Alzheimer's disease is the most common age-dependent dementia. Pathologically Alzheimer's disease is characterized by the invariant accumulation of senile plaques (1). Senile plaques are predominantly composed of amyloid β-peptide (Aβ). This is derived from the amyloid β-peptide precursor protein (βAPP) (2). βAPP is a type 1 transmembrane protein that matures during its transport to the cell surface by several post-translational modifications including N- and O-glycosylation (3), phosphorylation (3–5), sulfation (3), and endoproteolysis (2). Upon trafficking to the cell surface, βAPP can be reinternalized and targeted to endosomes (6, 7). βAPP can recycle from endosomes back to the cell surface (8) or be targeted to lysosomes (7) for its final degradation (7, 9). In polarized cells such as Madin-Darby canine kidney cells and hippocampal neurons, βAPP is transported to the basolateral compartment (10, 11) and to axons (12).

Amyloid β-peptide is generated by two sequential proteolytic cleavages mediated by β-secretase (BACE) and γ-secretase. BACE was recently identified as a membrane-associated aspartyl protease. We have now analyzed the maturation and pro-peptide cleavage of BACE. Pulse-chase experiments revealed that BACE is post-translationally modified during transport to the cell surface, which can be monitored by a significant increase in the molecular mass. The increase in molecular mass is caused by complex N-glycosylation. Treatment with tunicamycin and N-glycosidase F led to a BACE derivative with a molecular weight corresponding to an unmodified version. In contrast, the mature form of BACE was resistant to endoglycosidase H treatment. The cytoplasmic tail of BACE was required for efficient maturation and trafficking through the Golgi; a BACE variant lacking the cytoplasmic tail undergoes inefficient maturation. In contrast a soluble BACE variant that does not contain a membrane anchor matured more rapidly than full-length BACE. Pro-BACE was predominantly located within the endoplasmic reticulum. Pro-peptide cleavage occurred immediately before full maturation and trafficking through the Golgi.

During its transport to the cell surface, βAPP undergoes endoproteolytic cleavage. α-Secretase cleaves βAPP within its Aβ domain leading to the secretion of APPs-α in biological fluids and conditioned medium of cultured cells (3, 13). β-Secretase generates the N terminus of the Aβ domain (2), and this cleavage can occur in direct competition to the α-secretase cut (14–16). β-Secretase cleavage appears to occur within the Golgi (15, 16) as well as during reinternalization within endosomes (6, 7). The membrane-bound C-terminal fragment generated by the β-secretase cut is subsequently cleaved by the γ-secretase, which results in the physiological generation and secretion of Aβ (2).

Interestingly, point mutations associated with familial Alzheimer’s disease have been located at all three secretase cleavage sites, and these mutations pathologically affect endoproteolysis of βAPP (1). Recently major progress has been achieved in the identification of secretase activities involved in βAPP endoproteolysis. α-Secretase activity has been associated with metalloproteases of the ADAM-family (17, 18). γ-Secretase appears to be an aspartyl protease (19), which may be identical with presenilins (20).

β-Secretase (BACE is the β-site APP-cleaving enzyme) has recently been identified (21–25) and demonstrated to be an aspartyl protease. BACE is a membrane-bound type 1 protein, which appears to be synthesized as an inactive pro-enzyme. BACE is homologous to BACE-2 (23, 26, 27). Both proteases differ in their tissue-specific expression. Whereas BACE is abundantly expressed in brain (21, 23), BACE-2 shows lower expression in neuronal tissue but is widely expressed in peripheral tissue (23, 28). BACE appears to represent the major β-secretase activity (21–25, 28). Because very little is known about the maturation and proteolytic activation of BACE, it was necessary to investigate the maturation of BACE during its transport from the endoplasmic reticulum (ER) to the cell surface.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Cell Lines**—Human embryonic kidney 293 cells (HEK 293) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 490 μg/ml G418 (to select for βAPP expression), and 200 μg/ml zeocin (to select for BACE expression). HEK 293 cells stably expressing Swedish mutant βAPP or βAPP695 were described previously (7, 29).

**cDNA Cloning of BACE**—A full-length BACE cDNA was amplified by polymerase chain reaction from a human neuroblastoma (SK-N-MC) cDNA library in λZAP-Express-vector (obtained from Dr. Christian Behl, Max-Planck Institute for Psychiatry, Munich) using the GC-RICH-PCR-system (Roche Molecular Biochemicals) and subcloned into the EcoRI/XhoI site of pcDNA4/Myc-HisA (Invitrogen). The cDNA was sequenced for verification.

**Mutagenesis of BACE cDNAs**—The truncated BACE derivatives were generated by inserting stop codons after amino acid 454 (sBACE) or 477 (BACEΔC) using polymerase chain reaction. The cDNAs were...
subcloned into the EcoRI/XhoI site of pcDNA4/Myc-HisA. The pro-domain cleavage site mutant BACE-R45A was generated by polymerase chain reaction mutagenesis and subcloned into the EcoRI/XhoI site of pcDNA4/Myc-HisA. All cDNA constructs were sequenced to verify successful mutagenesis.

**Antibodies**—The following polyclonal antibodies to BACE were generated (see Fig. IA): antibody 7523 to amino acids 46–60, antibody GM 190 to amino acids 22–45, and antibody 7520 to amino acids 482–501. The synthetic peptides were coupled to keyhole limpet hemocyanin and used to immunize rabbits (Eurogentec, Seraing, Belgium). The monoclonal antibody 9E10 to the Myc epitope was obtained from the hybridoma bank.

**Metabolic Labeling and Immunoprecipitation of BACE**—To analyze expression of BACE, HEK 293 cells were starved for 1 h in methionine- and serum-free minimum Eagle's medium and subsequently were metabolically labeled with 700 μCi [35S]methionine (Promix, Amersham Pharmacia Biotech) in methionine- and serum-free minimum Eagle's medium for 5 min. Subsequently, cells were incubated in the presence of excess amounts of unlabeled methionine for the indicated time points. BACE was immunoprecipitated using previously described protocols (30).

**Tunicamycin, N-glycosidase F, and Endoglycosidase H Treatment**—Cells were pulse-labeled in the presence of 10 μg/ml of tunicamycin as described above. The cold chase was also performed in the presence or absence of tunicamycin. Cell lysates were immunoprecipitated with antibodies GM190 and 9E10. 500 μg of a protein extract from HEK 293 cells expressing Myc-tagged wtBACE were used for immunoprecipitation (30) of BACE with antibodies GM190 and 7523. Immunoprecipitates were divided into four aliquots and treated with and without 1 U N-glycosidase F or with and without 1 milliunit endoglycosidase H (endo H) for 1 h at 37 °C in the presence the buffers recommended by the supplier (Roche Molecular Biochemicals). Samples were loaded onto an SDS-polyacrylamide gel and analyzed by immunoblotting using the anti-Myc monoclonal antibody 9E10.

**Analysis of βAPP Metabolites**—HEK 293 cells expressing βAPP as well as BACE were grown to confluency. For the analysis of Aβ in conditioned medium, cells were metabolically labeled with 450 μCi [35S]methionine (Promix, Amersham Pharmacia Biotech) for 2 h and chased for 2 h in medium containing excess amounts of unlabeled methionine. Aβ and p3 were immunoprecipitated from conditioned medium with antibody 3926 (31) and were separated on 10–20% Tris-Tricine gels (Novex) and analyzed by fluorography.

**Immunocytochemistry**—Immunocytochemistry was carried out as described previously (7).

**RESULTS**

**Expression of Proteolytically Active BACE**—A BACE cDNA was isolated from a cDNA library generated from human brain. The cDNA was subcloned into an appropriate expression vector and was stably transfected into HEK 293 cells overexpressing wtβAPP (7). HEK 293 cell lines were used previously to identify BACE and to analyze its proteolytic function (21). The same cell line was also used to successfully investigate the effects of the Swedish mutation on β-secretase cleavage (29) thus demonstrating that HEK 293 cells represent a valid model system to study the cell biology of BACE. Cell lysates of untransfected HEK 293 cells or cell lines stably expressing BACE were prepared. BACE was immunoprecipitated with the polyclonal antibodies GM190 (targeting amino acids 22–45 corresponding to the pro-peptide sequence, Refs. 21, 22), 7523 (targeting amino acids 46–60), or antibody 7520 (targeting amino acids 482–501) (Fig. IA). Immunoprecipitated BACE was identified by Western blotting using the anti-Myc antibody 9E10. In lysates from transfected cells, a polypeptide corresponding to a molecular mass of about 70 kDa was detected by all three antibodies (Fig. IB). This is consistent with the molecular mass of BACE described previously (21–25). An additional higher molecular weight band was observed with antibody 7523 and antibody 7520 (Fig. IB). This band was only very weakly recognized by antibody GM190 (see also Figs. 4 and 5) but was preferentially identified by antibody 7523 (Fig. IB). The extremely weak staining of the higher molecular weight band by antibody GM190 may be caused by the very minor maturation of unprocessed BACE. Antibody 7520 raised to the C terminus equally identifies both bands. This indicates that the upper band lacks the pro-peptide and is preferentially recognized by the neo-epitope specific antibody 7523 after removal of the pro-peptide (see below). It is also important to note that GM190 identifies native BACE (after mild extraction of membranes) as well as the denatured protein (after SDS-polyacrylamide gel electrophoresis) or after paraformaldehyde fixation (see below).

We next analyzed the β-secretase activity of transfected BACE derivatives in HEK 293 cells (stably expressing βAPP). As shown before (21) co-expression of BACE together with wtβAPP results in a significant increase of Aβ production (Fig. IC), which is accompanied by a decrease of APPs-α (data not shown).

**Maturation of BACE**—We next analyzed the maturation of BACE derivatives in pulse-chase experiments. Three cell lines expressing different BACE cDNA constructs were investigated (Fig. IA). In addition to the above described cell line expressing the full-length BACE cDNA (wtBACE), cell lines were generated that express two truncated versions of BACE. Stop codons were inserted to generate BACE variants terminating after the transmembrane domain (at amino acid 477) or after amino acid 454 to produce BACEΔC and soluble BACE (sBACE), respectively (Fig. IA). Immunoprecipitation of Aβ revealed that wt
BACE, BACEΔC, and sBACE exhibited significant β-secretase activity (data not shown).

HEK 293 cells stably expressing wtBACE, BACEΔC, or sBACE were pulse-labeled for 5 min with [35S]methionine and chased for 0, 30, 60, 120, and 240 min in the presence of excess amounts of unlabeled methionine. Cell lysates were prepared after the indicated time points and immunoprecipitated with the monoclonal antibody to the Myc tag. Immunoprecipitates were separated by polyacrylamide gel electrophoresis and visualized by autoradiography. During the chase, wtBACE appeared to be post-translationally modified resulting in a significant increase in its molecular mass. Similar data were obtained for endogenous BACE (data not shown). After 120 min, wtBACE (Fig. 2A) efficiently matured whereas BACEΔC matured much slower (Fig. 2B). When sBACE was expressed, rapid maturation was observed (Fig. 2C, upper) and after 60 min, significant amounts of the mature polypeptide were secreted (Fig. 2C, lower).

The ratio of the mature polypeptide versus the immature protein was determined by phosphorimaging. This confirmed that BACEΔC matured slower than wtBACE whereas sBACE matured significantly faster than the membrane-associated BACE derivatives (Fig. 2D).

Because these results indicated that the cytoplasmic tail of BACE might be important for efficient trafficking, we determined the subcellular localization of wtBACE and BACEΔC by immunofluorescence. Cells grown on coverslips were fixed in 4% paraformaldehyde and permeabilized. BACE derivatives were detected using the antibody 7523. Consistent with previous results (21), wtBACE occurred within the Golgi and other vesicular compartments including the ER as well as the nuclear envelope (Fig. 3A). In contrast to wtBACE, BACEΔC accumulated within the ER (Fig. 3B). Taken together with the data shown in Fig. 2, these results demonstrate that BACE derivatives lacking the cytoplasmic tail fail to be efficiently transported out of the ER.

N-Glycosylation of BACE—The above described increase in the molecular mass of BACE that was observed during the chase period (Fig. 2) indicates that BACE might be glycosylated. BACE contains several potential N-linked glycosylation sites (21–25). To prove N-glycosylation, HEK 293 cells stably transfected with wtBACE were treated with tunicamycin, which blocks cotranslational N-glycosylation within the ER. Treatment of cells with tunicamycin completely inhibited the characteristic molecular weight shift observed in untreated cells and resulted in the generation of a BACE derivative corresponding to the unmodified core-protein (Fig. 4A). Moreover, treatment of immunoprecipitated BACE with N-glycosidase F also resulted in a derivative with a molecular mass of approximately 50 kDa, which corresponds to that of an unmodified BACE molecule (Fig. 4A).
were identified by immunoblotting using antibody 9E10. Note that maturation is completely inhibited and a BACE derivative with expected molecular weight of a non-modified version is not precipitated with antibody GM190 (see also Fig. 5). As shown in Fig. 4, B and C, endo H treatment fully removes sugars from the immature (pro-peptide-containing) form whereas the mature form was endo H resistant. Taken together these results demonstrate that the molecular weight increase of BACE observed during maturation is caused by post-translational N-glycosylation. Moreover, these data suggest that the mature endo-H resistant protein does not contain the pro-peptide (see below).

Pro-peptide Cleavage—BACE is produced as a pro-enzyme with a 24-amino acid pro-peptide attached to its N terminus (21–25). To follow endoproteolytic maturation of BACE, we performed pulse-chase experiments as described in Fig. 2. Aliquots of the cell lysates prepared after the indicated time points were immunoprecipitated either with antibody 7523 or with GM190 raised to the pro-peptide (see Fig. 1, A and B). Immunoprecipitation with antibody 7523 demonstrated the maturation of BACE during the cold chase described above (Fig. 5A). In contrast, antibody GM190 detected only a single polypeptide. Moreover, this polypeptide significantly decreased during the cold chase and was almost absent after 240 min, indicating pro-peptide removal (Fig. 5B). Because no N-glycosylation sites have been observed close to the potential cleavage site after amino acid 44 (21), it is very unlikely that full maturation of BACE may inhibit GM190 reactivity with the mature form. These data rather indicate that the pro-peptide is removed before or during BACE maturation.

To further prove the subcellular localization of the pro-enzyme, we performed double-immunocytochemical experiments. HEK 293 cells stably expressing wtBACE were double-stained with the monoclonal anti-Myc antibody and the polyclonal anti-pro-peptide antibody GM190. This revealed that the pro-enzyme is predominantly located within a tubular network corresponding to the ER as well as within the nuclear envelope (Fig. 5, C and D). In contrast, staining with the anti-Myc antibody showed a significant accumulation of BACE within a cellular compartment corresponding to the Golgi (Fig. 5C). This compartment showed little co-staining with the pro-peptide antibody GM 190 (compare Fig. 5C, upper versus lower). Taken together these data demonstrate that pro-BACE is efficiently processed during its passage through the secretory pathway.

The pro-domain of BACE contains a sequence motif $\text{Arg-Leu-Pro-Arg^{65}-Glu^{66}-Thr-Asp}$, where underlined amino acids

These results demonstrate that BACE is cotranslationally N-glycosylated and produces the 70-kDa immature BACE. During maturation N-linked sugars appear to be further modified finally leading to the mature 75-kDa protein observed late during the cold-chase period. Complex N-glycosylation associated with the molecular weight shift occurs after the release of BACE from the ER because brefeldin A completely inhibited the molecular weight shift observed to be associated with maturation (data not shown). To further prove that the increase in the molecular weight of BACE during maturation can be attributed to complex N-glycosylation, we treated cells with endo H. Lysates from cells treated with and without endo H were immunoprecipitated with antibody GM190 or 7520. Precipitated proteins were detected by immunoblotting using antibody 9E10. As shown in Fig. 4, A and C, endo H treatment fully removes sugars from the immature (pro-peptide-containing) form whereas the mature form was endo H resistant. Taken together these results demonstrate that the molecular weight increase of BACE observed during maturation is caused by post-translational N-glycosylation. Moreover, these data suggest that the mature endo-H resistant protein does not contain the pro-peptide (see below).

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The pro-domain of BACE contains a sequence motif $\text{Arg-Leu-Pro-Arg^{65}-Glu^{66}-Thr-Asp}$, where underlined amino acids
BACE were pulse-labeled with \([^{35}\text{S}]\)methionine and chased in the presence of excess amounts of unlabeled methionine for the indicated time points. Cell lysates were immunoprecipitated with antibody 7523 (A) or GM190 (B). Note that the pro-peptide antibody GM190 only detects the immature BACE species. C and D, double staining revealing the subcellular localization of the pro-enzyme within the ER. C, the anti-Myc antibody reveals the expected Golgi and vesicular staining described previously (21). In contrast, antibody GM190 detects the pro-enzyme predominantly within a tubular network corresponding to the ER as well as within the nuclear envelope. Arrows indicate the differential staining of the two antibodies. D, a double stain of cells with antibody GM190 and the anti-BIP antibody demonstrate that the pro-enzyme is predominantly located within the ER.

DISCUSSION

BACE is the major proteolytic activity involved in the \(\beta\)-secretase cleavage of \(\beta\)APP (21–25, 28). Thus this enzyme represents a prime target for compounds that are supposed to lower \(\beta\) production. However, little is known about the cell biology of BACE. Understanding its cellular trafficking, maturation, and endoproteolytic processing will provide the fundamental knowledge finally required to specifically interfere with BACE function. We therefore studied the maturation of BACE with a particular emphasis on glycosylation, pro-peptide cleavage, and trafficking as a function of the cytoplasmic tail. We found that BACE is initially synthesized as an \(\sim 70\)-kDa protein. This immature form of BACE is already \(N\)-glycosylated. Inhibition of \(N\)-glycosylation by tunicamycin \textit{in vitro} as well as enzymatic removal of these sugar moieties in \textit{vitro} reduced the molecular mass of BACE to \(\sim 50\) kDa that corresponds to the calculated molecular mass of the non-modified BACE molecule (21–25). During maturation, the immature BACE receives additional sugar moieties, which results in a further increase of the molecular mass to \(\sim 75\) kDa. The resulting 75-kDa species is endo H resistant, further supporting the conclusion that this polypeptide represents the fully matured enzyme. The modifications associated with the molecular weight shift appear to occur after the immature molecule has been released from the ER. Extensive \(N\)-linked glycosylation is supported by the identification of four potential \(N\)-linked glycosylation sites within the ectodomain of BACE (21–25).

Like many other proteases, BACE appears to be produced as a pro-enzyme (21–25). We found that the endoproteolytic removal of the pro-peptide occurs after BACE leaves the ER. This is supported by the finding that the pro-enzyme is absent from the mature, fully glycosylated, endo H resistant protein (Figs. 4B and 5). Moreover, an antibody raised to the pro-peptide of BACE detected the pro-enzyme predominantly within the ER and the nuclear envelope, whereas antibodies to the mature form detected BACE predominantly within the Golgi and other vesicular structures. These findings may also be interesting in regard to the identification of the enzymatic activity involved in pro-peptide removal. An obvious candidate for this cleavage may be furin, a member of a family of mammalian enzymes related to the yeast Kex2p and bacterial subtilisins (32).

Consistent with previous data (21, 23, 25), we found that BACE is transported through the secretory pathway. Interestingly, cellular transport of BACE appears to be dependent on its cytoplasmic tail. Removal of the cytoplasmic tail resulted in insufficient maturation and consequently in an accumulation of immature BACE within the ER and the nuclear envelope. These data suggest that the cytoplasmic tail of BACE contains signals that are required to guide BACE through the secretory pathway. The cytoplasmic tail of BACE contains an acidic domain. Such an acidic domain is also utilized by furin to regulate its subcellular localization (32). It will be interesting to investigate whether such signals influence \(\beta\)APP processing. Furthermore, interference with subcellular trafficking or pro-peptide cleavage may provide novel therapeutic targets for lowering the amyloid \(\beta\)-peptide production.

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