

## Phosphorylation Regulates Intracellular Trafficking of $\beta$ -Secretase\*

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**$\beta$ -Secretase (BACE) is a transmembrane aspartyl protease, which generates the N terminus of Alzheimer's disease amyloid  $\beta$ -peptide. Here, we report that BACE can be phosphorylated within its cytoplasmic domain at serine residue 498 by casein kinase 1. Phosphorylation exclusively occurs after full maturation of BACE by propeptide cleavage and complex *N*-glycosylation. Phosphorylation/dephosphorylation affects the subcellular localization of BACE. BACE wild type and an S498D mutant that mimics phosphorylated BACE are predominantly located within juxtannuclear Golgi compartments and endosomes, whereas nonphosphorylatable BACE S498A accumulates in peripheral EEA1-positive endosomes. Antibody uptake assays revealed that reinternalization of BACE from the cell surface is independent of its phosphorylation state. After reinternalization, BACE wild type as well as BACE S498D are efficiently retrieved from early endosomal compartments and further targeted to later endosomal compartments and/or the trans-Golgi network. In contrast, nonphosphorylatable BACE S498A is retained within early endosomes. Our results therefore demonstrate regulated trafficking of BACE within the secretory and endocytic pathway.**

Alzheimer's disease is the most common form of dementia and is pathologically characterized by the invariant accumulation of senile plaques and neurofibrillary tangles in certain areas of the brains of Alzheimer's disease patients (1). The major constituent of senile plaques is the amyloid  $\beta$ -peptide ( $A\beta$ ),<sup>1</sup> which derives from the  $\beta$ -amyloid precursor protein ( $\beta$ APP) by endoproteolytic processing (2).  $\beta$ -Secretase cleaves  $\beta$ APP at the N terminus of the  $A\beta$ -domain, resulting in the generation of soluble APP<sub>s</sub>- $\beta$  and a membrane-associated C-terminal fragment bearing the complete  $A\beta$ -domain. Subse-

quent cleavage of this fragment by  $\gamma$ -secretase, which appears to be identical with the presenilins, results in the release and secretion of  $A\beta$  (3).

Recently, an aspartyl protease with  $\beta$ -secretase activity was identified in human embryonic kidney (HEK) 293 cells and was initially called BACE ( $\beta$ -site APP-cleaving enzyme, Asp2, or memapsin 2) (4–8) (Fig. 1A). A close homologue was also identified and termed as BACE-2, Asp1, DRAP, or memapsin 1 (4, 5, 9).<sup>2</sup> Both enzymes are type I membrane proteins sharing significant homology with other members of the aspartyl protease family (5–8). While BACE-2 is predominantly expressed in peripheral tissues, BACE is highly expressed in neurons, the major site of  $A\beta$  generation. However, it appears that  $\beta$ APP is not the only substrate for BACE. In fact, mouse  $\beta$ APP is a very poor substrate for  $\beta$ -secretase activity (10). "Humanizing" the  $A\beta$  domain of mouse  $\beta$ APP by three amino acid substitutions resulted in efficient cleavage by  $\beta$ -secretase (10), suggesting that  $\beta$ APP may not be the exclusive substrate for BACE. However, other physiological substrates of BACE remain to be identified.

BACE is cotranslationally modified by *N*-glycosylation and further matures by complex glycosylation as well as proteolytic removal of its prodomain by a furin-like protease (11–14). The majority of BACE molecules are localized within Golgi and endosomal compartments, where they colocalize with  $\beta$ APP (4, 5, 7, 12, 14). The acidic pH optimum of BACE (5–8) indicates that it is predominantly active within late Golgi compartments and/or endosomes/lysosomes. This is consistent with previous findings demonstrating that  $\beta$ -secretase cleavage of  $\beta$ APP can occur in all of these acidic compartments (15–18).

It has recently been reported that BACE is reinternalized from the cell surface to early endosomes and can recycle back to the cell surface, a process that depends on a dileucine motif in the cytoplasmic tail of BACE (14). This signal is located close to a negatively charged domain, which contains a potential phosphorylation site. We found that BACE is indeed phosphorylated within its C-terminal domain and that the biological function of BACE phosphorylation resides in the regulation of the retrieval of reinternalized BACE from endosomes.

### MATERIALS AND METHODS

**Cell Culture and Transfection**—Human embryonic kidney (HEK) 293 and green monkey COS-7 cells were cultured in Dulbecco's modified Eagle's medium with Glutamax (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies). The cell lines stably overexpressing  $\beta$ APP<sub>695</sub> (19) and BACE carrying Myc epitopes at the C terminus (12) have been described previously. Transfection of cells with BACE cDNAs was carried out using Fugene reagent (Roche Molecular Biochemicals) according to the supplier's instructions. Single

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<sup>1</sup> The abbreviations used are:  $A\beta$ , amyloid  $\beta$ -peptide; BFA, brefeldin A;  $\beta$ APP,  $\beta$ -amyloid precursor protein; CK, casein kinase; CT, cytoplasmic tail; EEA1, early endosome antigen 1; GST, glutathione *S*-transferase; HD, hyphenaldisine; HEK, human embryonic kidney; PVDF, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis; BACE,  $\beta$ -secretase; endo H, endoglycosidase H; PBS, phosphate-buffered saline; ER, endoplasmic reticulum; TGN, trans-Golgi network; WT, wild type; PNGase F, peptide:*N*-glycanase F.

<sup>2</sup> For reasons of simplicity, we use the terms BACE and BACE-2 in this study.

cell clones were generated by selection in 200  $\mu\text{g/ml}$  zeocine (Invitrogen).

**cDNAs and Fusion Proteins**—The phosphorylation site mutants of BACE were generated by polymerase chain reaction techniques using the appropriate oligonucleotides. The resulting polymerase chain reaction fragments were subcloned into the *EcoRI/XhoI* restriction sites of pcDNA3.1 containing a zeocine resistance gene (Invitrogen). To generate fusion proteins of glutathione *S*-transferase and the cytoplasmic domain of BACE, the sequence of BACE encoding amino acids 476–501 was amplified by polymerase chain reaction using appropriate primers. The resulting fragments were subcloned into *EcoRI/XhoI* restriction sites of pGEX-5X-1 (Amersham Pharmacia Biotech), and fusion proteins were expressed in *Escherichia coli* DH5 $\alpha$  and purified on GSH-Sepharose according to the supplier's instructions.

**Antibodies, Metabolic Labeling, Immunoprecipitation, and Immunoblotting**—The polyclonal antibodies 7523, 7520, and GM190 recognizing the N terminus of BACE (amino acids 46–60), the C terminus (amino acids 482–501), and the propeptide (amino acids 22–45), respectively, have been described previously (12). Monoclonal  $\alpha$ -giantin antibodies and monoclonal  $\alpha$ - $\beta$ APP antibodies were generously provided by Drs. H.-P. Hauri and E. H. Koo, respectively. Monoclonal  $\alpha$ -EEA1 antibody was from Transduction Laboratories, and monoclonal antibody 9E10 developed by J. M. Bishop was obtained from the Developmental Studies Hybridoma Bank (University of Iowa).

To radiolabel cellular proteins, cells were incubated at 37 °C in methionine-free serum-free medium for 45 min. Cells were then incubated with the same medium supplemented with [<sup>35</sup>S]methionine/[<sup>35</sup>S]cysteine (Promix; Amersham Pharmacia Biotech) and kept at 37 °C for the times indicated in the respective experiments. For immunoprecipitations cells were lysed in buffer containing 1% Nonidet P-40 on ice for 10 min. Lysates were clarified by centrifugation for 10 min at 14,000  $\times g$  and immunoprecipitated for 3 h at 4 °C. After separation by SDS-PAGE, proteins were transferred to PVDF membrane (Imobilon; Millipore) and analyzed by autoradiography or phosphorimaging. Alternatively, BACE was detected by immunoblotting using the enhanced chemiluminescence technique (Amersham Pharmacia Biotech).

**In Vivo Phosphorylation**—HEK 293 cells were incubated 45 min in phosphate-free media (Sigma). Media were aspirated, and the respective fresh media were added containing 18 MBq/ml [<sup>32</sup>P]orthophosphate (Amersham Pharmacia Biotech). After 1 h at 37 °C, cells were incubated for an additional 1 h in the presence or absence of protein kinase activators/inhibitors or the phosphatase inhibitor okadaic acid at the concentrations indicated in the respective experiments. To inhibit *N*-glycosylation, cells were treated with 10  $\mu\text{g/ml}$  tunicamycin for 2 h prior to the addition of [<sup>32</sup>P]orthophosphate and during the labeling period. After labeling, media were aspirated, and the cells were washed twice with ice-cold PBS and immediately lysed on ice with lysis buffer containing 1% Nonidet P-40 for 10 min. Cell lysates were centrifuged for 10 min at 14,000  $\times g$ , and supernatants were immunoprecipitated with specific antibodies as indicated.

**In Vitro Phosphorylation Assays**—*In vitro* phosphorylation assays were carried out as described previously (20). Recombinant rat casein kinase (CK)-1  $\delta$  (New England Biolabs), recombinant  $\alpha$ -subunit of human CK-2 (New England Biolabs), and the catalytic subunit of protein kinase A purified from bovine heart (gift from Dr. V. Kinzel) were used for *in vitro* phosphorylation assays in a buffer containing 20 mM Tris, pH 7.5, 5 mM magnesium acetate, 5 mM dithiothreitol. Protein kinase C purified from rat brain (Biomol) was assayed in a similar buffer supplemented with 1  $\mu\text{M}$  phorbol 12,13-dibutyrate, 0.5 mM calcium chloride, and 100  $\mu\text{g/ml}$  phosphatidylserine under mixed micellar conditions. As substrates, fusion proteins of GST and the C terminus of BACE (see above) were used. Phosphorylation reactions were started by the addition of 10  $\mu\text{M}$  [ $\gamma$ -<sup>32</sup>P]ATP and allowed to proceed for 10 min at 32 °C. To control the kinase activities, parallel phosphorylation reactions were carried out using phosvitin (1 mg/ml; Sigma) or histone (0.5 mg/ml; Sigma) as protein substrates. Reactions were stopped by the addition of SDS sample buffer. Alternatively, cell extracts were used to phosphorylate fusion proteins of GST and BACE. HEK 293 cells were lysed in a buffer containing 20 mM Tris, pH 7.5, 5 mM magnesium acetate, 5 mM dithiothreitol, and 0.5% Triton X-100. Lysates were centrifuged for 10 min at 14,000  $\times g$ , and fusion proteins of GST and BACE-CT were added to the supernatant. Phosphorylation reactions were started by the addition of [ $\gamma$ -<sup>32</sup>P]ATP and allowed to proceed for 15 min in the presence or absence of 10  $\mu\text{M}$  hymenialdisine (HD) and 1  $\mu\text{M}$  okadaic acid. GST fusion proteins were isolated by precipitation with GSH-Sepharose (Amersham Pharmacia Biotech) for 2 h at 4 °C. Precipitates were washed five times with PBS and eluted by the addition of SDS sample buffer and separated by SDS-gel electrophoresis.

**Phosphoamino Acid Analysis**—Phosphoamino acid analysis was carried out by one-dimensional high voltage electrophoresis according to Jelinek and Weber (21). Radiolabeled proteins, electrotransferred onto PVDF membrane were hydrolyzed in 6 M HCl for 90 min at 110 °C. Subsequently, supernatants were dried in a SpeedVac concentrator, and pellets were dissolved in 8  $\mu\text{l}$  of pH 2.5 buffer (5.9% glacial acetic acid, 0.8% formic acid, 0.3% pyridine, 0.3 mM EDTA) and spotted onto 20  $\times$  20-cm cellulose thin layer chromatography plates (Merck) together with unlabeled phosphoamino acids (Ser(P), Thr(P), Tyr(P); 1  $\mu\text{g}$  each; Sigma). High voltage electrophoresis was carried out for 45 min at 20 mA. Radiolabeled phosphoamino acids were localized by autoradiography and identified by comparison with comigrating phosphoamino acids after ninhydrine staining.

**Deglycosylation Experiments**—BACE was immunoprecipitated from cell lysates as described above, and precipitates were incubated in the presence of endoglycosidase H (endo H; Roche Molecular Biochemicals) or PNGase F (Roche Molecular Biochemicals) for 14 h at 37 °C in the appropriate buffer. Reactions were stopped by the addition of SDS sample buffer, and reaction mixtures were separated by SDS gel electrophoresis.

**Immunocytochemistry**—Cells stably expressing BACE cDNAs were grown on polylysine-coated glass coverslips to 50–80% confluence. Cells were fixed in 4% paraformaldehyde/PBS at room temperature and processed for immunofluorescence as described previously (12). Bound primary antibodies were detected by Alexa 488- or Alexa 594-conjugated secondary antibodies (Molecular Probes, Inc., Eugene, OR). In some experiments, cells were incubated in the presence of 10  $\mu\text{g/ml}$  brefeldin A (BFA) for 30 min at 37 °C before fixation.

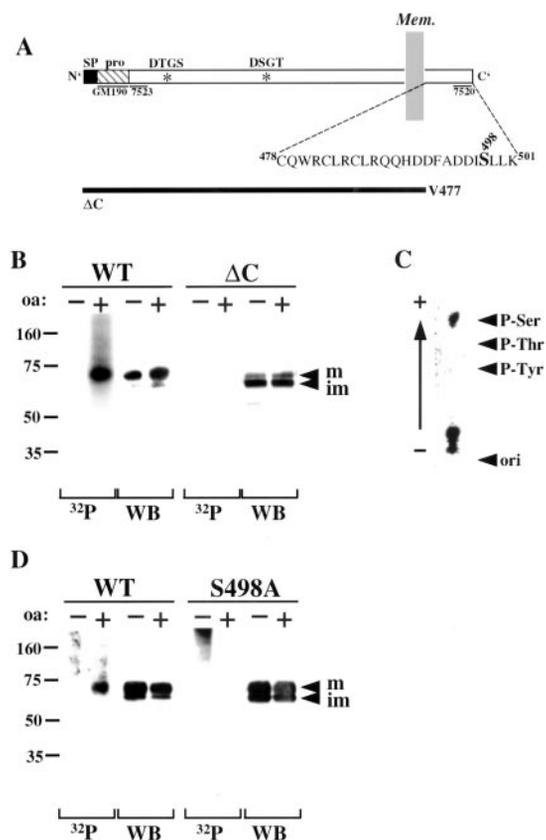
**Antibody Uptake Assays**—Cells grown on polylysine-coated glass coverslips were washed twice with ice-cold PBS and incubated for 20 min on ice in serum-free medium (Opti-MEM; Life Technologies, Inc.) containing the indicated antibodies. Cells were then washed three times with ice-cold PBS and subsequently incubated at 37 °C or 18 °C in Dulbecco's modified Eagle's medium with Glutamax supplemented with 10% fetal calf serum for various time periods. After two washes with PBS, cells were fixed in 4% paraformaldehyde/PBS and processed for immunofluorescence.

Cells were analyzed using a Leica DMRB fluorescence microscope, and photographs were taken with an RT monochrome spot camera (Diagnostic Instruments) and processed with Metaview program (Visitron Systems).

## RESULTS

**BACE Is Phosphorylated at Serine 498**—To investigate whether BACE is posttranslationally modified by phosphorylation, we used HEK 293 cells stably overexpressing human BACE. HEK 293 cells were used previously to identify BACE in a functional screening assay and to analyze its proteolytic function (7). The same cell line was also used to successfully investigate the effects of the Swedish  $\beta$ APP mutation on  $\beta$ -secretase cleavage (16), thus demonstrating that HEK 293 cells represent a valid model system to study the cell biology of BACE.

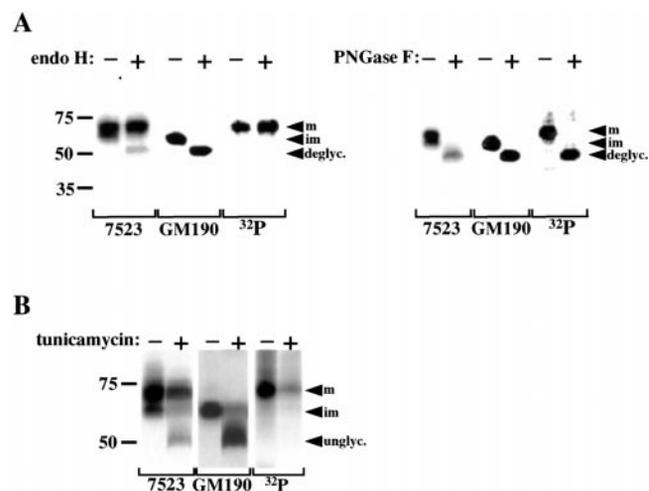
After *in vivo* labeling with [<sup>32</sup>P]orthophosphate, cell lysates were immunoprecipitated with antibody 7523 directed to an N-terminal sequence after the prodomain (Fig. 1A). Analysis of immunoprecipitated BACE by autoradiography revealed that BACE undergoes phosphorylation (Fig. 1B). Incorporation of [<sup>32</sup>P]orthophosphate is dependent on the presence of the phosphatase inhibitor okadaic acid (Fig. 1B). However, longer exposure of the autoradiograms revealed weakly radiolabeled BACE also in the absence of okadaic acid (data not shown). To prove if BACE is phosphorylated within its cytoplasmic tail or within its ectodomain as previously demonstrated for its substrate  $\beta$ APP (22–24), we generated a truncated derivative lacking the cytoplasmic domain (BACE $\Delta$ C; Fig. 1A (12)). Although high levels of BACE $\Delta$ C were expressed, the deletion of the cytoplasmic tail of BACE completely abolished phosphorylation (Fig. 1B). A soluble derivative of BACE lacking the transmembrane domain (12) is also not phosphorylated by cultured cells (data not shown), further supporting the finding that the ectodomain of BACE is not phosphorylated. These results therefore demonstrate exclusive phosphorylation of BACE within its cytoplasmic tail.



**FIG. 1. Phosphorylation of BACE at serine residue 498 within the cytoplasmic domain.** *A*, schematic of BACE. The aspartyl protease active site motifs (DTGS and DSGT) are indicated by asterisks. The signal peptide (SP) is indicated by a black box, and the propeptide (pro) is shown by a hatched box. The amino acid sequence of the cytoplasmic domain is given in single letter code, and the phosphorylation site (Ser<sup>498</sup>) is shown in boldface type. The epitopes of antibodies 7520, 7523, and GM190 are indicated. *B*, HEK cells stably expressing wild-type human BACE (WT) or a truncated derivative lacking the cytoplasmic domain ( $\Delta C$ ) were labeled with [<sup>32</sup>P]orthophosphate in the presence or absence of 0.2  $\mu M$  okadaic acid (oa). BACE was isolated by immunoprecipitation, separated by SDS-PAGE, and transferred to a PVDF membrane. Radiolabeled proteins were visualized by phosphorimaging (<sup>32</sup>P), and BACE was detected by Western immunoblot with antibody 7523 (WB). Mature (m) and immature (im) forms of BACE are indicated by arrowheads. *C*, phosphoamino acid analysis of radiolabeled BACE. The arrowheads indicate migrations of standard phosphoamino acids (P-Ser, P-Thr, P-Tyr) and origin of sample application (ori). *D*, phosphorylation of BACE WT and BACE S498A was analyzed as described for *B*. Substitution of Ser<sup>498</sup> by Ala completely abolished phosphate incorporation.

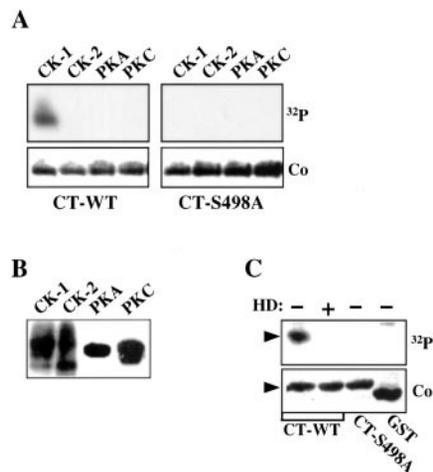
Phosphoamino acid analysis of <sup>32</sup>P-labeled BACE revealed that it is phosphorylated on serine residues (Fig. 1C). The cytoplasmic domain of BACE contains a single serine residue at position 498 close to the C terminus (Fig. 1A). To confirm that serine 498 is indeed phosphorylated *in vivo*, we generated a cell line stably expressing BACE containing a serine to alanine substitution (BACE S498A). This mutation completely blocks phosphorylation of BACE, thus demonstrating that serine 498 is the sole *in vivo* phosphorylation site (Fig. 1D).

**Phosphorylation Occurs Selectively after Full Maturation of BACE**—The results shown in Fig. 1 indicate that selectively mature BACE (70 kDa) is phosphorylated but not the immature 66-kDa form of BACE. If that is the case, phosphorylated BACE should be resistant to endo H treatment (12, 14). To prove this, cells expressing BACE carrying a Myc tag at the C terminus (12) were labeled with [<sup>32</sup>P]orthophosphate in the presence of okadaic acid, and BACE was isolated from cell lysates by immunoprecipitation with the  $\alpha$ -Myc antibody 9E10. Immunoprecipitates were incubated *in vitro* in the presence or



**FIG. 2. Selective phosphorylation of mature BACE.** *A*, cells stably expressing BACE WT were labeled with [<sup>32</sup>P]orthophosphate in the presence of 0.2  $\mu M$  okadaic acid. BACE was isolated by immunoprecipitation and incubated in the presence (+) or absence (-) of endo H or PNGase F. Proteins were then separated by SDS-PAGE and transferred to a PVDF membrane. After detection of <sup>32</sup>P-phosphorylated BACE by phosphorimaging (<sup>32</sup>P), membranes were subjected to immunoblotting with antibody 7523. To specifically detect immature forms of BACE, the same membrane was reprobed with antibody GM190 directed against the prodomain. The phosphorylated form of BACE is resistant to treatment with endo H, while it is sensitive to PNGase F. *B*, HEK cells were incubated with [<sup>32</sup>P]orthophosphate in the presence of 0.2  $\mu M$  okadaic acid and in the presence or absence of tunicamycin. BACE was isolated by immunoprecipitation, separated by SDS-PAGE, and transferred to a PVDF membrane. The membrane was first subjected to autoradiography to visualize <sup>32</sup>P-labeled proteins (<sup>32</sup>P) and then probed with antibody 7523 and with antibody GM190. Note that BACE is exclusively phosphorylated after full maturation by complex N-glycosylation and proteolytic removal of its prodomain.

absence of endo H or PNGase F and separated by SDS-PAGE. Immunoblot analysis with antibody 7523 revealed that endo H deglycosylated exclusively the immature 66-kDa form of BACE (Fig. 2A, left panel), consistent with the specificity of endo H for immature, biantennary or high mannose, N-linked glycans. We also identified the immature forms of BACE using antibody GM190 directed against the prodomain (12) and found that endo H selectively deglycosylates prodomain-containing immature forms of BACE (Fig. 2A, left panel). Autoradiography of the same membrane revealed that phosphorylated BACE is completely resistant to endo H treatment (Fig. 2A, left panel). In contrast to endo H, treatment with PNGase F, which removes all types of N-linked glycans, resulted in complete deglycosylation of both phosphorylated mature and unphosphorylated immature BACE as demonstrated by a significant molecular mass shift of <sup>32</sup>P-labeled BACE from 70 to 50 kDa (Fig. 2A, right panel). These results indicate that selectively fully mature BACE is phosphorylated. To confirm this in living cells, HEK 293 cells were labeled with [<sup>32</sup>P]orthophosphate in the presence or absence of tunicamycin to inhibit cotranslational N-glycosylation. BACE was immunoprecipitated with the  $\alpha$ -Myc antibody 9E10 and separated by SDS-PAGE. Consistent with previous results (12), tunicamycin treatment resulted in the accumulation of unglycosylated 50-kDa forms of BACE (Fig. 2B). Detection with antibody GM190 demonstrates accumulation of substantial levels of the unglycosylated prodomain-containing 50-kDa form of BACE after tunicamycin treatment (Fig. 2B). Autoradiography of the same membrane revealed that the unglycosylated form is not phosphorylated, in contrast to the fully mature BACE at 70 kDa (Fig. 2B). Taken together, these experiments demonstrate that phosphorylation of BACE occurs selectively after full maturation by removal of the prodomain and complex N-glycosylation.

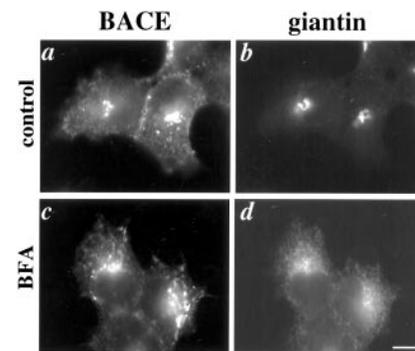


**FIG. 3. Casein kinase-1 phosphorylates BACE.** *A*, fusion proteins of GST carrying the WT (CT-WT; *left panels*) or the S498A mutant (CT-S498A; *right panels*) cytoplasmic domain of BACE were incubated with protein kinases CK-1, CK-2, A (PKA), and C (PKC) in the presence of [ $\gamma$ -<sup>32</sup>P]ATP. Reaction mixtures were separated by SDS-PAGE and transferred to PVDF membrane. Phosphorylated fusion proteins were detected by autoradiography (<sup>32</sup>P). The same membrane was stained with Coomassie (Co) to control protein loading. *B*, the catalytic activities of CK-1 and CK-2 were assayed using 1 mg/ml phosvitin, and that of protein kinase A was assayed with 0.5 mg/ml histone. The activity of rat brain protein kinase C was controlled by detection of autophosphorylation. *C*, lysates of HEK 293 cells were incubated with fusion protein CT-WT and [ $\gamma$ -<sup>32</sup>P]ATP in the presence or absence of the CK-1-selective inhibitor hymenialdisine (HD). As controls, the fusion protein CT-S498A or GST alone were used as substrates. After the phosphorylation reaction, fusion proteins were precipitated with GSH-Sepharose and separated by SDS-PAGE. Phosphorylated fusion protein was detected by autoradiography (<sup>32</sup>P). To prove equal loading, fusion proteins were stained with Coomassie (Co). The arrowheads indicate migration of GST fusion proteins. Hymenialdisine efficiently inhibits phosphorylation of CT-WT.

**Casein Kinase 1 Phosphorylates BACE**—To identify a protein kinase involved in phosphorylation of BACE, we first carried out *in vitro* phosphorylation assays using fusion proteins of GST with the cytoplasmic domain of BACE WT (CT-WT) or the S498A mutation (CT-S498A). The fusion proteins were incubated with four selected purified protein kinases in the presence of [ $\gamma$ -<sup>32</sup>P] ATP. Since the phosphorylation site of BACE is located within an acidic motif typical for substrate recognition by CKs (25) (see Fig. 1A), we used CK-1 and CK-2 in initial experiments. We also analyzed protein kinases A and C, since these enzymes have been shown to affect endoproteolytic processing of  $\beta$ APP (26–28). The fusion protein CT-WT was readily phosphorylated by CK-1, while CK-2, protein kinase A, and protein kinase C were not efficient (Fig. 3A, *left panel*), although all kinases were catalytically active under the assay conditions (Fig. 3B). CK-1-mediated phosphorylation of the cytoplasmic domain of BACE was completely abolished when CT-S498A was used as a substrate (Fig. 3A, *right panels*).

We next analyzed the involvement of CK-1 in the phosphorylation of BACE by cellular extracts. Lysates of HEK 293 cells were incubated with [ $\gamma$ -<sup>32</sup>P]ATP in the presence or absence of HD, which preferentially inhibits CK-1, glycogen synthase kinase 3 $\beta$ , and cyclin-dependent kinases (29), using the fusion protein CT-WT as substrate. CT-WT was readily phosphorylated by the cellular extracts in the absence of HD (Fig. 3C). In contrast, phosphorylation was completely blocked by HD. As expected, the fusion protein CT-S498A and GST alone were not phosphorylated by cellular extracts (Fig. 3C).

**Phosphorylation of the Cytoplasmic Domain Affects the Subcellular Localization of BACE**—We next examined the subcellular localization of BACE in stably transfected HEK 293 cells. Cells grown on glass coverslips were fixed and costained with

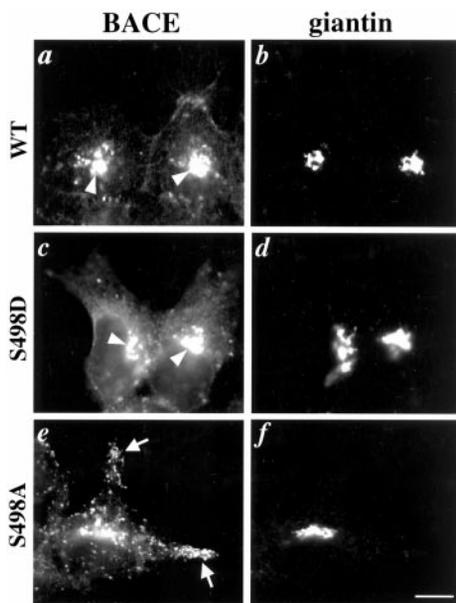


**FIG. 4. Subcellular localization of BACE.** HEK 293 cells stably transfected with cDNAs encoding BACE WT were grown on glass coverslips and incubated for 30 min at 37 °C in the presence (*c* and *d*) or absence (*a* and *b*) of brefeldin A (10  $\mu$ g/ml). Cells were fixed and costained with the polyclonal antibody 7523 (*a* and *c*) and the monoclonal anti-giantin antibody (*b* and *d*). Note the difference in the redistribution of BACE and giantin after BFA treatment. Bar, 10  $\mu$ m.

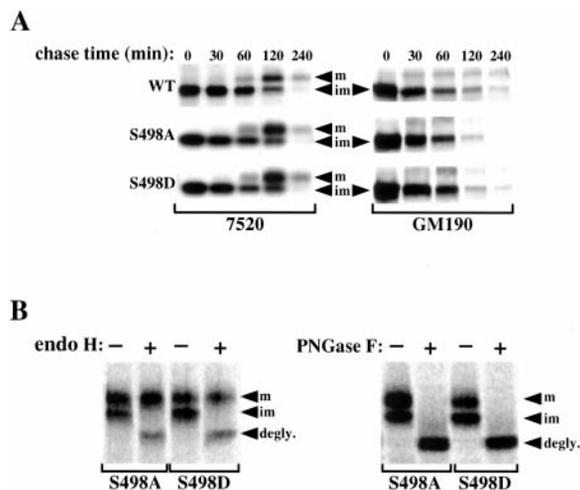
polyclonal antibody 7523 against BACE and with monoclonal antibody against giantin, a Golgi marker protein (30). BACE WT and giantin partially colocalize in juxtannuclear structures (Fig. 4, *a* and *b*). Consistent with previous results (4, 5, 7, 12, 14), additional staining was observed in juxtannuclear and vesicular structures that did not overlap with the Golgi marker giantin (Fig. 4, *a* and *b*). To prove whether this additional staining is due to localization of BACE in post-Golgi compartments, cells were treated with BFA for 30 min at 37 °C. BFA treatment is known to result in the fusion of Golgi compartments with the ER, while the trans-Golgi network (TGN) and other post-Golgi compartments fuse with endosomes to form vesicular/tubular structures (31). Treatment with BFA resulted in reticular staining of giantin, indicative for a redistribution of the Golgi marker protein giantin to the ER (Fig. 4d). In contrast, BACE was detected in juxtannuclear and peripheral vesicular structures after BFA treatment (Fig. 4c). These results indicate that BACE is not only localized in the ER and Golgi but also in post-Golgi compartments.

To examine if phosphorylation of BACE affects its accumulation in these compartments, we generated cDNA constructs encoding BACE derivatives in which serine residue 498 is substituted by alanine or by aspartate residues in order to mimic unphosphorylated or phosphorylated BACE molecules, respectively. HEK 293 cells stably expressing BACE WT, BACE S498D, or BACE S498A were permeabilized and costained with antibody 7523 and antibodies against giantin. As shown above (Fig. 4), BACE WT and giantin partially colocalize in juxtannuclear structures (Fig. 5, *a* and *b*). A very similar localization was observed for BACE S498D, which mimics phosphorylated BACE (Fig. 5, *c* and *d*). In contrast, nonphosphorylatable BACE S498A showed less juxtannuclear staining. Rather, BACE S498A accumulated in peripheral vesicular structures near the plasma membrane, particularly in cellular processes (Fig. 5e). Very similar data were obtained with independent cell clones and transiently transfected HEK 293 cells (data not shown). These data indicate that some aspects of subcellular sorting of BACE are affected by phosphorylation/dephosphorylation of serine residue 498.

To prove that the distinct staining pattern of the BACE mutants is not simply due to impaired maturation, we performed pulse-chase experiments. Immunoprecipitation with antibody 7520 against the C terminus of BACE demonstrate that the phosphorylation site mutants BACE S498A and BACE S498D mature with similar kinetics as the WT protein by complex *N*-glycosylation as indicated by the molecular mass shift from 66 to 70 kDa (Fig. 6A, *left panel*). To analyze proteo-



**FIG. 5. Phosphorylation state-dependent subcellular localization of BACE.** HEK 293 cells expressing BACE WT (*a* and *b*), the mutant derivatives BACE S498D (*c* and *d*), or BACE S498A (*e* and *f*) were grown on glass coverslips and then processed for immunocytochemistry. After fixation, cells were costained with polyclonal antibodies 7523 against BACE (*a*, *c*, and *e*) and monoclonal antibodies against giantin (*b*, *d*, and *f*). Note that BACE WT and BACE S498D showed similar subcellular distribution predominantly in juxtannuclear compartments (arrowheads) and partial colocalization with giantin. In contrast, BACE S498A revealed more vesicular staining in the periphery of the cell (arrows). Bar, 10  $\mu$ m.



**FIG. 6. Substitution of serine residue 498 does not affect maturation of BACE.** *A*, pulse-chase experiments with cells expressing BACE WT, S498A, and S498D. Cells stably expressing the respective cDNAs were pulse-labeled for 10 min with [ $^{35}$ S]methionine and chased in the presence of excess amounts of unlabeled methionine for the time points indicated. BACE was immunoprecipitated with antibody 7520 (left panel) or with antibody GM190 (right panel) and detected by autoradiography. *B*, BACE S498A and S498D were isolated by immunoprecipitation from cells labeled with [ $^{35}$ S]methionine and incubated in the presence or absence of endo H or PNGase F. After separation by SDS-PAGE, radiolabeled proteins were detected by autoradiography.

lytic cleavage of the prodomain of BACE, cell lysates were immunoprecipitated with antibody GM190 directed against the prodomain. Similar to our previous results (12), the prodomain is predominantly observed in the immature 66-kDa BACE species (Fig. 6A, right panel). The gradual disappearance of prodomain-containing forms indicates efficient maturation of BACE S498A and BACE S498D similar to BACE WT (Fig. 6A, right panel). Moreover, the mature 70-kDa forms of mutant BACE

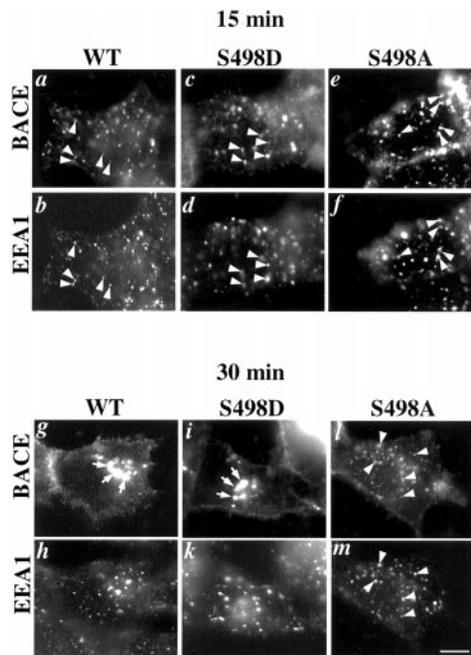
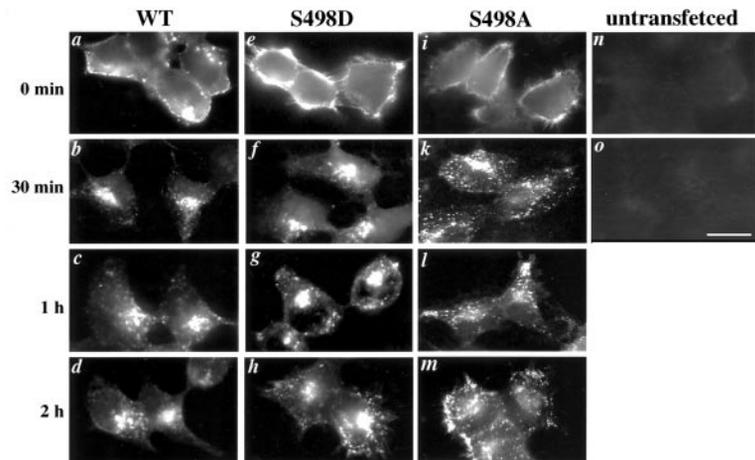
S498A and S498D are both resistant to endo H cleavage, while PNGase F efficiently deglycosylated the 70- and 66-kDa forms (Fig. 6B). These data demonstrate that the mutations in the cytoplasmic domain of BACE do not interfere with maturation by complex *N*-glycosylation, indicating unimpaired forward transport to late Golgi compartments and beyond. Therefore, the altered subcellular localization of the nonphosphorylated mutant BACE S498A is not due to impaired maturation.

**Phosphorylation Regulates Retrieval of BACE from Endocytosed Vesicles**—Recently, it was reported that cell surface-located BACE is internalized into endosomes (14). We therefore first investigated whether phosphorylation of BACE might regulate its reinternalization from the cell surface. HEK 293 cells stably expressing BACE WT or the phosphorylation site mutant BACE S498A or BACE S498D were incubated for 20 min on ice with antibody 7523 directed toward the ectodomain of BACE. After removal of nonspecifically bound antibodies, cells were returned to 37  $^{\circ}$ C for the time points indicated and processed for immunocytochemistry. At time point 0 min, cells expressing BACE WT or the S498D or S498A variants revealed strong staining of the cell surface (Fig. 7, *a*, *e*, and *i*). In contrast, untransfected HEK 293 cells were not labeled by antibody 7523, demonstrating specific detection of exogenous BACE at the cell surface (Fig. 7*n*). Staining of cells after 30 min at 37  $^{\circ}$ C revealed that all BACE variants are efficiently internalized from the cell surface (Fig. 7, *b*, *f*, and *k*). Again, untransfected cells were not stained, demonstrating that antibodies were not internalized by fluid phase endocytosis (Fig. 7*o*). Staining cells after 1 and 2 h at 37  $^{\circ}$ C revealed significant differences in the subcellular localization of internalized BACE S498A as compared with BACE WT and BACE S498D. Cells expressing BACE WT or the BACE S498D mutant that mimics phosphorylated BACE revealed intensive juxtannuclear localization indicative for late endosomal compartments and/or TGN (Fig. 7, *b*–*d* and *f*–*h*). In contrast, the nonphosphorylated mutant S498A showed predominant localization in vesicles near the plasma membrane, which occasionally appeared to accumulate in cellular processes (Fig. 7, *k*–*m*).

To identify the subcellular compartment to which BACE is targeted after internalization from the cell surface, we performed antibody uptake assays as described above. To detect early endosomes, cells were costained with monoclonal antibodies recognizing the early endosome antigen 1 (EEA1) (32). Staining of cells after 15 min showed internalization of BACE into some EEA1-positive compartments. No significant differences in the staining pattern between BACE WT and the phosphorylation site mutants (S498A and S498D) were detected at this time point, demonstrating that internalized BACE is targeted to early endosomal compartments independent of its phosphorylation state (Fig. 8, *a*–*f*). After 30 min, internalized BACE WT and BACE S498D were efficiently targeted to juxtannuclear structures (as described above; Fig. 8), showing less colocalization with the EEA1-positive compartments (Fig. 8, *g*–*k*). In contrast, the nonphosphorylated mutant BACE S498A showed less pronounced accumulation in juxtannuclear structures (as described above) but appeared to be retained at least partially in EEA1 positive endosomal compartments (Fig. 8, *l* and *m*).

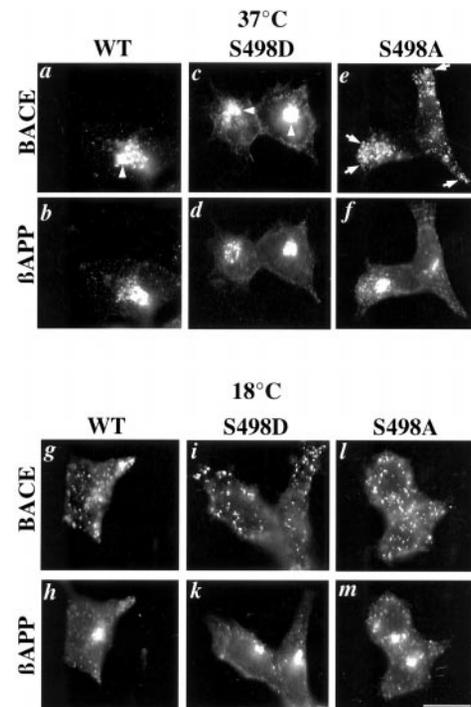
We next sought to identify the cellular trafficking step of BACE, which is regulated by its phosphorylation. It was shown previously that incubation of cells at 18  $^{\circ}$ C inhibits both forward transport from the ER to Golgi and retrograde transport from endocytosed vesicles to late endosomal compartments and the TGN but allows endocytosis (33, 34). Cells were incubated with antibody 7523 on ice as described above. After washing, the cells were returned to 37 or 18  $^{\circ}$ C and incubated for an

**FIG. 7. Phosphorylation regulates intracellular trafficking of BACE.** HEK 293 cells stably expressing BACE WT (*a–d*), BACE S498D (*e–h*), or BACE S498A (*i–m*) or untransfected cells (*n* and *o*) were incubated for 20 min with antibody 7523 on ice. Cells were washed and incubated at 37 °C for the time points indicated. Internalized antibodies 7523 were detected by Alexa 488-labeled anti-rabbit secondary antibody. *Bar*, 25  $\mu$ m.



**FIG. 8. BACE is internalized from the cell surface into EEA1 positive early endosomes.** HEK 293 cells stably expressing BACE WT (*a, b, g, and h*), BACE S498D (*c, d, i, and k*), or BACE S498A (*e, f, l, and m*) were incubated for 20 min with antibody 7523 on ice. Cells were washed and incubated at 37 °C for 15 min (*a–f*) or 30 min (*g–m*) before fixation. Internalized antibodies 7523 were detected by Alexa 488-labeled anti-rabbit secondary antibody (*a, c, e, g, i, and l*), and early endosomal compartments were detected with monoclonal antibodies against EEA1 (*b, d, f, h, k, and m*). Colocalization of BACE and EEA1 in early endosomal compartments is indicated by arrowheads (*a–f, l, and m*), while juxtannuclear structures containing BACE WT (*g*) or BACE S498D (*i*) are indicated by *arrows*. *Bar*, 10  $\mu$ m.

additional 1 h. Internalized BACE was then detected with fluorescence-labeled secondary antibody. After incubation at 37 °C, the above-described differences in the subcellular localization of BACE WT, BACE S498D, and BACE S498A were observed. While BACE WT and BACE S498D accumulated in juxtannuclear compartments, BACE S498A was predominantly localized in peripheral endocytosed vesicles (Fig. 9, *a, c, and e*), identified as early endosomes (see Fig. 8). Costaining of these cells with the monoclonal antibodies IG7/5A3 against  $\beta$ APP (35) revealed that internalized BACE WT and S498D partially colocalized with its protein substrate  $\beta$ APP in juxtannuclear structures, while internalized BACE S498A showed less colocalization with  $\beta$ APP (Fig. 9, *a–f*).



**FIG. 9. Phosphorylation-dependent retrieval of BACE from endocytosed vesicles.** HEK 293 cells stably expressing BACE WT (*a, b, g, and h*), BACE S498D (*c, d, i, and k*), or BACE S498A (*e, f, l, and m*) were incubated for 20 min with antibody 7523 on ice. Cells were washed and incubated at 37 °C (*a–f*) or 18 °C (*g–m*) for an additional 1 h. Cells were fixed, and internalized antibodies were visualized with Alexa 488-labeled anti-rabbit secondary antibody.  $\beta$ APP was detected with monoclonal antibodies IG7/5A3 and Alexa 594-labeled anti-mouse secondary antibody. The *arrowheads* indicate juxtannuclear localization of BACE WT and BACE S498D (*a and c*), and the *arrows* indicate localization of BACE S498A in peripheral vesicles (*e*). *Bar*, 25  $\mu$ m.

When cells were incubated at 18 °C, all variants of BACE were readily internalized, but, in contrast to incubation at 37 °C, transport of reinternalized BACE WT and BACE S498D to juxtannuclear structures was inhibited (Fig. 9, *g* and *i*). Rather, at 18 °C all variants of BACE accumulated in peripheral vesicles, and BACE WT and S498D showed a very similar localization as the nonphosphorylated S498A variant of BACE (Fig. 9, *g, i, and l*). Costaining of these cells with  $\beta$ APP antibodies demonstrates that localization of  $\beta$ APP in juxtannuclear structures was not impaired under these experimental conditions (Fig. 9, *g–m*).

## DISCUSSION

In this study, we analyzed the subcellular trafficking of BACE dependent on its phosphorylation state. A single phosphorylation site was identified by mutagenesis of serine residue 498 in the C-terminal domain of BACE (Fig. 1A). By testing several protein kinases *in vitro*, we found that CK-1 can phosphorylate BACE at the *in vivo* phosphorylation site at serine residue 498. HD, which preferentially inhibits CK-1 beside glycogen synthase kinase  $\beta$  and cyclin-dependent kinases (29), significantly reduced phosphorylation of the cytoplasmic domain of BACE in cellular extracts. The phosphorylation site identified at serine residue 498 is preceded by a stretch of acidic amino acid residues (Fig. 1A) representing a canonical recognition motif for CK-1, but not for glycogen synthase kinase  $\beta$  or cyclin-dependent kinases. Taken together, these data indicate that CK-1 or a CK-1-like kinase is involved in the phosphorylation of BACE *in vivo*. Phosphorylation occurs exclusively on the fully matured BACE after propeptide removal and complex *N*-glycosylation. This indicates that phosphorylation of BACE takes place selectively after its exit from the ER, presumably in Golgi or post-Golgi compartments. CK-1 occurs in several isoforms, and some have been shown to be associated with the plasma membrane and synaptic vesicles and to selectively phosphorylate a subset of membrane proteins (36). CK-1 is implicated in the regulation of vesicular trafficking in yeast presumably by phosphorylating components of clathrin adaptor proteins (37, 38). Our data suggest that a CK-1 isoform with a particular subcellular distribution (*i.e.* in late Golgi and/or endosomal compartments) might be responsible for the highly selective phosphorylation of mature BACE. It has been reported that CK-1 is significantly elevated in Alzheimer's disease brains (39, 40). Moreover, A $\beta$  has been shown to activate CK-1 *in vitro* (41). However, it remains to be determined if phosphorylation of BACE is altered during pathogenesis of Alzheimer's disease.

In order to investigate the cellular function of phosphorylation/dephosphorylation on the trafficking of BACE, we generated mutants in which the phosphorylation site at serine residue 498 has been substituted by an alanine or an aspartate residue to mimic nonphosphorylated and phosphorylated forms of BACE, respectively. The major advantage of this strategy is to circumvent the use of agents modulating kinase or phosphatase activities that might cause nonspecific or indirect effects (42, 43). BACE carrying the S498D substitution was predominantly localized in juxtannuclear compartments, where it partially colocalizes with the Golgi marker protein giantin. A very similar distribution was observed for BACE WT, which is consistent with previous results (7, 12, 14). In contrast, the non-phosphorylated form BACE S498A showed less localization in juxtannuclear structures but pronounced localization in vesicular compartments including EEA1 positive early endosomes. It was demonstrated previously that the complete deletion of the cytoplasmic domain of BACE results in its retention within the ER and in impaired maturation (12). However, the phosphorylation site mutants BACE S498A and BACE S498D mature normally by complex *N*-glycosylation and proteolytic removal of the prodomain as compared with BACE WT. Therefore, the significant differences in subcellular localization of the mutant variants of BACE are due to distinct sorting. Consistent with previous results (12, 14), we found that BACE is transported to the cell surface and that BACE is internalized into EEA1-positive early endosomal compartments. BACE WT as well as the mutant derivatives S498A and S498D were efficiently endocytosed, indicating that phosphorylation of BACE does not determine its reinternalization from the cell surface. Rather, we found that phosphorylation of BACE is functionally re-

quired for efficient retrieval of the enzyme from early endosomes to later endosomal and/or TGN compartments from which BACE might be recycled into the secretory pathway.

Interestingly, phosphorylation of reinternalized BACE affected its colocalization with its protein substrate  $\beta$ APP in juxtannuclear structures. However, overexpressing BACE WT or the phosphorylation site mutants led to increased secretion of A $\beta$  as compared with untransfected control cell lines (data not shown). This may reflect that expression of exogenous BACE leads to saturated levels of A $\beta$  generation, regardless of the differences in subcellular localization of the BACE variants. Indeed, previous studies demonstrated that  $\beta$ APP could be cleaved by  $\beta$ -secretase activity in distinct compartments, including endosomes and late Golgi compartments (16, 17, 44). We also demonstrate that reinternalization of BACE was not affected by the S498A or S498D mutation. In addition, all variants of BACE undergo normal maturation by complex *N*-glycosylation. These data indicate that at least two major sites of  $\beta$ -secretase activity, endosomes and secretory vesicles, can be reached by BACE independent of its phosphorylation state. However, it might be possible that regulation of subcellular trafficking of endogenously expressed BACE by phosphorylation affects proteolytic processing of other, yet unknown, protein substrates. In addition, we cannot yet exclude the possibility that phosphorylation may have subtle effects on  $\beta$ APP processing.

The regulatory mechanism of subcellular trafficking of BACE is highly reminiscent to that of furin. Retrieval of furin from endosomal to TGN compartments was also shown to be dependent on phosphorylation/dephosphorylation of its cytoplasmic domain (45). Therefore, the subcellular localization of BACE is regulated in a remarkably similar fashion like furin, a protease that has recently been demonstrated to be required for propeptide cleavage of BACE (11–13).

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## Phosphorylation Regulates Intracellular Trafficking of $\beta$ -Secretase

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