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Maturation and Endosomal Targeting of β-Site Amyloid Precursor Protein-cleaving Enzyme

THE ALZHEIMER'S DISEASE β-SECRETASE*

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The amyloidogenic Aβ peptide is liberated from the amyloid precursor protein (APP) by two proteolytic activities, β-secretase and γ-secretase. Recently, a type I membrane protein termed BACE (β-site APP cleaving enzyme) with characteristics of an aspartyl protease has been identified as the β-secretase. We undertook a series of biochemical and morphological investigations designed to characterize the basic properties of this protein. Initial studies indicated that BACE undergoes *N*-linked glycosylation at three of four potential sites. Metabolic pulse-chase experiments revealed that after core glycosylation, BACE is rapidly and efficiently transported to the Golgi apparatus and distal secretory pathway. BACE was also found to be quite stable, being turned over with a $t_{1/2}$ of ~16 h. Retention of BACE in the endoplasmic reticulum by introduction of a C-terminal dilysine motif prevented complex carbohydrate processing and demonstrated that propeptide cleavage occurs after exit from this organelle. BACE exhibited intramolecular disulfide bonding but did not form oligomeric structures by standard SDS-polyacrylamide gel electrophoresis analysis and sedimented as a monomer in sucrose velocity gradients. Immunofluorescence studies showed a largely vesicular staining pattern for BACE that colocalized well with endosomal, but not lysosomal, markers. Measurable levels of BACE were also detected on the plasma membrane by both immunostaining and cell surface biotinylation, and cycling of the protein between the cell membrane and the endosomes was documented. A cytoplasmic dileucine motif was found to be necessary for normal targeting of BACE to the endosomal system and accumulation of the protein in this intracellular site.

Alzheimer's disease (AD)[1] is a progressive neurodegenerative disorder characterized pathologically by the presence of parenchymal plaques composed largely of the amyloid β peptide (Aβ) (1). The deposition of Aβ is believed by many to initiate the crucial series of pathological events that ultimately lead to neuron loss and dementia (2, 3). Proteolytic processing of the amyloid precursor protein (APP) by two enzymatic activities, β- and γ-secretase, releases Aβ (4). The peptide is then thought to form insoluble aggregates, which amass in brain tissue eventually forming the pathognomonic lesions associated with the disease (5). The seemingly pivotal role played by Aβ in AD pathogenesis underscores the importance of thoroughly characterizing the proteases involved with the generation of the peptide. Presenilin 1 and presenilin 2 have been shown to mediate γ-secretase activity (6) and may themselves be actual γ-secretases (7, 8), although the latter has yet to be demonstrated definitively.

Recently, several reports have identified the type I integral membrane glycoprotein BACE (Asp 2, memapsin 2) as the putative β-secretase (9–13). BACE and its close relative BACE2 (Asp 1) together define a new class of membrane-bound aspartyl proteases (14). BACE itself constitutes the predominant β-secretase activity in human brain tissue (12). Overexpression of BACE leads to increased β-secretase activity while displaying appropriate cleavage site specificity on APP. In addition, purified BACE proteolyzes synthetic APP peptide substrates, with augmented activity toward those harboring the familial Alzheimer's disease (FAD)-associated KM → NL mutation (9, 11). Furthermore, immunostaining reveals the presence of BACE in endosomes, one of several intracellular sites where Aβ is thought to be produced (9).

Although the evidence that BACE is the Alzheimer's disease β-secretase is quite compelling, little is known about its behavior *in vivo*. More specifically, the cellular compartments where BACE is active and presumably interacts with APP remain largely unestablished as does the nature of the interaction between the two proteins. Reports have localized the generation of Aβ to the endosomal system (15), as well as to the trans-Golgi network (TGN) (16–18) and the endoplasmic reticulum/intermediate compartment (19–22). BACE could presumably act on APP in any or all of these regions. To begin to address these issues more adequately, we undertook a series of biochemical and morphological investigations designed to characterize the basic properties of BACE. In this report, we demonstrate BACE to be a stable, monomeric protein that, after

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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; ER, endoplasmic reticulum; TGN, trans-Golgi network; HA, influenza hemagglutinin; Man-6-PR, mannose 6-phosphate receptor; BN-1, BACE N-terminal antibody 1; BC-1, BACE C-terminal antibody 1; PNGase F, peptide:*N*-glycosidase F; Endo H, endoglycosidase H; BME, β-mercaptoethanol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

core glycosylation in the ER, is rapidly and efficiently transported to the Golgi apparatus before targeting to the endosomal system. Cycling of BACE between endosomes and the cell surface is shown. We also demonstrate that a dileucine motif on the cytoplasmic tail of BACE plays an important role in the normal trafficking of the protein to endocytic compartments.

EXPERIMENTAL PROCEDURES

Generation and Expression of BACE Constructs—Wild type BACE was generated by reverse transcriptase-polymerase chain reaction, cloned into pcDNA3.1, and its sequence was confirmed. All other BACE constructs were generated by oligonucleotide-based mutagenesis after which they were T-A-cloned into the pTarget™ eukaryotic expression vector (Promega). Expression in 293 and HeLa cells was obtained using Geneporter transfection reagent (Gene Therapy Systems, San Diego CA) with a DNA/Geneporter ratio of approximately 1 μg/10 μl. 293 and HeLa cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% penicillin/streptomycin.

Antibodies—N-terminal (ETDEEPEEPGRRGSFVEC) and C-terminal (LRQQHDDFADDISLL) BACE peptides were generated and used to produce rabbit polyclonal antisera. BACE constructs containing a C-terminal influenza hemagglutinin antigenic tag (HA) were detected using either a polyclonal (polyclonal antibody HA11; Covance, Richmond, CA) or a monoclonal antibody (mAb HA11; Covance) directed against the HA epitope. Immunoprecipitations and Western blots were performed by standard methods.

Metabolic Labeling and Pulse-Chase Experiments—293 cells in 6-well plates (~1 million cells per well) were preincubated in methionine/cysteine-free (starve) medium for 30 min after which they were incubated in starve medium supplemented with 0.5 mCi of [^{35}S]methionine/cysteine per well. For pulse-chase studies, cells expressing BACE-HA were metabolically labeled as described above after which they were quenched in starve medium supplemented with a 100 molar excess of cold methionine/cysteine. The cells were then chased in normal growth medium for a variable amount of time after which they were washed, lysed in radioimmunoprecipitation assay (RIPA) buffer (1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 50 mM Tris, pH 8, 150 mM NaCl), and immunoprecipitated with mAb HA11. Conditions were carefully defined to ensure quantitative immunoprecipitations using mAb HA11. Immunoprecipitates were eluted by boiling in 1× Laemmli sample buffer plus 0.34 M β-mercaptoethanol for 3–5 min. Samples were then electrophoresed on 8% polyacrylamide gels, transferred to nitrocellulose, and exposed to a PhosphorImager screen for 24–48 h. PhosphorImager screens were read on a Storm 860 Scanner and bands were quantified in ImageQuant version 1.11.

Endoglycosidase H Digestions—Immunoprecipitates from pulse-chase experiments were eluted by boiling in 25 μl of 0.1% SDS, 0.1 M BME for 5 min. Each sample was then divided into two 12.5-μl aliquots, one of which was treated with 7.5 μl of 0.5 M sodium citrate, pH 5.5, 37.5 μl of H_2O, 5 μl of 1% phenylmethylsulfonyl fluoride in isopropyl alcohol, and 5 μl of 0.5 units/ml Endo H (Sigma), whereas the other was treated with the same reaction mixture with H_2O substituted for Endo H. The digestions were incubated overnight at 30 °C and stopped by the addition of 6× Laemmli sample buffer plus 2 M BME. Samples were then electrophoresed on 8% polyacrylamide gels, transferred to nitrocellulose, and exposed to a PhosphorImager screen for 36–48 h.

PNGase F Digestions—293 cells expressing BACE-HA or BACE-HAKK were metabolically labeled for 2.5 h, lysed in RIPA buffer, and immunoprecipitated with mAb HA11. Immunoprecipitates were eluted by boiling in 25 μl of 0.1% SDS, 0.1 M BME for 5 min, and separated into 10-μl aliquots. To each aliquot was then added 3 μl of 0.5 M Tris·Cl, pH 8.6, 5 μl of H_2O, 2 μl of 10% Nonidet P-40, and 5 μl of 250 milliunits/ml PNGase F (Glyco). For negative controls, 0.5 M Tris·Cl, pH 8.6, was added instead of PNGase F. Reactions were incubated at 37 °C for a variable times after which they were stopped by the addition of 6× Laemmli sample buffer plus 2 M BME. Samples were then electrophoresed on 8% polyacrylamide gels, dried down, and exposed to a PhosphorImager screen for 36–48 h.

Velocity Gradient Sedimentation—293 cells in 35-mm dishes expressing BACE-HA were lysed in either 1% Triton X-100 or 50 mM CHAPS lysis buffer (each with 50 mM Tris, pH 7.6, 150 mM NaCl, and 2 mM EDTA). Lysates were rocked for approximately 1 h on ice after which they were loaded on 5–20% continuous sucrose gradients containing either 0.1% Triton X-100 or 25 mM CHAPS. Gradients were spun in an SW40 ultracentrifuge rotor at 4 °C for 20 h at 40,000 rpm. They were then spit into 15 fractions of roughly 750 μl. 20–30 μl of each fraction were electrophoresed on 8% polyacrylamide gels, transferred to nitrocellulose, and blotted with mAb HA11.

Fluorescence Microscopy—HeLa cells on coverslips expressing the appropriate BACE construct were fixed and permeabilized in ice-cold methanol. They were then washed with PBS at room temperature and processed with the appropriate primary antibody diluted in PBS supplemented with 2% fetal bovine serum (FBS) and 0.02% saponin. BACE-HA was detected with polyclonal antibody HA11. A monoclonal antibody directed against the transferrin receptor was used to detect endosomes (mAb B3/25; Roche Molecular Biochemicals), whereas lysosomes were detected with monoclonal antibodies directed against either LAMP 1 or LAMP 2 (mAb B3/25 or mAb H4B4 respectively; Developmental Studies Hybridoma Bank, the University of Iowa, Iowa City, IA). The cells were then incubated with fluorescent-conjugated secondary antibodies also diluted in PBS with 2% FBS and 0.02% saponin. Secondary antibodies were obtained from Molecular Probes (Eugene, OR) and consisted of either the Alexa Fluor 488 goat anti-rabbit or Alexa Fluor 594 goat anti-mouse. After antibody staining, coverslips were mounted on slides with Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, AL) and sealed with nail polish. Fluorescence was examined at 60× with either a Nikon E600 microscope utilizing UV illumination or with a Bio-Rad MCR 1024 confocal microscope.

For surface staining, cells expressing the appropriate BACE construct were fixed in 3.7% formaldehyde after which they were either permeabilized with 0.1% Triton X-100 for 5 min at room temperature or left untreated. The cells were then washed 3 times with PBS and incubated with either mAb HA11 or BN-1 diluted in PBS supplemented with 2% FBS. An Alexa Fluor 488 goat anti-mouse secondary was used for these procedures. Coverslips were mounted and observed as described above.

Transferrin uptake experiments were performed as follows. Cells expressing BACE-HA were incubated in serum-free medium for 15 min at 37 °C, after which they were treated with the same medium supplemented with biotinylated transferrin (50 μg/ml) for 30 min. Cells were then fixed with 3.7% formaldehyde, permeabilized, and treated with the appropriate primary and secondary antibodies. BACE-HA was visualized with mAb HA11 while a streptavidin-fluorescein isothiocyanate secondary (PharMingen, San Diego, CA) was used to detect biotinylated transferrin.

Cell Surface Biotinylation—Unless otherwise stated, all reagents for biotinylation procedures were kept on ice. 293 cells in 35-mm dishes expressing the appropriate BACE construct were placed on ice and washed twice with KRPH buffer (128 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, 5 mM Na_2HPO_4, 20 mM HEPES, pH 7.4). Cells were then incubated in KRPH supplemented with 0.5 mg/ml EZ-Link™ Sulfo-NHS-SS-Biotin (Pierce) for 30 min after which they were washed three times with KRPH plus 20 mM glycine. The final wash was allowed to sit for 15 min. The cells were then washed twice more with KRPH, lysed in RIPA buffer, and immunoprecipitated with mAb HA11. Immunoprecipitates were eluted by boiling in 1× Laemmli samples buffer without BME, electrophoresed on 8% polyacrylamide gels, transferred to nitrocellulose, and blotted with ^{125}I-streptavidin (Amersham Pharmacia Biotech). The blots were then exposed to a PhosphorImager screen for 24–36 h.

Internalization and Recycling Studies—For internalization experiments, cells were biotinylated as described above. However, instead of proceeding directly to lysis, the cells were returned to pre-warmed 37 °C growth media for a variable amount of time after which they were washed twice with KRPH and incubated in cleavage buffer (50 mM glutathione (Sigma), 90 mM NaCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, 0.2% bovine serum albumin, pH 8.6) for two intervals of 15 min each. After cleavage, the cells were washed twice with KRPH plus 20 mM glycine. The second wash was allowed to sit for 15 min. The cells were then washed twice more with KRPH and lysed in RIPA buffer. For detecting BACE recycling to the cell surface, this procedure was modified slightly. After the final KRPH wash, cells were returned once more to pre-warmed 37 °C growth media for either 30 or 60 min after which they were washed, treated with cleavage buffer, and lysed in a manner identical to internalization experiments. As a control for protein degradation and cell loss, one dish of cells was not treated with cleavage buffer a second time before lysis. Immunoprecipitations and Western blots proceeded as described previously for cell surface biotinylation experiments.

RESULTS

BACE Is Glycosylated at 3 of 4 Potential N-Linked Sites and Exists as a Mixture of Mature and Immature Species—BACE is

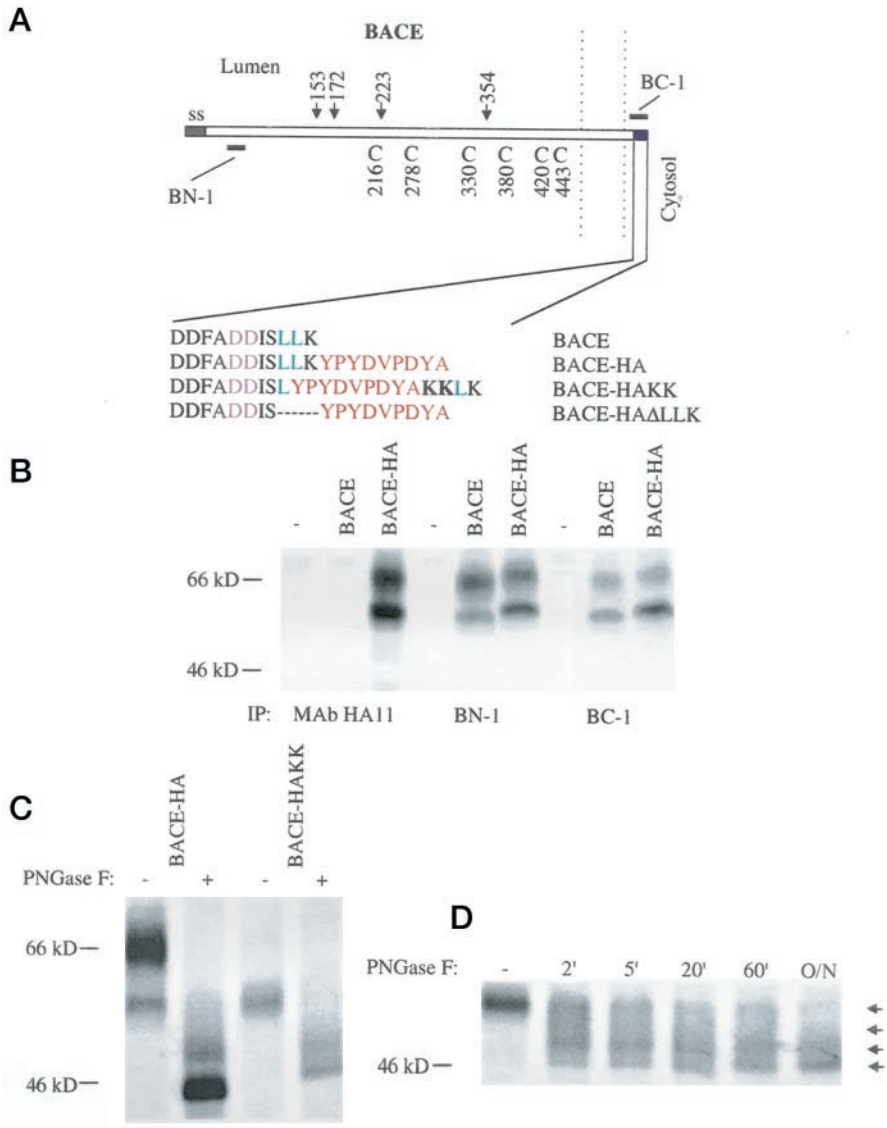

FIG. 1. **Expression and *N*-linked glycosylation of BACE.** *A,* schematic representation of BACE constructs used. *Arrows* indicate potential sites for *N*-linked glycosylation, and lumenal cysteine residues are denoted by *C*. The putative signal sequence is marked *ss*. Regions used to design antibodies are also indicated (*BN-1* and *BC-1*). The C-terminal region has been enlarged to highlight mutagenesis strategies. The HA tag is shown in *red* and the dilysine ER retention motif is shown in *bold.* The cytoplasmic dileucine motif and its upstream acidic residues are shown in *green* and *purple,* respectively. *B,* 293 cells transfected with either BACE, BACE-HA, or empty vector (−) were metabolically labeled for 2.5 h, lysed, and immunoprecipitated with either mAb HA11, BN-1, or BC-1 as indicated. *C,* 293 cells expressing either BACE-HA or BACE-HAKK were metabolically labeled for 2.5 h, lysed, and immunoprecipitated with mAb HA11. Immunoprecipitates were eluted and either digested with PNGase F overnight (+) or left untreated (−). *D,* radiolabeled, immunoprecipitated BACE-HAKK was either left untreated (−) or digested with PNGase F for the indicated amount of time. *Arrows* denote fully glycosylated BACE-HAKK and three deglycosylated species.

a type I integral membrane protein containing 4 potential *N*-linked glycosylation sites and 6 extracellular/lumenal cysteine residues potentially involved in inter- or intramolecular disulfide bonding. To facilitate our characterization of BACE, an influenza hemagglutinin (HA) antigenic tag was introduced to the C terminus of the protein (BACE-HA) (Fig. 1*A*). In addition, antisera were raised against N- and C-terminal peptide sequences (Fig. 1*A, BN-1* and *BC-1*). Both BACE and BACE-HA were found by sandwich enzyme-linked immunosorbent assay to increase Aβ secretion by ~50% in cells stably expressing APPwt (data not shown), consistent with published reports (9) and confirming the biological activity of our constructs.

For biochemical studies, BACE constructs were expressed transiently in human embryonic kidney 293 cells using pcDNA3 vectors. These cells were used because they are easily transfectable and, due to the absence of the SV40T antigen, yield more modest levels of BACE expression than can be obtained in 293T cells. Immunoprecipitation of metabolically labeled BACE from 293 cells with either BN-1, BC-1, or mAb HA11 showed that both BACE and BACE-HA migrated as a tight ~58-kDa band coupled with a more diffuse ~66-kDa band (Fig. 1*B*). When a dilysine ER retention motif was placed on the C terminus of BACE-HA (BACE-HAKK) (Fig. 1*A*), the resulting protein was effectively sequestered in the ER (Fig. 4*B*). Immunoprecipitation of BACE-HAKK revealed that the ~66-kDa band was no longer apparent (see Fig. 1*C, lanes 1* and *3*), suggesting that the ~58-kDa band represents the immature ER resident species, whereas the ~66-kDa band corresponds to a mature form of BACE that has undergone further modification, most likely in the Golgi apparatus.

Previous work has shown BACE to be *N*-linked glycosylated (9). We confirmed this finding by subjecting radiolabeled BACE-HA and BACE-HAKK to digestion with peptide:*N*-glycosidase F (PNGase F), an enzyme that effectively removes all *N*-linked oligosaccharide side chains from glycoproteins. PNGase F treatment reduced the majority of both BACE-HA and BACE-HAKK down to a single ~46-kDa species, indicating the presence of *N*-linked sugars on the translated protein (Fig. 1*C*). It should be noted that PNGase F-digested BACE-HA migrated slightly faster than PNGase F-digested BACE-HAKK, which is retained in the ER. The reason for this is not clear, but a previous study has suggested that BACE undergoes a propeptide cleavage event (13). Our results suggest that this cleavage occurs only after BACE exits the ER. The number of *N*-linked sites on BACE that were core-glycosylated was estimated by treating radiolabeled BACE-HAKK with PNGase F for different amounts of time (Fig. 1*D*). BACE-HAKK was used to simplify the banding pattern since this ER-restricted form of BACE exists only as the 58-kDa immature species (Fig. 1*C*).

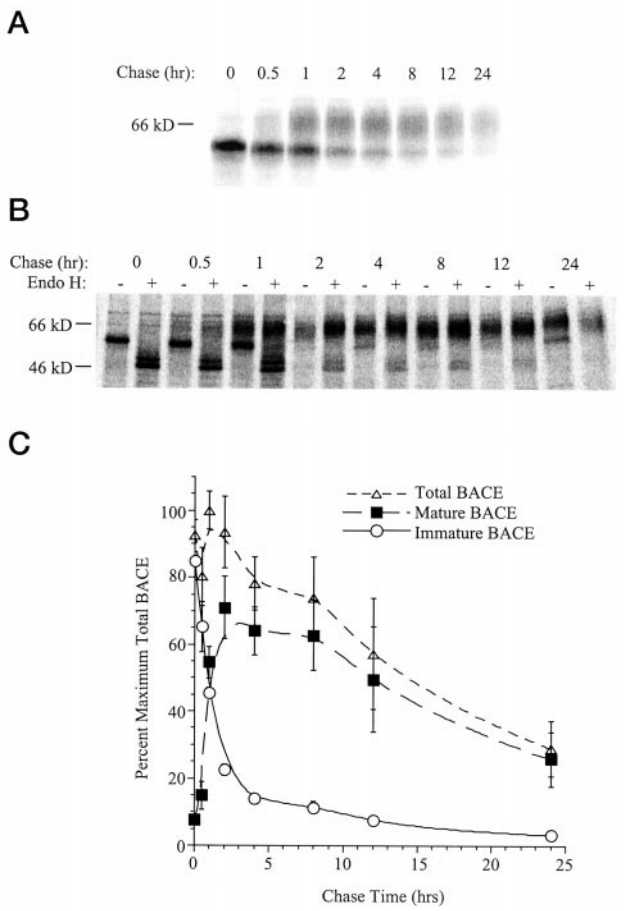

FIG. 2. **Maturation and turnover of BACE.** 293 cells expressing BACE-HA were metabolically labeled for 30 min and chased in cold medium for the indicated amount of time. Cells were then lysed and immunoprecipitated with mAb HA11. Samples were eluted and either electrophoresed directly (*A*) or split into two aliquots, one of which was digested with Endo H overnight (lanes indicated with a +) (*B*). BACE maturation from an immature species to a mature, Endo H-resistant species is shown. *C,* plot showing the kinetics of BACE maturation and turnover. Immature, mature, and total BACE-HA are expressed as a percentage of maximum total BACE-HA (1-h time point).

SDS-PAGE analysis of the resulting reaction mixtures revealed the presence of four bands, each roughly 4 kDa apart, most likely representing fully glycosylated BACE-HAKK and a triad of deglycosylated species with either one, two, or three oligosaccharide side chains removed. This suggests that three out of four potential *N*-linked sites on BACE are utilized during glycosylation in the ER.

Kinetics and Efficiency of BACE Transport—In order to gain a better understanding of BACE maturation, we performed a series of metabolic pulse-chase experiments. 293 cells transfected with BACE-HA were metabolically labeled with [^{35}S]methionine/cysteine and chased in non-radioactive media for various times up to 24 h. Subsequent cell lysis and quantitative immunoprecipitation with mAb-HA11 followed by SDS-PAGE analysis showed that the ~58-kDa immature species quickly and efficiently matured into the more diffusely migrating ~66-kDa species at later time points (Fig. 2*A*). That the ~66-kDa species does indeed correspond to a Golgi-modified form of BACE was confirmed by treating radiolabeled protein collected from pulse-chase studies with endoglycosidase H (Endo H), an enzyme that removes immature but not medial Golgi-processed *N*-linked oligosaccharide side chains. Thus, Golgi modification of *N*-linked sugars renders them resistant to Endo H cleavage. We found that while immature ~58-kDa BACE-HA was sensitive to Endo H, mature ~66-kDa BACE-HA was resistant (Fig. 2*B*). This finding verified our presumption that BACE maturation involves transport to the Golgi apparatus where all three oligosaccharide side chains of the protein are modified.

Quantification of pulse-chase data compiled from multiple experiments allowed us to measure the kinetics of BACE maturation and turnover. BACE transport from the ER occurred rapidly and efficiently. Mature Golgi-modified BACE-HA was initially detected at the 30-min time point, and by the 2-h time point constituted ~75% of total protein (Fig. 2*C*). By this time, a small amount of immature BACE-HA remained, although it clearly represented a minority species. We also found BACE to be a highly stable protein with a $t_{1/2}$ of ~16 h. The kinetics of BACE turnover was not significantly affected by either lactacystin, a specific proteasome inhibitor, or chloroquine, an agent that disrupts the acidification of endosomes and lysosomes (data not shown).

BACE Forms Heat-stable Disulfide Bonds and Exists Primarily as a Monomer—Many cellular and viral membrane proteins form homo- or hetero-oligomeric complexes, the formation of which is a prerequisite for exit from the ER as well as for normal protein function (23). Therefore, we considered the possibility that BACE might form stable complexes with itself and/or other proteins. To address this, we conducted a series of investigations designed to isolate higher molecular weight species immunoreactive for BACE. Lysates from 293 cells expressing BACE-HA were analyzed by Western blot with mAb HA11 after incubation at either 37, 55, or 95 °C in the presence or absence of β-mercaptoethanol (BME) (Fig. 3*A*). In the presence of reducing agent, BACE migrated as the characteristic doublet described previously with no evidence of higher molecular weight complexes, regardless of temperature. In the absence of reducing agent, BACE migrated more quickly as a single band, as would be expected of a protein containing intramolecular disulfide bonds. At lower temperatures, a small amount of BACE migrated as a higher molecular weight species. Although this could reflect the presence of BACE oligomers that exhibit some degree of SDS resistance, it could also be due to SDS-induced aggregation. Therefore, we employed an additional technique to determine if BACE forms oligomeric complexes.

Velocity gradient sedimentation was used as an alternative strategy to search for high molecular weight BACE complexes. 293 cells expressing BACE-HA were solubilized in either Triton X-100 or CHAPS lysis buffer. The lysates were then loaded on continuous sucrose velocity gradients, centrifuged for 20 h, and fractionated. Aliquots from each fraction were screened for BACE-HA by Western blot with mAb HA11 (Fig. 3*B*). In the presence of either Triton X-100 or CHAPS, BACE sedimented in a single, low density peak. The presence of high molecular weight complexes would have led to significant amounts of BACE in higher density fractions. Thus, this result is consistent with BACE existing primarily as a monomer.

BACE Co-localizes Well with Endosomal, but Not Lysosomal, Markers—To characterize accurately the cellular distribution of BACE, we performed a series of immunofluorescence experiments using both conventional and confocal microscopy. HeLa cells expressing a variety of BACE constructs were fixed and permeabilized. They were then stained with either BN-1, mAb-HA11, or a rabbit polyclonal antisera recognizing the HA epitope. Costaining with antibodies directed against a variety of organelle markers was also performed. BACE-HA demonstrated a highly punctate staining pattern reminiscent of vesicular compartments (Fig. 4*A*). An identical distribution was observed for BACE lacking the HA antigenic tag (data not shown). By contrast, cells expressing BACE-HAKK exhibited primarily nuclear envelope and ER immunoreactivity (Fig. 4*B*).

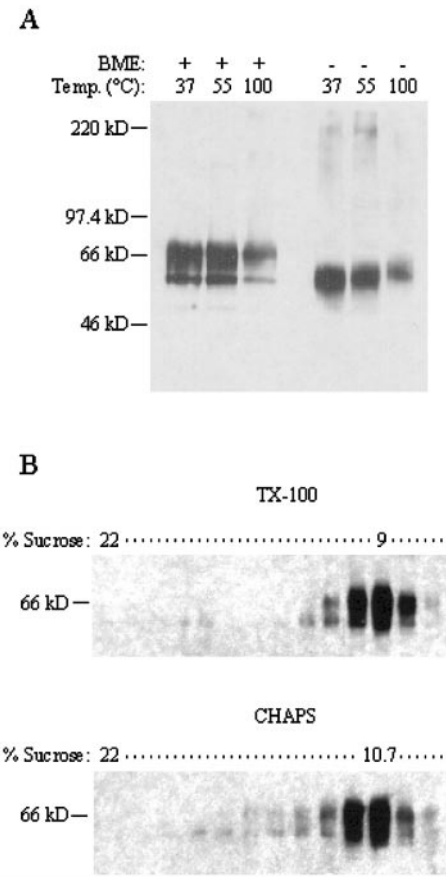

FIG. 3. **Analysis of BACE oligomerization.** *A,* lysates from 293 cells expressing BACE-HA were treated with an appropriate amount of 6× Laemmli sample buffer either with or without 2 M BME. Samples were then incubated at either 37, 55, or 100 °C before electrophoresis and Western blot with mAb HA11. Non-reduced BACE protein migrates more quickly indicating the presence of intramolecular disulfide bonds. *B,* 293 cells expressing BACE-HA were lysed in either Triton X-100 (*TX-100*) or CHAPS lysis buffer. Lysates were spun on continuous sucrose velocity gradients that were then fractionated from *bottom* to *top*. Aliquots of each fraction were screened for BACE-HA by Western blot with mAb HA11. The sucrose concentrations of the bottom, top, and peak fractions are indicated. BACE appears to sediment primarily as a monomer.

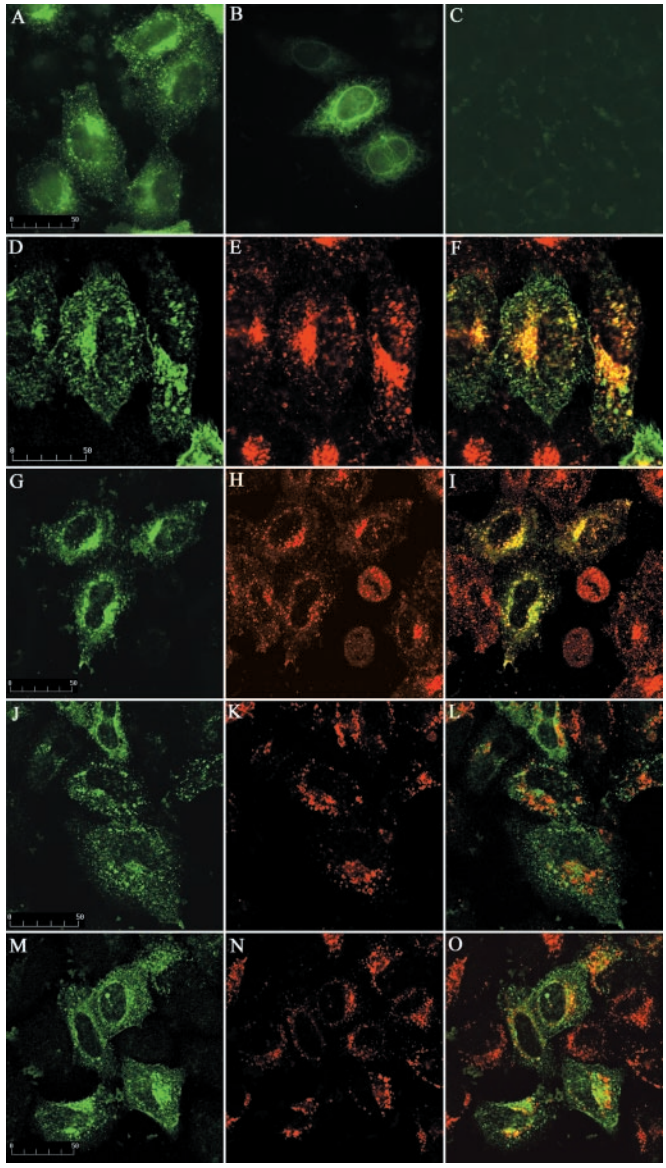

FIG. 4. **Morphology of BACE.** Immunofluorescent microscopy using an anti-HA antibody was used to visualize HeLa cells expressing BACE-HA (*A*), BACE-HAKK (*B*), or empty vector (*C*). All cells were fixed and permeabilized prior to staining. *D–O* were observed with a confocal microscope. BACE-HA is shown in *green*, and organelle markers are shown in *red.* Merged images on the *far right* indicate the extent of colocalization (*yellow*). BACE colocalizes well with endosomes as demonstrated with either an anti-transferrin receptor antibody (*D–F*) or endocytosed biotinylated transferrin (*G–I*). Colocalization with the lysosomal markers LAMP1 (*J–L*) or LAMP2 (*M–O*) is much less apparent. *Scale bar,* 50 μm.

BACE-HA staining colocalized well with that of the transferrin receptor, a well known endosomal marker (Fig. 4, *D–F*). Colocalization with early (Fig. 4, *J–L*) and late (Fig. 4, *M–O*) lysosomal markers, however, was much less apparent.

BACE did not entirely costain with our endosomal marker, although the extent of signal overlap was quite notable. We therefore considered the possibility that our anti-transferrin receptor antibody only recognized a portion of the endosomal population. We addressed this by treating BACE-HA-transfected HeLa cells with biotinylated transferrin. After a 30-min incubation to allow for adequate uptake, the cells were fixed, permeabilized, and stained with mAb HA11 and streptavidin-tagged with a fluorescent label. This experiment revealed much more extensive BACE colocalization with endosomes (Fig. 4, *G–I*).

BACE Cycles between the Cell Surface and Endosomes—The cellular distribution of BACE by immunofluorescence raised the question of how the protein is targeted to endosomes from the secretory pathway. Examination of the cytoplasmic tail of BACE revealed the presence of a C-terminal DD*XX*LL motif. Dileucine-based sorting motifs have been associated with endosomal/lysosomal targeting from the cell surface as well as from the TGN (24), and upstream acidic residues like aspartic acid have been shown to be necessary for proper functioning of this signal sequence (25). Thus, we hypothesized that BACE is primarily trafficked to the endosomal system either by internalization from the plasma membrane via clathrin-coated pits or by direct transfer from the Golgi apparatus.

We began our investigations by first asking whether BACE is present on the cell surface. BACE-HA-transfected HeLa cells were fixed and either permeabilized with Triton X-100 or left untreated. Both groups of cells were then stained with either mAb HA11, which recognizes the cytoplasmic HA tag, or BN-1, which is directed against the extracellular domain of BACE. Although Triton X-100-pretreated cells were immunoreactive to both mAb HA11 and BN-1, unpermeabilized cells stained positively only with BN-1 (Fig. 5, *A–D*). Thus, in cells with an intact plasma membrane, BACE could be recognized only by an

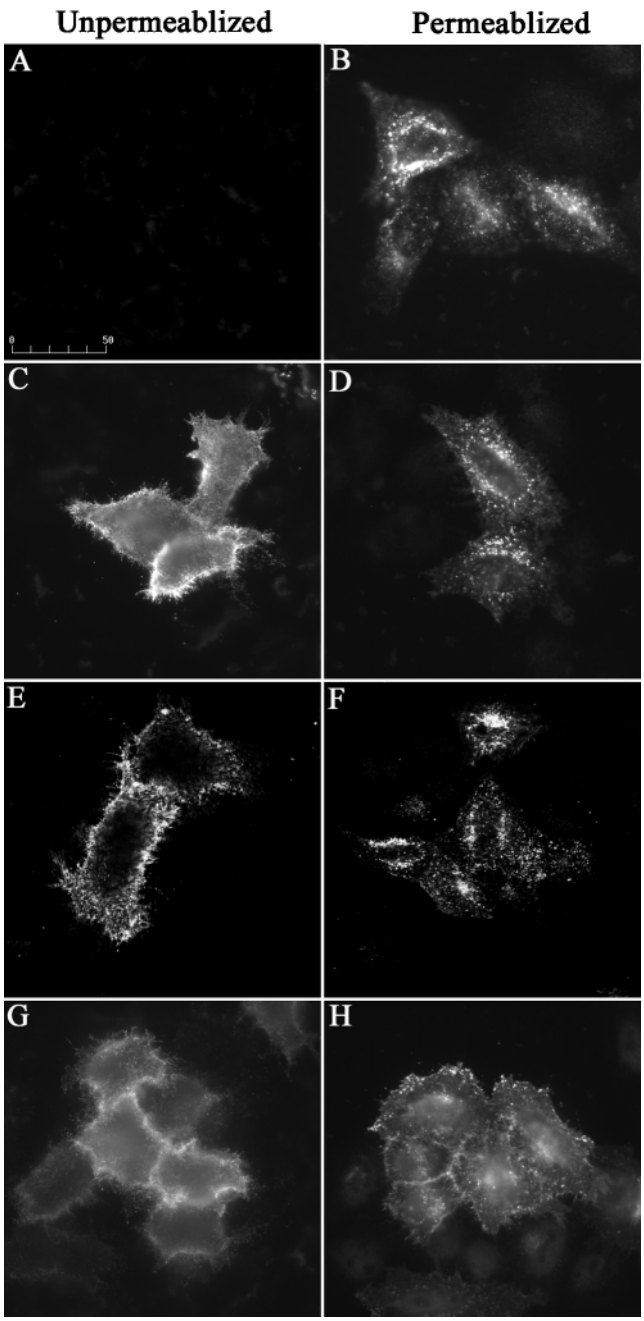

FIG. 5. **BACE is present on the cell surface.** HeLa cells expressing BACE-HA were fixed with formaldehyde after which they were either stained immediately (*1st column*) or first permeabilized with Triton X-100 (*2nd column*). The cells were stained with either mAb HA11 (*A* and *B*) or BN-1 (*C* and *D*). mAb HA11 recognizes the cytoplasmic tail of BACE and thus only stains permeabilized cells. By contrast BN-1 recognizes the lumenal/extracellular portion of BACE and gives a strong cell surface staining pattern in unpermeabilized cells along with the typical vesicular pattern in permeabilized cells. Confocal microscopy was also used to show similar results with BN-1 (*E* and *F*). Cells expressing BACE-HAΔLLK were also stained with BN-1 and show an enhanced cell surface pattern that is relatively maintained after cell permeabilization (*G* and *H*). *Scale bar,* 50 μm.

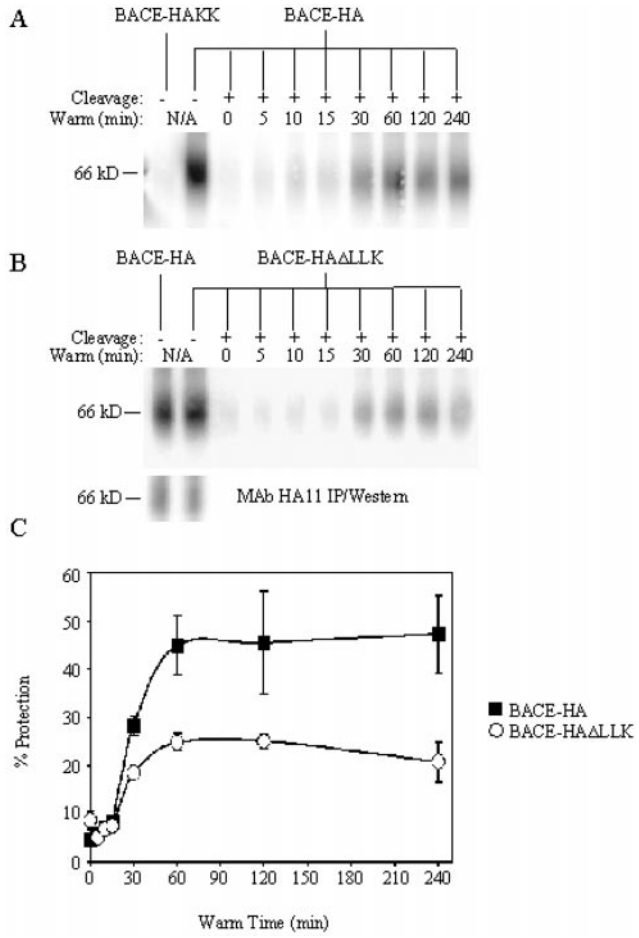

FIG. 6. **Endocytosis measurements on BACE.** 293 cells expressing the appropriate BACE construct were subjected to cell surface biotinylation, lysed, and immunoprecipitated with mAb HA11. For endocytosis measurements, cells were warmed to 37 °C after biotinylation and incubated for the indicated amount of time after which biotin still present on the cell surface was removed with glutathione (cleavage). The cells were then lysed and immunoprecipitated with mAb HA11. Immunoprecipitates were electrophoresed and blotted with ^{125}I-streptavidin. *A,* BACE is present in significant quantities on the cell surface and is internalized at a slow but measurable rate. ER-retained BACE-HAKK is included as a negative control (*1st lane*). *B,* BACE-HAΔLLK is internalized less effectively at steady state than BACE-HA and accumulates on the cell surface to a greater extent. BACE-HA is shown for comparison (*1st lane*). *C,* plot comparing the endocytosis rates of BACE-HA and BACE-HAΔLLK. Internalization is expressed as the percentage of initial biotinylated BACE-HA protected from glutathione.

antibody directed against its extracellular domain, whereas the lack of immunoreactivity to an antibody directed against its intracellular domain verified that the integrity of the plasma membrane itself had been maintained. Furthermore, the observed BN-1 staining in unpermeabilized cells highlighted cell processes and adhesion points, providing a characteristic cell surface pattern. Confocal micrographs of these same cells demonstrated an annular distribution of immunoreactivity only present on the cell membrane (Fig. 5, *E* and *F*). These results together provided strong evidence that BACE spends at least part of its lifetime on the cell surface.

The presence of BACE on the plasma membrane was verified by cell surface biotinylation experiments. 293 cells transfected with BACE-HA or BACE-HAKK were subjected to cell surface biotinylation at 4 °C, washed, and lysed. BACE protein was then immunoprecipitated with mAb HA11, resolved by SDS-PAGE, and blotted with ^{125}I-streptavidin. The mature ~66-kDa form of BACE-HA gave a robust signal by this method, indicating the presence of a significant population of the protein on the cell surface. Furthermore, the ER resident species BACE-HAKK was undetectable, controlling for the possibility that intracellular proteins were unintentionally biotinylated through the course of the experiment (Fig. 6*A, lanes 1* and *2*). A similar strategy was employed to examine the rate of BACE internalization from the plasma membrane. BACE-HA-transfected 293 cells were biotinylated and washed as described previously. However, they were then warmed to 37 °C in media

and incubated for times ranging from 0 to 240 min after which the remaining cell surface biotin was cleaved off with the non-cell-permeable reducing agent glutathione. Subsequent lysis, immunoprecipitation, and Western blot showed that, as incubation time between biotinylation and cleavage steps increased, a larger and larger fraction of biotinylated BACE-HA became protected from reducing agent (Fig. 6*A*). This finding suggested that BACE was indeed being internalized from the cell surface.

The kinetics of BACE endocytosis was determined by quantifying the band intensities from several biotinylation experiments (Fig. 6*C*). We found that during the 1st h of incubation at 37 °C, BACE-HA internalization proceeded at a rate of approximately 1% per min before reaching an equilibrium at only ~47% of initially biotinylated protein. This raised the possibility that BACE cycles between the cell surface and endosomes, a process by which internalized biotinylated BACE could be returned to the plasma membrane thereby becoming susceptible once again to glutathione-mediated cleavage. To test for the presence of recycling, 293 cells expressing BACE-HA were biotinylated, warmed for 1 h, and treated with glutathione as before. However, instead of lysis, the cells were then subjected to an additional 37 °C incubation lasting either 30 min or 1 h, after which they were again treated with glutathione. We reasoned that if biotinylated BACE returned to the plasma membrane during this second incubation, it would again become susceptible to glutathione-mediated cleavage leading to a diminished signal by Western blot. We found that the amount of biotinylated BACE protected from reducing agent did indeed decrease during the second incubation by as much as 34.6 ± 1.8% (Fig. 7*A, 7C*). Furthermore, cells subjected to an additional 1-h incubation without a second glutathione treatment exhibited a much smaller decline in the amount of biotinylated BACE, providing an effective control for variables such as protein degradation and cell loss (Fig. 7*A, lane 6*). These findings show that BACE cycles between the plasma membrane and the endosomal system.

Deletion of the Dileucine Motif on the Cytoplasmic Tail of BACE Affects the Endosomal Targeting of the Protein—To investigate the role of the cytoplasmic dileucine motif in BACE trafficking, we generated a construct in which the last three amino acid residues of the protein, including the crucial pair of leucines, were deleted and replaced with an HA tag (BACE-HAΔLLK) (Fig. 1*A*). The cellular distribution of this species was then studied by immunofluorescence and compared with data obtained for BACE-HA. In unpermeabilized cells, BACE-HAΔLLK exhibited a cell surface staining pattern similar to that of BACE-HA (Fig. 5, *C* and *G*). However, in permeabilized cells, where BACE-HA immunoreactivity became mostly vesicular, BACE-HAΔLLK continued to demonstrate predominantly cell surface staining (Fig. 5, *D* and *H*). This finding suggested that elimination of the cytoplasmic dileucine motif on BACE significantly hampers endosomal targeting resulting in a noticeable redistribution of the protein to the plasma membrane.

The altered trafficking of BACE-HAΔLLK was confirmed biochemically. In biotinylation experiments, the amount of BACE protein on the surface of BACE-HAΔLLK-expressing cells was found to exceed that on the surface of BACE-HA-expressing cells by 21.9 ± 5.1% after normalization of total biotinylated BACE to cellular BACE levels by Western blot (Fig. 6*B, lanes 1* and *2*). This discrepancy provided additional evidence that BACE-HAΔLLK targeting to endosomes is impaired leading to increased accumulation on the plasma membrane. In endocytosis experiments BACE-HAΔLLK displayed similar internalization kinetics to BACE-HA at early time points. However, the amount of BACE-HAΔLLK protected from reducing agent eventually plateaued at a level only half that reached by BACE-HA (Fig. 6, *B* and *C*). This finding suggested that, in the absence of its cytoplasmic dileucine motif, BACE is mistrafficked between the cell surface and the endosomal system such that the protein is either less readily internalized or more readily recycled. To address these possibilities, we performed recycling studies on BACE-HAΔLLK analogous to those conducted for BACE-HA. In these experiments, internalized BACE-HAΔLLK was found to return to the cell surface at twice the rate of BACE-HA (Fig. 7, *B* and *C*). Thus, eliminating the cytoplasmic dileucine motif of BACE appears to disrupt both the endosomal targeting and the recycling kinetics of the protein.

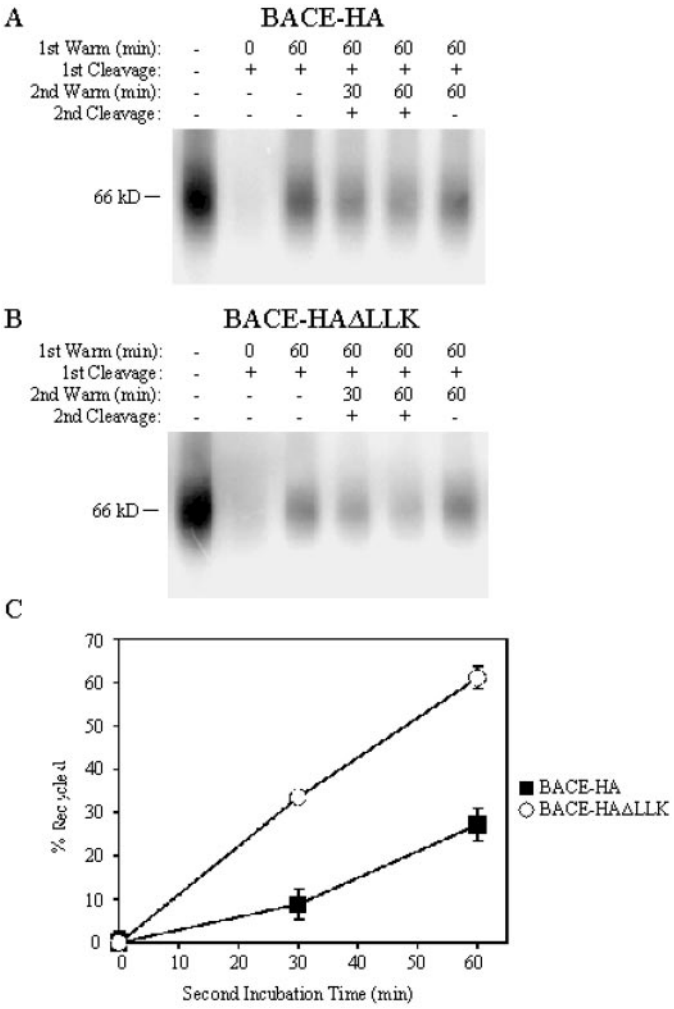

FIG. 7. **Recycling studies on BACE.** 293 cells expressing either BACE-HA (*A*) or BACE-HAΔLLK (*B*) were subjected to cell surface biotinylation, and the amount of biotinylated BACE protected from glutathione after a 1-h, 37 °C incubation was determined as before. Some cells were then subjected to an additional incubation at 37 °C for the time indicated followed by a second glutathione treatment (*2nd cleavage*). In both cases the amount of protected biotinylated BACE protein decreased in these samples suggesting significant recycling to the cell surface. In each experiment, the second glutathione treatment was omitted for one sample (*last lane*) to control for protein degradation and cell loss. *C,* recycling data plotted demonstrating that BACE-HAΔLLK is recycled twice as fast as BACE-HA.

DISCUSSION

The membrane-bound aspartyl protease BACE has been identified as the β-secretase responsible for cleavage of APP (9–13). In this study, we sought to characterize better the normal cell biology of BACE through a series of biochemical and morphological investigations. Our findings suggest that

BACE is a stable protein that rapidly and efficiently transits through the secretory pathway before being targeted largely to the endosomal system. Through confocal analysis, we confirmed earlier immunofluorescence studies that had localized BACE to endosomes and not lysosomes (9). The fact that BACE does not appear to accumulate in lysosomes is consistent with the long half-life of the protein and the lack of effect chloroquine had on its turnover.

While in the ER, BACE acquired intramolecular disulfide bonds and was core-glycosylated at three out of four potential sites. The efficiency with which an *N*-linked site is utilized has been shown to depend in part on the identity of the amino acid directly following the N*X*(S/T) sequon, in the so-called Y position (26). This is especially true if the *N*-linked site in question bears the N*X*S as opposed to the N*X*T sequon as is the case for three of four *N*-linked sites on BACE. Examination of the BACE sequence reveals a glutamic acid residue in the Y position of the third *N*-linked site and a phenylalanine residue in the Y position of the fourth. *N*-Linked sites bearing these sequences have been shown to be glycosylated with efficiencies of only 30 and 40%, respectively. Thus, it is likely that one of these two sites is not core-glycosylated. All oligosaccharide side chains were processed in the Golgi as judged by their complete resistance to Endo H digestion. Retention of BACE in the ER by the introduction of a cytoplasmic dilysine motif not only prevented carbohydrate modification as expected but also made it possible to determine that ER-restricted BACE migrated slightly more slowly than the wild type molecule even after removal of all *N*-linked carbohydrates. Since both species were recognized by a C-terminal HA epitope tag, this suggests that BACE undergoes an N-terminal propeptide cleavage as previously suggested (13) but only after transport from the ER.

We investigated the possibility that BACE assembles into stable oligomeric structures through the course of its life span. The formation of such complexes has been observed for a variety of proteins and, in some cases, is essential for normal function (23). For instance, oligomerization of the T-cell receptor after interaction with antigen bound to major histocompatability complex is thought to be a necessary event for downstream signaling and immunosurveillance (27, 28). Within the field of AD-related research, the N- and C-terminal fragments of presenilin 1 have been shown to be components of a high molecular weight complex containing β-catenin that has been implicated in apoptosis (29–32). In our studies, BACE did not appear to oligomerize to an appreciable degree in the presence of either 1% Triton X-100 or 50 mM CHAPS. However, we cannot exclude the possibility that BACE does form complexes that are simply not detergent-stable.

The mechanism by which BACE accumulates in the endosomal system was analyzed by immunofluorescence as well as cell surface biotinylation. These studies revealed that a significant population of BACE cycles between the plasma membrane and endocytic compartments. Given the long half-life of BACE and its recycling rate, a typical BACE molecule must cycle between the cell surface and the endosomal system many times through the course of its life span. A variety of cell surface proteins are actively targeted for internalization and recycling by motifs in their cytoplasmic domains. The transferrin receptor for instance, whose endocytosis is mediated by a well characterized C-terminal Y*XX*Z motif, is internalized at a rate of 11–13%/min (33, 34). More recently, the dileucine motif-dependent endocytosis of cluster of differentiation antigen 4 has been found to exhibit an internalization rate between 5 and 19%/min depending on the phosphorylation state of a cytoplasmic serine residue (35). By contrast, the measured internalization rate of BACE-HA (~1%/min) does not exceed most estimates for bulk flow of proteins from the plasma membrane to underlying vesicular compartments (36, 37). Whether the rate at which BACE is internalized is cell type-dependent is not known nor are the cellular factors that might regulate its endocytosis.

Passive internalization from the cell surface cannot, by itself, account for the marked endosomal accumulation of BACE seen by immunofluorescence. For this to occur, BACE must remain internalized for a prolonged period prior to returning to the cell surface. Alternatively, initial targeting of BACE to endocytic compartments could occur by direct transfer from the TGN, a process that would bypass the plasma membrane entirely. This type of trafficking has been observed for several proteins involved in lysosomal sorting and degradation (38). For example, the Man-6-PR is targeted directly from the TGN to late endosomes after which it cycles to and from both the cell surface and the Golgi apparatus. The endosomal targeting of the Man-6-PR, but not its internalization from the plasma membrane, has been found to require a cytoplasmic dileucine motif similar to that of BACE (39). The glucose transporter GLUT4 is also targeted by a cytoplasmic dileucine motif to endosomes directly from the TGN after which it cycles to and from the cell surface in response to exogenous insulin levels (40–42).

The importance of the dileucine motif on the C terminus of BACE was analyzed by mutagenesis. Upon deletion of the motif, the normal endosomal distribution of BACE was modified such that a greater proportion of the protein was resident on the cell surface, with less being internalized at steady state. Eliminating the cytoplasmic dileucine motif of BACE probably has little effect on the actual efficiency of endocytosis of the protein, given our measures of endocytosis kinetics and the fact that the internalization rate of BACE-HA resembles what would be predicted from simple bulk flow of plasma membrane. Instead, the altered distribution of BACE lacking the dileucine motif is more likely due to the protein being less effectively sequestered in endosomes after internalization. We observed that, in the absence of its C-terminal dileucine motif, BACE is recycled from the endosomal system to the cell surface at twice its normal rate. This change in BACE trafficking results in more of the protein on the cell surface and less in endosomes at steady state. In addition and perhaps more importantly, elimination of the cytoplasmic dileucine motif most likely impairs the initial targeting of BACE from the TGN to endosomes allowing the protein to pass directly to the plasma membrane where it accumulates. A similar change in cellular distribution was observed for the Man-6-PR after mutagenesis of its C-terminal dileucine motif (39). Thus, the cytoplasmic dileucine motif of BACE appears to play an important role in the trafficking of the protein to endocytic compartments. That BACE should be targeted to and retained in endosomes is consistent with its classification as an aspartyl protease. A protein whose activity is strongest at mildly acidic pH would be expected to function most effectively in the endosomal system.

Another group has recently completed an investigation of BACE maturation and trafficking (43). They report similar findings to ours regarding the glycosylation and propeptide cleavage of BACE in the secretory pathway. In addition, they demonstrate by pulse-chase analysis that the cytoplasmic tail of BACE is important for normal trafficking. We show in this paper that the accumulation of BACE in the endosomal system appears to be governed largely by a cytoplasmic dileucine motif. In this way, we extend the conclusions of the previous study by determining explicitly the mechanisms by which BACE is targeted to endocytic vesicles.

Identifying the cellular compartments where BACE is normally active and where it exerts its pathogenic effects on APP

is of great importance for the development of therapeutic strategies to combat AD. As stated previously, Aβ production has been found to occur in the endosomal/lysosomal system, the ER, and the TGN (15–22). Whether BACE actually cleaves APP in all of these compartments, however, is not certain. We and others (9) have demonstrated that BACE accumulates primarily in endosomes. However, BACE is also present, at least in an itinerant fashion, all along the secretory pathway providing several alternative sites for interaction with APP. As an aspartyl protease, BACE is most active at mildly acidic pH (9, 13) making endosomes perhaps the most likely location for the amyloidogenic activity of the protein. Nevertheless, reports have also demonstrated β-secretase activity in both the ER- and Golgi-derived vesicles (16, 19). Studying the effects that targeting mutants like BACE-KK and BACE-ΔLLK have on APP processing should provide valuable insights into these issues as well as the normal physiological role of this protein.

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