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Characterization of Recombinant, Soluble β-Secretase from an Insect Cell Expression System

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ABSTRACT

The β-site amyloid precursor protein-cleaving enzyme (BACE) cleaves the amyloid precursor protein to produce the N terminus of the amyloid β peptide, a major component of the plaques found in the brains of Alzheimer's disease patients. Sequence analysis of BACE indicates that the protein contains the consensus sequences found in most known aspartyl proteases, but otherwise has only modest homology with aspartyl proteases of known three-dimensional structure (i.e., pepsin, renin, or cathepsin D). Because BACE has been shown to be one of the two proteolytic activities responsible for the production of the Aβ peptide, this enzyme is a prime target for the design of therapeutic agents aimed at reducing Aβ for the treatment of Alzheimer's disease. Toward this ultimate goal, we have expressed a recombinant, truncated human BACE in a *Drosophila melanogaster* S2 cell expression system to generate high levels of secreted BACE protein. The protein was convenient to purify and was enzymatically active and specific for cleaving the β-secretase site of human APP, as demonstrated with soluble APP as the substrate in novel sandwich enzyme-linked immunosorbent assay and Western blot assays. Further kinetic analysis revealed no catalytic differences between this recombinant, secreted BACE, and brain BACE. Both showed a strong preference for substrates that contained the Swedish mutation, where NL is substituted for KM immediately upstream of the cleavage site, relative to the wild-type sequence, and both showed the same extent of inhibition by a peptide-based inhibitor. The capability to produce large quantities of BACE enzyme will facilitate protein structure determination and inhibitor development efforts that may lead to the evolution of useful Alzheimer's disease treatments.

Alzheimer's disease (AD) is a neurodegenerative disorder that is characterized by neuronal loss in the brain and the presence of amyloid plaques and neurofibrillary tangles (Selkoe, 1991). The major components of amyloid plaque cores have been identified as two small peptide fragments derived from the amyloid precursor protein (APP), Aβ42 and Aβ40 (Glenner and Wong, 1984; Robakis et al., 1987; Tanzi et al., 1987; Miller et al., 1993). APP itself is a type I integral membrane protein with the Aβ segment, which begins at D672 in the longest isoform, spanning the boundary of the exocytoplasmic region (28 amino acids) and the transmembrane domain (12–14 amino acids). Aβ is generated from APP by the proteolytic activity of the enzymes β- and γ-secretase, which produce the amino- and carboxyl-terminal ends of Aβ, respectively (Fig. 1) (see Checler, 1995). β-Secretase cleavage also generates a soluble N-terminal fragment from APP (sAPPβ) (Seubert et al., 1993). Another enzyme, α-secretase, cleaves APP at a position within the Aβ sequence to produce a soluble APPα (sAPPα) (Esch et al., 1990). During the course of AD, Aβ produced by the enzymes β- and γ-secretase accumulates extracellularly in vivo and forms large, insoluble amyloid fibrils that elicit both cytotoxic and inflammatory responses after deposition in the brain (Cummings et al., 1996; Yankner, 1996). Thus, understanding the enzymes responsible for the production of this toxic peptide is crucial to finding a therapeutic intervention point in AD (Selkoe, 1997; De Strooper and Konig, 1999).

Whereas several studies suggested that known proteases like cathepsin D or caspase had activity similar to β-secretase, it remained unclear whether these proteases were directly responsible for the overproduction of Aβ found in AD

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ABBREVIATIONS: AD, Alzheimer's disease; APP, amyloid precursor protein; Aβ, amyloid β peptide (those mentioned herein consist of 40 or 42 amino acids); sAPP, soluble APP; BACE, β-site amyloid precursor protein cleaving enzyme; ELISA, enzyme-linked immunosorbent assay; HPLC, high-pressure liquid chromatography; AChE, acetylcholinesterase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; TBS-T, Tris-buffered saline/Tween-20; HRP, horseradish peroxidase; CHO, Chinese hamster ovary; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DNP, dinitrophenol.

(Chevallier et al., 1997; Gervais et al., 1999). Recently, however, using genomic database searching, expression cloning, and basic biochemical methods, the protein corresponding to the β-secretase enzyme has been identified conclusively (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999; Lin et al., 2000). The gene for human β-site APP-cleaving enzyme (BACE, also called Asp 2 and Memapsin 2) encodes a 501-residue protein with an N-terminal signal sequence of 21 amino acids followed by a pro-protein domain consisting of residues 22 to 45 (Vassar et al., 1999) that is proteolytically removed to generate the mature BACE. BACE is also an integral membrane protein that contains a predicted transmembrane domain of 17 residues followed by a short cytosolic C-terminal tail of 24 amino acids (Fig. 1). Sequence analyses indicated that BACE belongs to a subfamily of both membrane-bound and soluble proteases and contains the classical consensus active site motif found in aspartyl proteases (D T/S G T/S) at positions 93 to 96 and 289 to 292. The entire BACE sequence displays only mild homology with currently known aspartyl proteases (approximately 30% identity and 37% similarity with members of the mammalian pepsin family), with the highest homology found in the central portion of the extracellular domain of BACE. A variety of experimental evidence, including enzyme overexpression in tissue culture, mutation of the active site aspartates, antisense reduction of enzyme expression, and in vitro assays with various synthetic APP fragments or substrate peptides, confirmed that this enzyme was indeed responsible for APP processing at the β-site (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999; Lin et al., 2000).

With the discovery and initial characterization of BACE, researchers now have a clearly defined target for the design of therapeutic drugs for AD. In theory, drugs that reduce or block BACE activity would lower Aβ levels in the brain and thus slow the formation of amyloid plaques and the progression of AD (Yankner, 1996; De Strooper and Konig, 1999). However, a great deal of information concerning the structural and functional features of this enzyme is still required to reach the ultimate goal of specific drug design. To obtain this information, we have developed a *Drosophila melanogaster* S2 expression system for the production of a secreted form of recombinant human β-secretase (SecBACE) in quantities sufficient for structural resolution by X-ray crystallography and biochemical analysis in functional assays. This system has been previously used for the production of *D.*

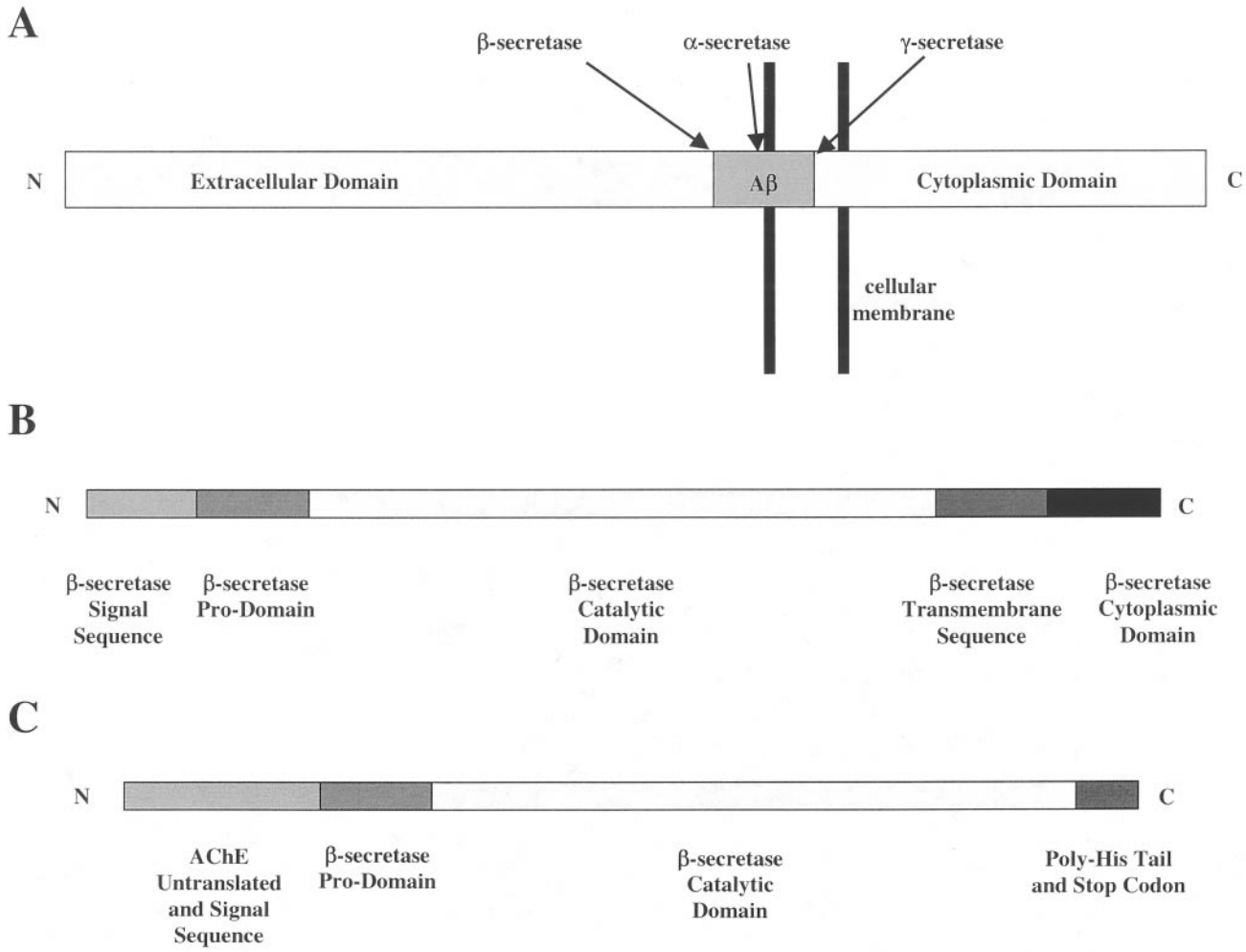

Fig. 1. Schematic of APP and β-secretase proteins. A, structure of APP as found in the cell membrane with secretase cleavage sites and Aβ sequence indicated. B, structure of wild-type β-secretase. C, structure of recombinant SecBACE containing the β-secretase catalytic domain truncated before the transmembrane segment of the enzyme. The AChE untranslated DNA, signal sequence, His-tag and stop codon were incorporated using PCR methods (see *Experimental Procedures*).

melanogaster acetylcholinesterase (AChE), human AChE, and an immunoglobulin fragment fusion protein (Incardona and Rosenberry, 1996; Eckman et al., 1999; Mallender et al., 1999; Harel et al., 2000). Our results from a novel ELISA-based assay and an HPLC-based kinetic assay for β-secretase activity demonstrated that SecBACE protein purified from tissue culture media was enzymatically active and capable of cleaving the β-secretase sequence within APP.

Experimental Procedures

Recombinant Human β-Secretase. A fragment of human BACE cDNA (~1.6 kilobases) was amplified from a human brain cDNA library (Edge Biosystems, Gaithersburg, MD) with PCR primers (obtained from Life Technologies, Rockville, MD; forward primer AGCTCCCTCTCCTGAGAAG and reverse primer TCAGTGGTGGTGGTGGTGGTGCTTCAGCAGGGAGATGTC) and Pfu polymerase (Stratagene, LaJolla, CA). From this BACE cDNA, which contained the entire BACE protein coding domain, a further truncated cDNA was amplified that corresponded to a recombinant, secreted form of human BACE, SecBACE. This amplification used primers that annealed to regions in the BACE pro-domain and near the transmembrane segment and Pfu polymerase (Fig. 1). Specifically, the primer for the 5′ end of SecBACE consisted of a short segment of 5′ untranslated DNA and signal sequence from the human AChE gene fused to the 5′ end of the BACE pro-domain. This primer was prepared by PCR amplification of the AChE DNA sequence with a 3′ primer that included the 5′ end of the BACE pro-domain as an overhanging 3′ sequence. This PCR product was purified by low-melting temperature agarose gel electrophoresis (FMC, Rockland, ME). For amplification from the 3′ end of SecBACE, a primer was designed that would truncate the enzyme upstream from the transmembrane segment (residue Ala459) and insert a six-residue His-tag, translation stop codon and *Nhe*I cut site. These primers amplified a 1.4-kilobase SecBACE construct that was cloned into *Sma*I-digested pTZ18u (Mead et al., 1986). The SecBACE gene cassette identity and sequence was confirmed by DNA sequencing carried out at the Mayo Clinic Molecular Core Facility (Rochester, MN). The SecBACE gene cassette was cut out of pTZ18u as an *Eco*RI-*Nhe*I fragment and cloned into *Eco*RI (partial digest)-*Nhe*I digested pTZ18u containing the human AChE Asp718 gene cassette from pPacSecHuman (Mallender et al., 1999). Cloning of the SecBACE fragment into this vector fused the SecBACE sequence with the *D. melanogaster* AChE 3′ untranslated DNA normally used in our S2 cell expression system (Eckman et al., 1999; Mallender et al., 1999). The reconstituted SecBACE gene cassette was cloned into the pPac expression vector as an Asp718 fragment. After confirmation of cassette identity and orientation by restriction endonuclease digestion, pPacSecBACE plasmid DNA was transfected into *D. melanogaster* S2 cells using previous protocols (Eckman et al., 1999; Mallender et al., 1999, 2000). S2 cells were cotransfected with the pPacHph vector that encodes the gene for hygromycin B resistance. After several weeks of selection with hygromycin B, monoclonal S2 cell lines were established using soft agar cloning techniques that are described elsewhere (Eckman et al., 1999; Mallender et al., 1999; Mallender et al., 2000). Individual cells lines were weaned into *D. melanogaster* Serum-Free Media (Life Technologies, Gaithersburg, MD) supplemented with L-glutamine and appropriate antibiotics.

Purification of Secreted SecBACE from Insect Cell Culture Media. After accumulation in tissue culture media for 10 to 14 days, the expressed SecBACE was purified by passage over a nickel-agarose affinity column (Ni-agarose; Qiagen, Valencia, CA) with a modified version of the protocol recommended for purification under nondenaturing conditions. Briefly, tissue culture media was dialyzed extensively against 20 mM sodium phosphate buffer, pH 8.0, 300 mM sodium chloride, and 10 mM imidazole (buffer 1) and passed over a Ni-agarose column that was pre-equilibrated with buffer 1. The column was then washed with the following buffers: buffer 1, buffer 2, 20 mM sodium phosphate, pH 8.0, 1 M sodium chloride, 1% Triton X-100, and 10 mM imidazole; and buffer 3, 20 mM sodium phosphate, pH 6.5, 300 mM sodium chloride, and 10 mM imidazole. The purified SecBACE protein was eluted with Buffer 1 containing 250 mM imidazole. SecBACE protein was further purified by ion exchange chromatography on a Bio-Rad (Hercules, CA) BioScale Q5 column. Chromatography was carried out in 20 mM HEPES buffer, pH 7.4, with a sodium chloride elution gradient. Protein concentrations of purified SecBACE fractions were determined with a bicinchoninic acid assay kit (Pierce, Rockford, IL).

SDS-PAGE and Western Blot Analysis of SecBACE Protein. Analysis of SecBACE protein in cell culture media, initial SecBACE purified fractions (from Ni-agarose) or completely purified material was carried out by SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). After electrophoresis, proteins were either visualized with SyproRuby fluorescent stain (Molecular Probes, Eugene, OR) or transferred to PVDF membranes (Immobilon P; Millipore, Bedford, MA) using a Protein II Mini Trans-Blot cell apparatus (Bio-Rad, Hercules, CA). After blocking with 5% newborn calf serum/5% nonfat dry milk in TBS-T (50 mM Tris, pH 8, 150 mM sodium chloride, 0.05% Tween-20), the blots were probed with anti-His tag antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) diluted 1:10,000 in TBS-T with 5% newborn calf serum. After extensive washing with TBS-T, bound antibody was detected with HRP-labeled goat anti-mouse antibodies (Life Technologies, Rockville, MD) and visualized with the Super Signal WestDura Extended Duration Substrate kit (Pierce). Fluorescent (from SDS-PAGE gels) or chemiluminescent (from Western blots) bands were detected and recorded using a FluorChem Digital Imaging System (Alpha Innotech, San Leandro, CA).

Amino Acid Sequence Analysis of SecBACE. N-terminal sequence analysis of purified SecBACE protein was carried out at the Mayo Clinic Rochester Protein Core Facility. Protein samples for amino acid analysis were run on SDS-PAGE gels and transferred to PVDF membranes (see above). After visualization, protein bands were cut from the blots and subjected to sequencing.

Assay of SecBACE Enzymatic Activity by ELISA. SecBACE activity was monitored using sAPP and Aβ sequence-specific antibodies in a sandwich ELISA assay. ELISA wells (Maxisorp Immuno Plates; Nunc, Naperville, IL) were precoated with polyclonal goat antibody reagent 207 [20 μg/ml in 0.1 M sodium carbonate buffer (Suzuki et al., 1994)] and blocked with 1% Block Ace in PBS (50 mM phosphate buffer, pH 8, 150 mM sodium chloride), respectively. Antibody reagent 207 recognizes the extracellular domain of soluble APP and thus serves as a capture antibody for all forms of APP (α- or β-secretase cleaved soluble APP). Soluble APP was obtained from transiently tranfected CHO cell lines produced with FuGene6 (Roche, Nutley, NJ) transfection reagent (using manufacturer suggested conditions) and a pAG3 vector containing the APP 695wt-HIS tag construct (Murphy et al., 1999). Transfected CHO cells were transferred to serum-free media CHO-S-SFM II (Life Technologies) and sAPP was allowed to accumulate for 48 h before collection and assay. Samples of SecBACE protein were incubated with sAPP in BACE reaction buffered (100 mM sodium acetate, pH 4.5; 100 mM sodium chloride; 0.06% Triton X-100) for 12 to 18 h at 37°C, after which the reactions were stopped by the addition of an equal volume of 100 mM Tris base, pH 12. Aliquots (200 μl) were transferred to precoated ELISA wells containing 50 μl of EC buffer [20 mM sodium phosphate, 2 mM EDTA, 0.4 M sodium chloride, 0.2% BSA, 0.05% CHAPS, 0.4% Block Ace, 0.05% sodium azide (Suzuki et al., 1994)]. After overnight incubation at 4°C, wells were washed extensively with PBS and bound sAPPα was detected with the BAN50 antibody (Takeda, Japan, Suzuki et al., 1994) that recognizes the N-terminal portion of Aβ and thus can recognize the C terminus of sAPPα. After extensive PBS washing, bound BAN50 antibody was detected with HRP-labeled goat anti-mouse $F(ab)_2$ antibodies (Amersham Pharmacia Biotech) and TMB Microwell Peroxidase Substrate System (KPL,

Gaithersburg, MD). Developed ELISA plates were read on a SpectraMAX plus microplate reader (Molecular Devices, Sunnyvale, CA). This assay may slightly underestimate the extent of sAPPα cleavage to sAPPβ because a small fraction of antibody 207 may recognize the 16-residue peptide also produced by this cleavage.

A similar method for assessing SecBACE activity involved a Western blot protease assay. After incubation of SecBACE and sAPPα as described above, samples were run on SDS-PAGE, transferred to PVDF membranes, and probed with 207 and BAN50 antibodies to determine the amounts of total sAPP and sAPPα present in each sample, respectively (as outlined in the previous section). Bound 207 and BAN50 antibodies were visualized with HRP-labeled rabbit anti-goat antibodies (DAKO, Ltd., Denmark) or HRP-labeled goat anti-mouse F(ab)$_2$ antibodies (respectively) and an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech). This SecBACE assay was carried out in the presence and absence of a 1x concentration of complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN).

Assay of SecBACE Enzymatic Activity by HPLC. All peptides used in this study were synthesized by QCB (Hopkinton, MA). Their purity (>95%) and identity were verified by a combination of reverse-phase HPLC and mass spectral analysis. To monitor SecBACE activity by HPLC, four peptide substrates were prepared representing 9- and 29-residue peptides centered about the cleavage sites of the wild-type and Swedish mutant versions of APP (Citron et al., 1992; Mullan et al., 1992; Cai et al., 1993). To monitor reaction progress, each peptide incorporated a dinitrophenol (DNP) moiety appended to the ϵ-amino group of the lysine residue at P8′. As reported previously with other BACE constructs, the wild-type APP peptides were poor substrates, requiring large amounts of enzyme and prolonged incubation times to measure product formation. In contrast, the Swedish mutant peptides, where NL is substituted for KM immediately upstream of the cleavage site, were much better substrates and were therefore used for detailed kinetic analysis. The sequences for the long (29 amino acids) and short (9 amino acids) Swedish mutant peptides were as follows: AcTTRPGSGLTNIKTEEISEVNL-DAEFRHDK(DNP) and AcEVNL-DAEFK(DNP), where a hyphen in N-D marks the cleavage site. Reactions were performed at 25°C in 50 mM sodium acetate, pH 4.5, containing 0.25 mg/ml BSA, variable concentrations of the substrate (as indicated in the text and figure captions) and 30 nM recombinant enzyme. In initial experiments, aliquots of the reaction mixture were removed at different time points (to assure the linearity of the reaction course), boiled to stop the reaction, and injected onto a C18 Bondpack reverse phase column (Waters Millipore, Milford, MA). The substrate and product peaks were separated by 2 min with a 30-min linear gradient of 0 to 100% acetonitrile with 0.1% of trifluoroacetic acid. Cleavage of the correct peptide bond by SecBACE was confirmed by collecting the product peak and subjecting this to LC-MS analysis. Reaction time courses were linear for at least 20 min under all conditions tested. Therefore, all subsequent experiments were performed with a single reading after a 15-min reaction time.

Inhibition studies were performed at a substrate concentration of 20 μM and 1–10,000 nM concentrations of the statine-based inhibitor Ac-KTEEISEVN(Statine)VAEF-COOH previously reported (Sinha et al., 1999). The IC_{50} value was calculated using the Langmuir isotherm equation (Copeland, 2000),

$$\% \text{ Inhibition} = 100\left(1 - \frac{A_i}{A_0}\right) = \frac{100}{1 + \left(\frac{IC_{50}}{[I]}\right)^n}$$

where A_i and A_0 are product peak areas in the presence and absence of inhibitor, respectively, [I] is the inhibitor concentration, IC_{50} is the inhibitor concentration resulting in 50% inhibition, and n is the Hill slope.

Results

Production of SecBACE Protein from *D. melanogaster* S2 Cells. The SecBACE construct was prepared from the BACE coding region by addition of the AChE signal sequence, truncation before the BACE transmembrane segment, and addition of the C-terminal His-tag and stop codon with both PCR and conventional cloning techniques. After insertion of this construct into the pPac *Drosophila* expression vector and selection of stable SecBACE transfectants, protein was allowed to accumulate in the culture media for 10 to 14 days before analysis. Initial cultures were grown in media supplemented with 10% FBS, but the presence of serum proteins interfered with Western blotting of media samples and contributed to protein impurities after preliminary Ni-agarose purification experiments (data not shown). Eventual transfer of the cells into serum-free media eliminated these difficulties. SDS-PAGE and Western blots probed with anti-His antibodies indicated that SecBACE was a 56- to 57-kDa protein (Fig. 2). After Ni-agarose affinity chromatography, SDS-PAGE and Western blot analyses showed that SecBACE was greater than 75% pure. Further purification of SecBACE (>95% pure by SDS-PAGE and Western blot) was achieved by ion exchange chromatography with a multistep sodium chloride elution gradient (Fig. 3). N-terminal amino acid analysis of the 56- to 57-kDa protein band revealed that it consisted of a mixture of properly processed, mature SecBACE (~60%) and pro-SecBACE (~40%) that retains the pro-protein domain (data not shown).

Proteolytic Activity of SecBACE. SecBACE activity was analyzed with sAPP as a substrate and APP sequence-specific antibodies in two different assay formats that assessed the amount of cleavage at the β-secretase recognition site. In the first assay, a sandwich ELISA protocol was used to detect the reduction in sAPPα after incubation with samples containing SecBACE. Any reduction in signal in this assay represents proteolytic removal of the C-terminal 16-residue segment between the β- and α-secretase cleavage sites. Because the BAN50 antibody recognizes only this segment, it cannot detect sAPPβ on the ELISA wells precoated with antibody 207. This assay confirmed that tissue culture media containing SecBACE and purified SecBACE fractions from Ni-agarose columns and ion exchange columns were effective at recognizing and cleaving APP (Fig. 4A). In the second assay, the same sequence-specific antibodies were used to monitor SecBACE-mediated cleavage of APP in a Western blot assay. After incubation of SecBACE and sAPP, samples were run on SDS-PAGE, transferred to PVDF membranes, and probed with either the 207 antibody reagent or BAN50. Incubation with SecBACE did not cause a reduction in the amount of total APP detected with the 207 antibody, ruling out any nonspecific proteolysis of the substrate protein. Results with BAN50 (specific for the C terminus of APPα) showed that SecBACE reduced the amount of sAPPα in a concentration-dependent manner (Fig. 4B). Collectively, these results confirmed that SecBACE is proteolytically active and specific for the APP β-secretase cleavage site.

Specific SecBACE activity was measured in an HPLC assay with APP Swedish mutant peptides of two different lengths. No saturation was observed with either of the substrates at concentrations up to 40 μM, suggesting high K_m values for both substrates under the current conditions. Val-

ues of k_{cat}/K_m were calculated from the slopes of the linear substrate-concentration dependence plots (Fig. 5A), yielding estimates of $(3.0 \pm 0.2) \times 10^4$ M^{-1} s^{-1} and $(1.3 \pm 0.1) \times 10^4$ M^{-1} s^{-1} for the 29- and 9-amino acid substrates, respectively. The lack of significant difference in specific activities using different length peptides suggests that most of the effective interactions in the enzyme-substrate complex do not extend beyond the P4-P5′ site. Our value of k_{cat}/K_m for the 29-residue substrate is in good agreement with a specific activity of 900 nmol/min/mg reported for similar substrate with a purified BACE-IgG fusion construct expressed in human 293T cells (Vassar et al., 1999). Assuming a molecular mass of 57 kDa for our secBACE and a substrate concentration of 30 μM (comparable with that used by Vassar et al. (1999)), our k_{cat}/K_m of 3×10^4 M^{-1} s^{-1} corresponds to a specific activity of 950 nmol/min/mg.

SecBACE is inhibited by the statine-based inhibitor described previously (Sinha et al., 1999) with IC_{50} values of 43 ± 4 and 51 ± 6 nM (Fig. 5B) in assays using the 29- and 9-residue substrates, respectively. These values are in good agreement with the value of 30 nM reported for BACE purified from human brain (Sinha et al., 1999), suggesting that neither truncation of the enzyme at the C terminus nor potential differences in post-translational modification between mammalian and insect expression systems (e.g., glycosylation) impact significantly the enzymatic properties of human BACE.

Discussion

In this article, we report the production and initial characterization of a recombinant, secreted form of human β-secretase

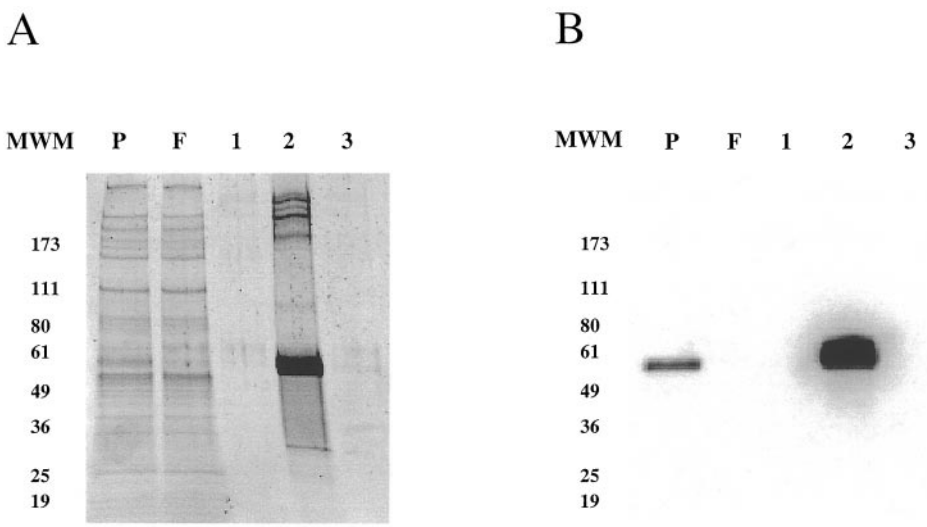

Fig. 2. Ni-agarose purification of SecBACE from S2 cell culture media. A, SDS-PAGE gel of SecBACE samples accumulated in serum-free culture medium before and after purification by Ni-agarose. Lane MWM, molecular mass standards in kilodaltons; P, media onput; F, media flow through over column; 1 to 3, high imidazole elution fractions containing purified SecBACE following washing protocol (see *Experimental Procedures*). The gel was stained for total protein. B, Western blot of an identical SDS-PAGE gel probed with antibodies specific for His-tagged proteins.

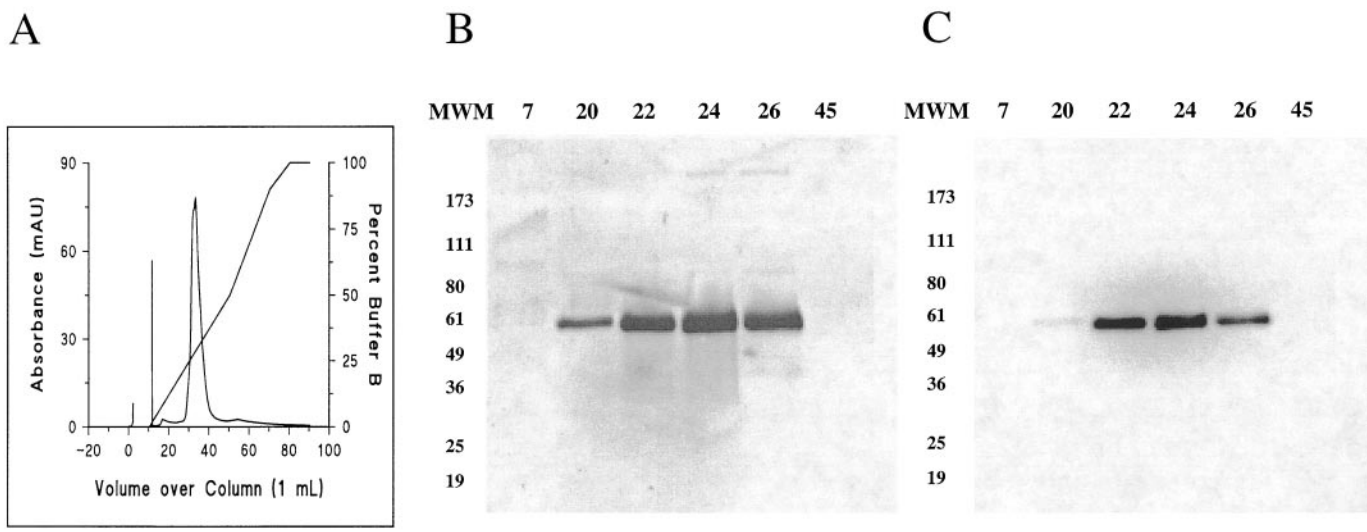

Fig. 3. Ion-exchange FPLC purification of Ni-agarose-purified SecBACE. A, SecBACE purified on Ni-agarose as in Fig. 2 was subjected to anion exchange chromatography. The percentage buffer B (0.5 M sodium chloride) and protein absorbance (mAU) values are displayed for 1-ml fractions eluting from the column. B, samples taken from the indicated fractions in A were run on SDS-PAGE, transferred to PVDF membranes, and stained for protein with SYPRO Ruby. C, the blot in B was probed with antibodies specific for His-tagged proteins. FPLC purification yielded nearly completely pure preparations of SecBACE protein.

enzyme, SecBACE, produced from a *D. melanogaster* S2 expression system. This enzyme plays a key role in the pathogenesis of AD, and information is needed on its structural and functional characteristics if specific inhibitors with therapeutic potential are to be designed. Our expression system, previously used for the high-level expression of other recombinant proteins (Incar-

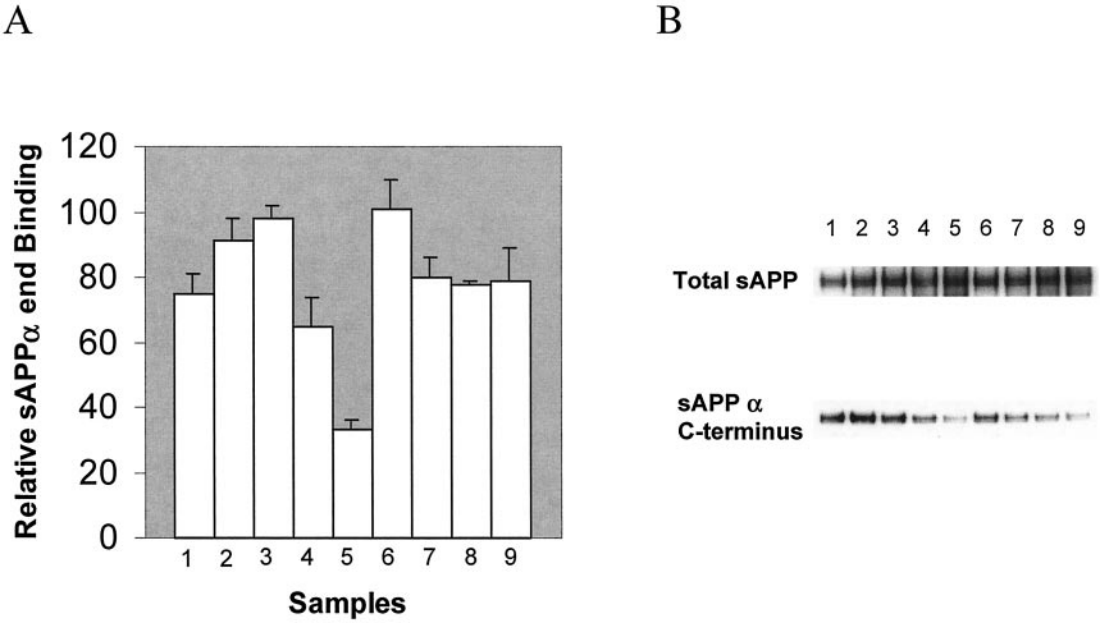

Fig. 4. Proteolytic activity of SecBACE using sAPP as a substrate. A, sandwich ELISA assay detecting active SecBACE in fractions from Ni-agarose and FPLC purifications. Reduction in total sAPPα captured on 207 antibody-coated ELISA wells after incubation with various SecBACE samples was quantified with the BAN50 antibody (specific for the C terminus of sAPPα or the N terminus of Aβ). Samples 1 to 5 correspond to media onput, media flow although, high salt wash, and 250 mM imidazole elution fractions 1 and 2 (respectively) from a Ni-agarose purification of SecBACE. Sample 6 consists of a reaction containing only sAPP and thus serves as the control for the experiment. Samples 7 to 9 correspond to equal amounts of protein from peak fractions of FPLC-purified SecBACE from elution fraction 2 (sample 5). Only samples in which recombinant SecBACE was detected by SDS-PAGE and Western blot showed the ability to cleave sAPP. B, Western blot assay detecting SecBACE cleavage of sAPPα specifically at the β-secretase cleavage site. Similar to the assay above, reaction samples composed of sAPP incubated with SecBACE were run on SDS-PAGE gels and transferred to PVDF membranes. Blots were probed with either antibody 207 (detecting Total sAPP) or BAN 50 (detecting sAPPα C-terminus). Lane 1 contains a sAPP-only control reaction. Lanes 2 to 5 contain sAPP incubated with increasing amounts of purified SecBACE protein (approximately 1, 2, 5, and 10 μg). Lanes 6 to 9 contain samples identical to those in lanes 2 to 5, except that these samples also were incubated with a complete protease inhibitor cocktail specific for many aspartyl, serine, cysteine, and metallo proteases, demonstrating the relative insensitivity of SecBACE to many known protease inhibitors.

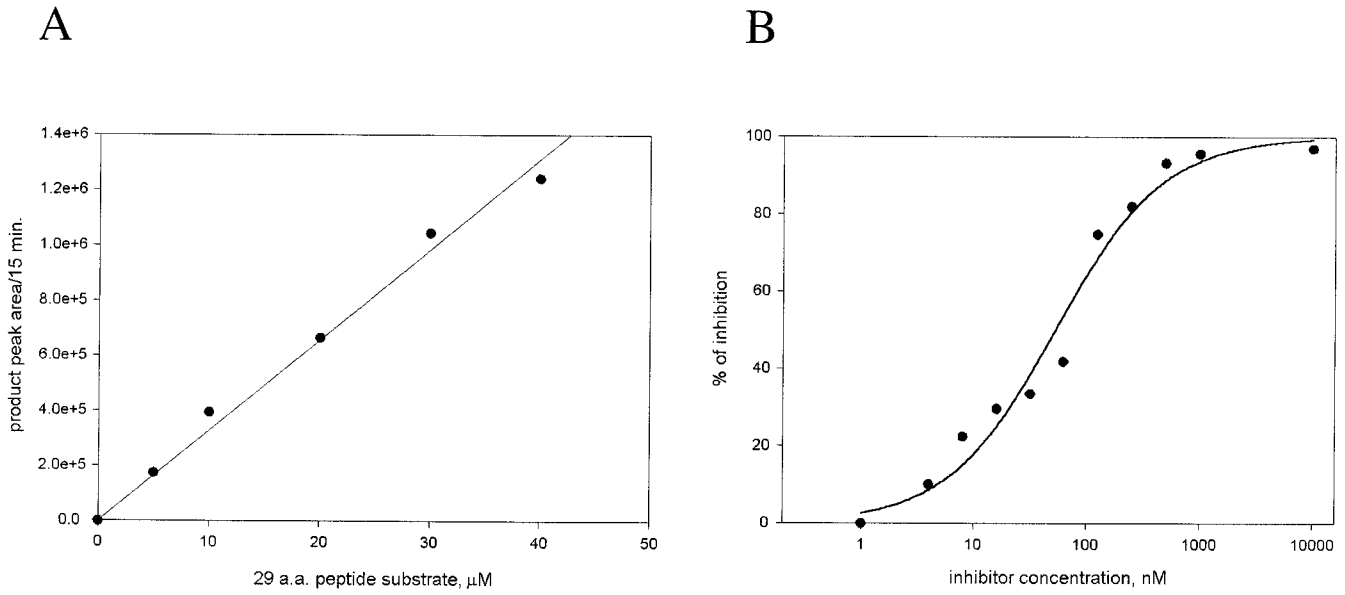

Fig. 5. Kinetic analyses of SecBACE. A, velocity of product formation as a function of substrate (29-residue peptide) concentration for SecBACE-catalyzed peptide cleavage. Conditions: 30 nM SecBACE in 50 mM acetate buffer, pH 4.5, with 0.25 mg/ml BSA and the indicated concentrations of peptide substrate. B, inhibition of the β-secretase activity of SecBACE by a statine-based inhibitor. The data shown were generated using the 29-residue peptide substrate at a constant concentration of 20 μM. The curve drawn through the data is the nonlinear least-squares fit to eq. 1 and yields the following fitting parameters: IC_{50}, 51 $\pm$ 6 nM; Hill slope, 0.9 $\pm$ 0.1.

dona and Rosenberry, 1996; Eckman et al., 1999; Harel et al., 2000; Mallender et al., 1999, 2000), yields between 2 and 5 mg of purified SecBACE per liter of medium after 12 days of continuous culture. This quantity of enzyme is essential to support future studies focused on the development of BACE specific inhibitors (i.e., kinetic analyses, structure determination, high-throughput screening of inhibitors). Initially, a 56- to 57-kDa band was identified from Ni-agarose purification fractions that were reactive with anti-His antibodies in Western blots. Based on the primary sequence of SecBACE, a 51-kDa protein was expected. The BACE sequence, however, does contain four putative N-glycosylation sites (Asn153, Asn172, Asn 223, and Asn354) that are all present in the SecBACE construct. Insect cells have been shown to be proficient in glycosylating recombinant proteins from other species, often with the fucosylated paucomannosidic structure $(GlcNAc)_2(Mannose)_3Fucose$ (1039 Da) (Rudd et al., 2000), and glycosylations of this size have been observed specifically for human AChE produced from our S2 cell system (Israel Silman, personal communication). N-glycosylation of all 4 sites in SecBACE produced from *D. melanogaster* with structures of a similar size would result in a 4-kDa increase in apparent protein size on SDS-PAGE. Glycosylation of these sites in BACE produced from mammalian cell lines was recently confirmed (Haniu et al., 2000). Additionally, amino acid sequence analysis of SecBACE indicated that the protein was not completely processed and that a fraction of the secreted material retained the enzyme pro-domain. It is possible that the sequence or structure of the SecBACE pro-domain reduces the ability of *D. melanogaster* pro-domain convertase enzymes to properly process the enzyme to its mature form. Unlike BACE produced in *Escherichia coli* in a previous study (Lin et al., 2000), incubation of SecBACE protein at acidic pH for up to 24 h had no effect on the amount of pro-SecBACE protein present (data not shown).

Proteolytic activity was initially assessed using sAPPα as the target substrate. Other studies have used either synthetic peptides (Vassar et al., 1999; Yan et al., 1999; Lin et al., 2000;) or recombinant APP-reporter protein fusion constructs (Sinha et al., 1999) as substrates in in vitro BACE activity assays. Our assay format has the advantage that sAPP is a more physiologically relevant substrate, and it is amenable to both kinetic studies and high-throughput screening methods. With the Western blot format of this assay, we confirmed the ability of SecBACE to cleave sAPPα without further proteolysis of the APP substrate. Indeed, both assays serve as direct evidence that a purified recombinant BACE protein is capable of proteolysis of wild-type sAPPα into sAPPβ in a defined in vitro environment. Furthermore, a protease inhibitor cocktail specific for all known classes of proteases (aspartyl, serine, cysteine, and metallo) was unable to block SecBACE activity in this assay format. Despite its sequence classification as an aspartyl protease, this result replicates the finding of others that BACE must be a unique protease because of its broad spectrum inhibitor insensitivity (Yamazaki and Ihara, 1998; Vassar et al., 1999).

More detailed kinetic studies using short synthetic peptides revealed that SecBACE expressed from insect cells catalyzes β-specific cleavage with a strong preference for substrates incorporating the Swedish mutations of APP. The affinity of our recombinant enzyme for a statine-based peptide inhibitor is comparable with that of native enzyme purified from brain (Sinha et al., 1999), confirming that the BACE active site structure is faithfully recapitulated in the soluble, recombinant enzyme. Thus, the conformation of the extramembrane globular domain of BACE seems to be minimally affected by removal of the membrane-anchoring domain of the natural enzyme. The rate of substrate turnover (k_{cat}/K_m) is about 10-fold higher for SecBACE than for a similar construct that was expressed in *E. coli* and refolded from insoluble inclusion bodies (Lin et al., 2000). The discrepancy between the kinetics of the *E. coli*- and insect-expressed enzymes may reflect slight differences in the substrates used [one additional amino acid at the N terminus and underivatized R residue instead of K-(DNP) at the C-terminus in the substrate used for the *E. coli* expressed enzyme]; more probably, however, it reflects possible complications during protein refolding that diminish the fraction of active enzyme in the final samples (Lin et al., 2000). Additionally, we have found that, like many enzymes, purified BACE has a propensity to adsorb to the surfaces of vessels, such as microtiter plate wells, and become inactivated (data not shown). The inclusion of 0.25 mg/ml BSA in our reaction buffer ameliorates this problem. This effect may also contribute to the differences in apparent activity observed between our enzyme and the *E. coli*-expressed enzyme reported earlier (Lin et al., 2000).

In an attempt to gain some information concerning the putative structure for the BACE catalytic domain, homology modeling studies were undertaken using the SWISS-MODEL server (http://www.expasy.ch/swissmod/SWISS-MODEL.html) and Swiss-PDBViewer 3.5 software (Glaxo Wellcome Experimental Research, United Kingdom) (Peitsch, 1995, 1996; Guex and Peitsch, 1997) (data not shown). In agreement with previous reports on modeling of BACE, our homology models of the BACE catalytic domain conformed reasonably well with the overall protein fold of the pepsin family of aspartyl proteases (Huang et al., 2000; Sauder et al., 2000). However, the six Cys residues in the catalytic domain of BACE were not all able to form disulfide bonds in our models, probably because BACE shows poor sequence homology with the pepsin family of proteases. This structural uncertainty has recently been overcome, because the three-dimensional structure of BACE has been determined using molecular replacement methods with human pepsin as the search model (Hong et al., 2000).

In conclusion, this report summarizes our progress on the development of a high-level expression system for the production of secreted, human β-secretase and the initial characterization of the recombinant protein, SecBACE. The role of this enzyme in normal physiological function has yet to be resolved. Nevertheless, BACE remains an attractive target for intervention in the amyloid cascade and pathogenesis of AD. Our ability to produce and purify large quantities of active enzyme will lead to additional detailed structural studies. When complemented with analyses of enzyme reaction kinetics, substrate specificity, and computer models, these studies will permit the design of enzyme-specific inhibitors that may serve as an amyloid-reducing therapy for the treatment of AD.

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