A Splice Variant of \(\beta\)-Secretase Deficient in the Amyloidogenic Processing of the Amyloid Precursor Protein*

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\(\beta\)-Secretase (BACE) initiates the amyloidogenic processing of the amyloid precursor protein leading to the generation of the \(\beta\)-amyloid, the main component of Alzheimer’s disease senile plaques. BACE is a type I transmembrane aspartyl protease of 501 amino acids. Here we describe a novel BACE mRNA lacking 132 base pairs that is expressed in the pancreas but not in the brain. Sequence alignment indicates that the deleted fragment matches the terminal two-thirds of exon 3. The new BACE variant is short of a 44-amino acid region located between the two catalytic aspartyl residues. Accordingly, a 50-kDa form of BACE (BACE457) is detected in the human pancreas. When expressed in cells, BACE457 colocalizes with the marker for the endoplasmic reticulum BiP. Moreover, BACE457 remains in a proenzymatic and endoglycosidase H-sensitive state, suggesting that its transport along the secretory pathway is blocked at the level of the endoplasmic reticulum. Notably, this novel form of BACE does not contribute to the processing of the amyloid precursor protein. Our findings suggest that tissue-specific splicing of the BACE mRNA may explain the observation that in the human pancreas robust transcription of the BACE gene does not translate into recovered enzymatic activity.

The \(\beta\)-amyloid peptide (A\(\beta\)) is the main component of the senile plaques found in the brain of Alzheimer’s disease patients (1, 2). Amyloidogenic processing of the amyloid precursor protein (APP) is initiated by \(\beta\)-secretase cleavage generating a 100-kDa soluble form of the ectodomain (sAPP\(\beta\)) and a 12-kDa membrane-associated intermediate of 99 amino acids (C99). The latter is then processed by \(\gamma\)-secretase to A\(\beta\) (3). \(\beta\)-Secretase cleaves typically after Met\(^{596}\) of the APP\(_{99}\) isoform generating the amino terminus of A\(\beta\) at Asp\(^{1}\) (4). Substitution of Met\(^{596}\) of APP with a Leu increases whereas a Val decreases cleavage rate by the \(\beta\)-secretase (5). Indeed, the Swedish variant of APP Lys\(^{595}\)-Met\(^{596}\) to Asn-Leu (APP\(_{SWE}\)) linked to Alzheimer’s disease is a better substrate for \(\beta\)-secretase (6, 7).

Sensitivity to alkalinizing agents indicates an acidic pH value optimum for \(\beta\)-secretase as found in Golgi-derived vesicles or in the endosomal/lysosomal compartment (8, 9). This is consistent with cellular studies demonstrating the involvement of the endocytic pathway in A\(\beta\) generation (10, 11).

Recently, BACE was identified as an enzyme having all the characteristics expected for \(\beta\)-secretase (12–16). The BACE gene is encoded on chromosome 11 (11q23.2) (17, 18) and was shown to generate transcripts whose expression is robust in pancreas, moderate in brain, and low in several peripheral tissues (12–14, 16). BACE is a type I transmembrane aspartyl protease of 501 amino acids (15). All six ectodomain cysteine residues appear to be involved in disulfide bonds tethering the catalytic domain in an atypical pattern for an aspartyl protease (19). Maturation of BACE occurs by propeptide removal and complex N-glycosylation at four Asn residues (12, 13, 19), both of which take place almost simultaneously after exit from the endoplasmic reticulum. Immunohistochemistry studies localized BACE at the same intracellular sites as APP, consistent with Golgi and endosomal distribution (12, 15, 16, 20). When expressed in cells, mature BACE has a half-life of more than 9 h (19).

BACE overexpression in cultured cells increases the generation of sAPP\(\beta\), C99, and A\(\beta\) (12–16). Notably, the amount of full-length APP remains unchanged under these conditions, whereas processing of APP by the nonamyloidogenic pathway is reduced, i.e. cleavage after Lys\(^{16}\) of A\(\beta\) by \(\alpha\)-secretase. In contrast, \(\beta\)-secretase antisense application leads to opposite effects (12, 14). Purified recombinant BACE cleaves more efficiently a substrate carrying the Swedish mutation when compared with the wild-type peptide (12, 14). The aspartyl protease inhibitor pepstatin, as well as several inhibitors for the other protease classes, were found inactive (12–14, 16). The pH value optimum in the slight acidic range (4.5–5.5) is consistent with the involvement of an intracellular acidic compartment in \(\beta\)-secretase processing (12–14, 16).

\(\textbf{In situ}\) hybridization studies demonstrated homogenous expression of the BACE mRNA in neurons of all brain regions, whereas glial cells appeared to express little or no BACE (12). Accordingly, BACE protein is detected in neurons but not glial cells (15). BACE enzymatic activity is extracted from human brain (13) in line with the observation that \(\beta\)-secretase activity is enriched in cells of central nervous system origin (21). In contrast, little or no BACE activity is detected in peripheral tissues including the human pancreas, although high expression of its mRNA is found in this tissue (13). Here we show that an endoplasmic reticulum-retained pancreatic form of BACE resulting from differential mRNA splicing is deficient in APP processing.
**EXPERIMENTAL PROCEDURES**

**Antibodies**—The rabbit polyclonal antisera GM190 (20), 815, and 818 were raised against synthetic antigens corresponding to peptides 22–45, 46–61 (with an additional carboxyl-terminal cysteine), and 484–501 of BACE501. Antisera 815 and 818 were affinity purified using commercially available reagents with the corresponding covalently coupled peptide. All antisera reacted equally well against BACE501 and BACE457. The monoclonal antibody β1 was raised as described previously (9). The mouse monoclonal antibodies 6E10 and α-GRP 78 are commercially available (Senetek; StressGen Biotechnologies Corp.).

**Polymerase Chain Reaction and Plasmid Construction**—The BACE501 and the BACE457 cDNAs were amplified by seminested polymerase chain reaction from a human brain cDNA library. For the first amplification, the primers BetaBHS2 (GGGATCCACCATGGCCCAAGCCC) and BetaR33 (GGGGAATTCACTTCAGCAGGGAGATGTGATGCATCAG) were used. For the second reaction, the primer pair BetaBHS2 (GGGATCCACCATGCGCAGAGGAGGATGTGATGCATCAG) and BetaR33 was chosen. Subsequently the cDNA fragments were subcloned into the BamHI/EcoRI sites of an appropriate vector for cytomegalovirus-regulated mammalian expression. For specific detection of BACE501 or BACE457, primers overlapping the respective exon/exon boundaries were chosen. Primers 501s (TGGCCAGGCTCAGGAGG) or 457s (GGGACCTGCCT) were used in combination with primer BetaR33 in a seminested polymerase chain reaction on human pancreas and brain cDNA libraries (CLONTECH), as well as on cDNA synthesized from total human pancreas poly(A)+ RNA (CLONTECH). Pancreatic cDNA and poly(A)+ RNA were obtained from different pools of human tissues. cDNA synthesis was performed with Molekum murine leukemia virus reverse transcriptase (Life Technologies, Inc.) using the random priming method according to the manufacturer’s protocol. The BACE501 and BACE457 plasmids were used as positive or negative template controls.

**Cell Extracts and Pancreas Homogenates for Western Blot Analysis**—Cultured cells were extracted 48 h post-transfection in RIPA buffer (10 mM Tris, pH 7.5, 150 mM sodium chloride, 1% Triton X-100, 1% sodium-deoxycholate, 0.5% SDS) containing protease inhibitors (Roche Molecular Biochemicals). Tissue samples from human cortex or pancreas were homogenized in homogenization buffer (10 mM Tris, pH 7.5, 150 mM sodium chloride, 1% EDTA) and centrifuged at 4 °C for 10 min at 10,000 × g. Subsequently the supernatants were centrifuged at 4 °C for 90 min at 100,000 × g. The new supernatants were discarded, and the pellets were resuspended in RIPA buffer by ultrasonic treatment. For PNGase F treatment pancreas homogenate or cell extracts were incubated with PNGase F overnight at 30 °C in 100 mM Tris/HCl, pH 7.5, 150 mM sodium chloride, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium-deoxycholate, 1% SDS) and centrifuged at 4 °C for 90 min at 100,000 × g.

**Cell Culturing, Transient Transfection, Metabolic Labeling, and Immunoprecipitation**—HEK-293 and COS-7 cells were cultured in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin. Transient transfections were performed using the SuperFect transfection reagent (Qiagen) according to the manufacturer’s protocol. For metabolic labeling, cells were starved for 30 min in Met/Cys-free DMEM (Sigma) supplemented with 5% dialyzed FCS and 1% penicillin/streptomycin. Subsequently, cells were pulse-labeled for 10 min with 100 μCi/ml [35]S-cysteine (Amersham Pharmacia Biotech). For Endo H (Roche Molecular Biochemicals) treatment, immunoprecipitated BACE501 or BACE457 was eluted from protein A-Sepharose with a 10-min, 100 °C treatment in 0.5% SDS, 1% β-mercaptoethanol. Each sample was split into two aliquots. Sodium citrate (to a final concentration of 50 mM, pH 5.5) and, if indicated, 5 milliunits of Endo H were added to the samples and incubated for 1 h at 37 °C.

**Immunocytochemistry**—Transfected cells were plated on poly-lysine-coated chamber slides (Costar). For immunostaining, cells were fixed in 3% paraformaldehyde or 5% methanol/acetic acid (95/5) at −20 °C. After blocking with 3% bovine serum albumin in phosphate-buffered saline, cells were incubated with the antibodies α-GRP 78 (anti-BiP) at 1:200 dilution, 815 at 1:500 dilution, and 818 at 1:2000 dilution. Alexa-488 and Alexa-594 secondary antibodies (Molecular Probes) were used at 1:500 dilution. Stained cells were embedded in Mowiol (Calbiochem).

**FIG. 1.** Expression of a differentially spliced BACE mRNA in the human pancreas. Shown are DNA fragments separated on an agarose gel and obtained by seminested polymerase chain reaction using BACE457-specific primers (left panel) and BACE501-specific primers (right panel) with total human brain or two different pancreas cDNAs as templates. The BACE501 plasmid, the BACE457 plasmid, and water (control) served as template in specificity control reactions. The migration of DNA size markers is given on the right.

**FIG. 2.** Schematic representation of the 44-amino acid deletion generating BACE457. Shown is the alignment of prorenin, pepsinogen A, and BACE with the location of the exon/exon boundaries for these asparyl proteases. The BACE501 open reading frame is distributed over nine exons of the indicated size in base pairs. Vertical dotted lines frame the 132 base pairs missing in BACE457 and leading to an in-frame deletion of 44 amino acids. The deletion is located between the two catalytic asparyl residues (D) and also eliminates two of the four N-glycosylation sites (asterisks) present in BACE501.
Bioinformatic Approach—To assemble the genomic sequence of BACE, component genomic fragments representing the BACE exons were identified by searching GenBank™ with the cDNA sequence. High-throughput genomic sequencing (HTGS) draft sequences AC020997 and AP001822 were found to include sequences representing the full-length cDNA. These entries were split into their component contigs. An iterative method alternating sequence search and assembly steps was then applied to these contigs to build the intervening introns.

RESULTS

To address the discrepancy observed in pancreas, i.e. efficient transcription of the BACE mRNA in the absence of recovered enzymatic activity, we searched for differential mRNA splicing. Initially, we amplified the BACE cDNA from a human brain cDNA library by nested polymerase chain reaction. Restriction mapping was performed with restriction enzymes expected to digest the full-length BACE cDNA. Using this strategy, we identified a DNA fragment that was not digested by NcoI and StuI (not shown). This suggested the existence of a BACE transcript lacking a fragment of at least 120 base pairs. We then designed several polymerase chain reaction primers specific for the region around the NcoI and StuI sites. Accordingly, we confirmed the existence of a differentially spliced BACE mRNA in human pancreas that was not detectable in human brain (Fig. 1). The amplification of the splice variant was confirmed using a cDNA pancreatic library, as well as using a distinct poly(A+) RNA pool. In contrast to this, the full-length form of BACE was expressed in brain but was not detected in pancreas (Fig. 1). Sequencing analysis indicated that 132 base pairs were missing in this cDNA compared with that of full-length BACE. To validate these findings, we screened the GenBank™ data base to gain information on the structure of the human BACE gene. We found that the BACE open reading frame was distributed over nine exons covering about 26 kilobases of the human genome (Fig. 2). The first intron accounted for about 18.6 kilobases. All other introns ranged in length between 1526 bases (intron 2) and 240 bases (intron 7). The 132 base pairs missing in the proposed differentially spliced mRNA appeared to match the terminal two-thirds of exon 3 (Fig. 2). Alignment of the exon/exon boundaries between several members of the human aspartyl protease family at the protein level showed no conserved pattern (Fig. 2). Interestingly, the only exon/exon boundary that was located in a conserved position corresponded to the 5' start of the differentially spliced intron. Lack of the 132 base pairs would cause an in-frame deletion of 44 amino acids in the full-length BACE (hereafter referred to as BACE457; see Fig. 2). Moreover, the deletion in BACE457 would eliminate in the full-length BACE (BACE501) two of the four consensus sites for N-glycosylation (Fig. 2). Based on this, BACE457 should migrate faster than BACE501 when separated by polyacrylamide gel electrophoresis. In fact, recombinant BACE457 expressed in HEK-293 cells was revealed as an immunoreactive band with a apparent molecular mass of 50 kDa using the specific antibody 815 raised against the amino terminus of BACE (Fig. 3A, upper panel, lane 2). In contrast, BACE501 migrated mainly as a diffuse band of about 65 kDa (Fig. 3A, upper panel, lane 1). Analysis of membrane protein isolated from three distinct human pancreatic samples revealed a specific immunoreactive protein comigrating with the recombinant BACE457 (Fig. 3A, lower panel).
BACE501 are transiently transfected with the BACE501 and BACE457 plasmids or mock-transfected with an empty vector. BACE501, expressed in HEK-293 cells overexpressing the human APP precursor protein, is retained in the early secretory pathway, the splice variant BACE457 is deficient in the processing of the amyloid precursor protein. HEK-293 cells overexpressing the human APP precursor protein were transiently transfected with the BACE501 and BACE457 plasmids or mock-transfected with an empty vector. BACE501 (lane 1), but not BACE457 (lane 2), caused an increase in the steady-state levels of C99, the processing product of APP generated by β-secretase, when compared with mock-transfected cells. C99 was visualized specifically after immunoprecipitation with β1 and immunoblotting with 6E10.

We have identified a differentially spliced form of BACE. The novel variant of BACE was found in the pancreas but was not detected in the brain. In contrast, we observed opposite expression pattern of full-length BACE. In the human pancreas, we demonstrate BACE457 expression to exist at the level of the endoplasmic reticulum. To further explore this hypothesis, we analyzed whether the propeptide of BACE457 was cleaved off, a process shown to occur after exit from the endoplasmic reticulum for BACE501. As expected (19, 22), propeptide removal and full glycosylation of BACE501 were completed in about 3.5 h when analyzed in a pulse-chase experiment (Fig. 4, upper panel). On the other hand, we found that virtually all of BACE457 remained in the proenzymatic form over a period of 16 h (Fig. 4, lower panel). The presence of the propeptide would reduce the length of BACE457 to 20 and not 44 amino acids when compared with BACE501. This may explain the small difference observed in the apparent molecular masses (48 and 46 kDa, respectively) between the two deglycosylated forms. Immunofluorescence studies revealed that BACE501 is present on the cell surface (Fig. 5, panel 1), as well as along the secretory pathway, i.e. in the endoplasmic reticulum and Golgi apparatus (Fig. 5, panels 2 and 3) when expressed in cells. Strengthening the evidence that BACE457 is retained in the early secretory pathway, the splice variant was not detected on the cell surface (Fig. 5, panel 4) but found exclusively in the endoplasmic reticulum where it colocalized with the specific marker BiP (Fig. 5, panels 2 and 3) when expressed in cells. Strengthening the evidence that BACE457 is retained in the early secretory pathway, the splice variant was not detected on the cell surface (Fig. 5, panel 4) but found exclusively in the endoplasmic reticulum where it colocalized with the specific marker BiP (Fig. 5, panels 2 and 3) when expressed in cells.

**DISCUSSION**

We have identified a differentially spliced form of BACE. The novel variant of BACE was found in the pancreas but was not detected in the brain. In contrast, we observed an opposite expression pattern of full-length BACE. In the human pancreas, we demonstrate BACE457 expression to exist at the level of the endoplasmic reticulum.
mRNA and at the protein level. Previous studies (12–15) have demonstrated transcription of BACE in pancreas in the absence of recovered enzymatic activity. The novel splice form suggests an explanation for this apparent discrepancy. BACE457 lacks 44 amino acids located between the two catalytic aspartyl residues and containing two of four putative glycosylation sites. Such a deletion can be expected to cause misfolding of the protein, explaining the observed retention within the endoplasmic reticulum, e.g. by a quality control mechanism. With the strategy of analysis chosen, we cannot exclude at this point that additional splice variants of BACE may exist.

Full glycosylation and activation by propeptide removal are post-translational modifications acquired during maturation and transport of BACE to a subcellular compartment where APP processing occurs. Failure to detect said modifications for the pancreatic variant is consistent with the observation that BACE457 was not found to contribute to the amyloidogenic process for a gene encoding an aspartyl protease, might indicate that the BACE gene is derived by convergent evolution and pepsinogen A, as well as the first description of differential splicing for a gene encoding an aspartyl protease family members (13) suggests an explanation for this apparent discrepancy.

An Inactive Pancreatic form of BACE

We also report the structure of the BACE gene. Both a novel pattern of exon/exon boundaries when compared with prorenin and pepsinogen A, as well as the first description of differential splicing for a gene encoding an aspartyl protease, might indicate that the BACE gene is derived by convergent evolution rather than by a gene duplication mechanism. The homology between BACE and the aspartyl protease family members (13) appears to span the open reading frame encoded by exon 2 to exon 8 and excluding exon 1 and exon 9. Exon 1 encodes the signal peptide, the propeptide, and the poorly conserved amino terminus of mature BACE. Exon 9 encodes the characteristic carboxyl terminus of BACE that includes the transmembrane spanning domain and the cytosolic tail.

The expression of an inactive form of BACE in pancreas, as opposed to having the gene repressed transcriptionally, suggests an unknown function of this form of BACE that may be independent of proteolytic activity. Alternatively, because propeptide removal may not be required for activity, a difference in substrate specificity between the two differentially spliced forms of BACE cannot yet be excluded.

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