned media from H4-B cells treated with either proteasomal or lysosomal inhibitors by Western blot analysis with the anti-BACE46–65 antibody. Levels of secreted BACE were similar in the media from treated and untreated cells (Fig. 4). These results rule out the possibility that the reduction in BACE levels following treatment with lysosomal inhibitors is due to inhibition of BACE shedding and/or secretion.

Treatment with Lysosomal Inhibitors Cause Accumulation of BACE in the Lysosomal Compartments—To determine the cellular compartments where BACE accumulates following inhibition of lysosomal proteases, we carried out immunofluorescence microscopy. It has been previously shown by immunofluorescence microscopy that ectopically expressed BACE is localized primarily in the TGN and the endosomal system (9, 24, 25). However, significant quantities of the glycoprotein are also present in the endoplasmic reticulum, at the cell surface (9, 26, 27), and to a lesser extent in the lysosomes (9, 24). Ectopically expressed BACE demonstrated a highly punctuate staining pattern reminiscent of vesicular compartments in H4-B cells (Fig. 5, A, D, and G). Using various subcellular markers, we confirmed that BACE co-localizes with TGN (syntaxin 6), early endosomes (EEA1), and partially with lysosomes when lysosomal hydrolases are inhibited.

It has been previously shown that dextran, hydrophilic polysaccharides synthesized by *Leuconostoc* bacteria, are internalized in cells by fluid-phase endocytosis and delivered through endosomal compartments to the lysosomes, where they accumulate due to their acid hydrolase-resistant nature (19). Dextran accumulates in the lysosomes after 8 h of treatment (28). Therefore, H4-B cells were treated with dextran conjugated with Texas Red (TR-dextran) for 8 h. Ectopically expressed BACE exhibits partial localization with TR-dextran-positive vacuoles, with a pattern similar to the one observed for LAMP2 staining (Fig. 5, J–L). However, both NH4Cl and chloroquine treatment resulted in a dramatic accumulation of BACE in TR-dextran-positive vacuoles (Fig. 5, M–R). These results indicate that BACE accumulates in the lysosomes when lysosomal hydrolases are inhibited.

Mutation of the COOH-terminal BACE Di-leucine Sorting Signal Reduces the Accumulation of BACE in the Lysosomes—Di-leucine-based sorting motifs have been associated with endosomal/lysosomal targeting from the cell surface as well as from the TGN (29). It has been previously shown that dextran, hydrophilic polysaccharides synthesized by *Leuconostoc* bacteria, are internalized in cells by fluid-phase endocytosis and delivered through endosomal compartments to the lysosomes, where they accumulate due to their acid hydrolase-resistant nature (19). Dextran accumulates in the lysosomes after 8 h of treatment (28). Therefore, H4-B cells were treated with dextran conjugated with Texas Red (TR-dextran) for 8 h. Ectopically expressed BACE exhibits partial localization with TR-dextran-positive vacuoles, with a pattern similar to the one observed for LAMP2 staining (Fig. 5, J–L). However, both NH4Cl and chloroquine treatment resulted in a dramatic accumulation of BACE in TR-dextran-positive vacuoles (Fig. 5, M–R). These results indicate that BACE accumulates in the lysosomes when lysosomal hydrolases are inhibited.

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V5-tagged or BACE-LL/AA-V5-tagged cDNAs in which Leu499 and Leu500 in the COOH-terminal sequence (DDISLLK) were replaced by alanines (Fig. 6A). Double staining with an anti-V5 and anti-LAMP2 antibody revealed that BACE accumulates in organelles LAMP2 positive following chloroquine treatment (Fig. 6B, panels d–f) with a pattern similar to the one observed following TR-dextran uptake (Fig. 5, M–R). Conversely, the BACE-LL/AA mutant, which is retained at the cell membrane (Fig. 6B, panels g–j), only partially co-localized with TR-dextran-positive vacuoles with a pattern similar to the one observed for LAMP2 staining (J–L). However, NH₄Cl or chloroquine treatment induced a dramatic accumulation of BACE in the TR-dextran-positive vacuoles (M–R).

**DISCUSSION**

We have found that lysosomal inhibitors (chloroquine, NH₄Cl), but not proteasomal inhibitors (lactacystin, MG132) lead to the accumulation of both endogenous and ectopically expressed BACE in a variety of cell types, including primary cortical neurons. These data are somewhat discrepant but reconcilable with a previous report (15) of an increase in BACE protein levels following treatment with proteasomal inhibitors in SH-SY5Y cells overexpressing BACE. It is not clear from this study that endogenous BACE would normally be degraded by the proteasome. Moreover, in the same study, the authors did not test for the effects of lysosomal inhibitors on BACE degradation. This is a critical control to confirm that the observed effects were actually due to the inhibition of the proteasome as opposed to cathepsins, which are also inhibited by some proteasomal inhibitors (18).
Employing both a fluid-phase marker (TR-dextran) and LAMP2 staining, we showed that BACE accumulates in late endosomes/lysosomes following inhibition of lysosomal hydrolases. These data indicate that BACE is transported to late endosomal/lysosomal compartments where it can then be degraded via the lysosomal pathway. We also found that mutagenesis of the di-leucine motif in the COOH-terminal sequence (DDISLLK) were replaced by alanines. B. Naïve H4 cells were transiently transfected with either BACE-WT/V5 (panels a–f) or BACE-LL/AA mutant-V5 (panels g–i), and then they were incubated for 24 h in the absence (panels a–c) or presence of chloroquine (50 μM) (panels d–f and j–l). Following fixation and permeabilization, the cells were labeled with monoclonal antibody against LAMP2 or polyclonal antibody against the V5 tag.

Interestingly, GGA1, -2, and -3 (Golgi-localized γ-ear-containing ARF binding proteins) have been shown to bind the BACE acidic di-leucine motif (10, 31). Moreover, phosphorylation of serine 498 in BACE motif greatly reduces the accumulation of BACE in LAMP2-positive compartments following inhibition of lysosomal hydrolases. These data indicate that the di-leucine motif not only plays a role in BACE endocytosis but also in sorting BACE to the lysosomes.

These studies suggest that increased BACE protein levels in AD patients may be partly due to decreased degradation. In this case, our current data would implicate a role for impaired lysosomal degradation as a potential underlying mechanism. Further studies will be necessary to determine the extent to which impairment of lysosomal degradation may contribute to increased BACE protein levels and activity in AD pathogenesis.

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FIGURE 6. Mutation of the COOH-terminal BACE di-leucine sorting signal reduces the accumulation of BACE in LAMP2-positive compartments following inhibition of lysosomal hydrolases. A, schematic representation of the BACE-LL/AA mutants in which Leu199 and Leu500 in the COOH-terminal sequence (DDISLLK) were replaced by alanines. B, Naïve H4 cells were transiently transfected with either BACE-WT-V5 (panels a–f) or BACE-LL/AA mutant-V5 (panels g–i), and then they were incubated for 24 h in the absence (panels a–c) or presence of chloroquine (50 μM) (panels d–f and j–l). Following fixation and permeabilization, the cells were labeled with monoclonal antibody against LAMP2 or polyclonal antibody against the V5 tag.
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