Dimerization of β-Site β-Amyloid Precursor Protein-cleaving Enzyme*

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Cleavage of the β-amylloid precursor protein (APP) by the aspartyl protease β-site APP-cleaving enzyme (BACE) is the first step in the generation of the amyloid β-peptide, which is deposited in the brain of Alzheimer’s disease patients. Whereas the subsequent cleavage by γ-secretase was shown to originate from the cooperation of a multicomponent complex, it is currently unknown whether in a cellular environment BACE is enzymatically active as a monomer or in concert with other proteins. Using blue native gel electrophoresis we found that endogenous and overexpressed BACE has a molecular mass of 140 kDa instead of the expected mass of 70 kDa under denaturing conditions. This suggests that under native conditions BACE exists as a homodimer. Homodimerization was confirmed by co-immunoprecipitation of full-length BACE carrying different epitope tags. In contrast, the soluble active BACE ectodomain was exclusively present as a monomer both under native and denaturing conditions. A domain analysis revealed that the BACE ectodomain dimerized as long as it was attached to the membrane, whereas the cytoplasmic domain and the transmembrane domain were dispensable for dimerization. By adding a KXXX-endoplasmic reticulum retention signal to BACE, we demonstrate that dimerization of BACE occurs already before full maturation and pro-peptide cleavage. Furthermore, kinetic analysis of the purified native BACE dimer revealed a higher affinity and turnover rate in comparison to the monomeric soluble BACE. Dimerization of BACE might, thus, facilitate binding and cleavage of physiological substrates.

Current evidence strongly supports that generation, aggregation, and deposition of amyloid β-peptide (Aβ)1 in brains of Alzheimer’s disease (AD) patients is an invariant pathological feature of this devastating neurodegenerative disease (1, 2). Aβ is generated from the β-amylloid precursor protein (βAPP) by endoproteolytic processing. Two sequential cleavages, first by β-secretase and then by γ-secretase, are required to liberate Aβ (2, 3). The gene encoding the β-secretase BACE (β-site APP-cleaving enzyme) was identified as a type I transmembrane protease (4–8). It contains two active site motifs in its luminal domain harboring the signature sequence of aspartic proteases DT/SGT/S (4–8). For full maturation, BACE is transported through the secretory pathway, subjected to complex glycosylation, and processed by a furin-like pro-protein convertase (9–13). The pro-domain of BACE assists proper folding of BACE but does not confer strict zymogen-like activity (14). On a cellular level BACE is found mainly in acidic compartments such as the trans-Golgi network and endosomes (9, 10, 13) where it is associated with rafts by palmitoylation (15, 16). BACE is highly homologous to BACE-2, but different cleavage sites were identified within the APP substrate for both enzymes (17–19). Besides APP, additional substrates for BACE have been described, like the sialyltransferase ST6Gal I (20, 21), the adhesion protein P-selectin glycoprotein ligand-1 (PSOL-1) (22), and APP-like proteins (23). In addition, Aβ itself complements the substrate profile of this protease (24–26).

The complete absence of Aβ synthesis in knockout mice conclusively identified BACE as the sole β-secretase (27–30). The knock-out of the BACE gene in Tg2576 mice, which overexpress human APP, leading to excessive cerebral Aβ generation, results in a behavioral and electrophysiological rescue of the Aβ-dependent hippocampal memory deficits (31). Moreover, the apparent absence of an overt phenotype of BACE-deficient mice qualifies this protease as an ideal drug target for the therapy of AD (32) even more so, since BACE is apparently up-regulated in sporadic AD cases (33, 34).

The structure of the soluble BACE ectodomain was resolved in a complex with high affinity inhibitors showing a monomer with great similarity to the pepsin-like aspartic proteases (35, 36). Several peptidomimetic inhibitors were designed based on the substrate specificity and were described to inhibit BACE at nanomolar concentrations (37–40). However, because of the essential long term treatment of AD, BACE inhibitors have to

BACE; BACE-N1, soluble C-terminal truncated BACE; BACE-pA, BACE-polyalanine; HSQ 295, embryonic kidney 293 cells; BN-PAGE, blue native PAGE; TRITC, tetramethylrhodamine isothiocyanate; HA, hemaggulutinin; ER, endoplasmatic reticulum; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; HPLC, high performance liquid chromatography.

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be highly selective. This is complicated by the aforementioned structural similarities to other proteases as well as the extended substrate pocket (35). Loose substrate specificity of **BACE in vitro** (41) is also reflected by the variable cleavage sites of the known substrates *in vivo* (20–23). Whereas other aspartic proteases, like pepsin, renin, and cathepsin D exist as soluble monomers, cathepsin E is the only known aspartic protease that exists as a homodimer consisting of two fully catalytically active monomers (42). Dimerization of cathepsin E results in an increased pH and temperature stability, which is consistent with its biological function in endosomal vesicles (43–45).

For membrane-bound BACE it remains unclear whether it is monomeric or associates with itself or heterologous proteins, as it was shown for the presenilins, the catalytically active component within the γ-secretase complex (3, 46). Moreover, a participation of native BACE in a larger complex could alter its enzymatic properties (39, 47). Because BACE is the rate-limiting enzyme of Aβ production (4, 31), a detailed understanding of its molecular architecture and function is of great importance.

To clarify whether membrane-bound BACE functions in cooperation with itself or other proteins, we investigated BACE under native conditions. We demonstrate dimerization of native BACE and show that membrane attachment is a prerequisite for the dimerization of the catalytic ectodomain. A comparison of the kinetic parameters of purified dimeric to monomeric BACE reveals an increased affinity and a higher turnover rate of the dimer for a Swedish mutant Aβ production (42). Dimerization of cathepsin E remains unclear whether it is monomeric or associates with itself or heterologous proteins, as it was shown for the presenilins, the catalytically active component within the γ-secretase complex (3, 46). Moreover, a participation of native BACE in a larger complex could alter its enzymatic properties (39, 47). Because BACE is the rate-limiting enzyme of Aβ production (4, 31), a detailed understanding of its molecular architecture and function is of great importance.

**EXPERIMENTAL PROCEDURES**

**Mouse Lines and Tissue Preparation**—The construct used to generate the transgenic BACE overexpressing mouse line contains a human BACE cDNA insertion in the Thy-1 cassette at the XhoI site of the pTCα plasmid (48). Transgenic mice were generated by pronuclear injection of DBA/C57Bl6 embryos (N. Smyth, University of Cologne). Brain from P5 BACE-deficient (22) and control littermates were used for endogenous BACE-1 expression analysis. Mice were sacrificed at an age of 3 months, and tissue was snap-frozen in liquid nitrogen. Geno-

**Cloning of BACE Expression Constructs**—Full-length BACE (BACE-FL) was tagged at its C terminus with an Myc-His tag in pdNA4/Zeoc (+) vector (Invitrogen) or was fused to the hemagglutinin tag in the pCDNA3.1/Hygro (+) vector (Invitrogen) using the GC-Rich-PCR kit (Roche Applied Science). For the purification of soluble BACE a Myc-His tag was fused to the soluble ectodomain of BACE truncated at amino acid 454 and cloned into the pcDNA4Myc/His/A vector (9). In the BACE-polyalanine (BACE-pa) construct the wild-type transmembrane domain (amino acids 445–477) was replaced by a stretch of 23 alanines. To establish a GPI-linked BACE-NT (BACE-GPI) a Myc-His tag was fused to the soluble ectodomain of BACE and a polymerase chain reaction (PCR) was used (50) to amplify a...
acetate buffer with 0.25 mg/ml bovine serum albumin, pH 4.5, at 26 °C. The inner filter effect was measured and found to be not significant up to a substrate concentration of 30 μM. MnSO₄ concentrations were adjusted in all assays and did not exceed 5%. Reactions were started by the addition of enzyme, and the increase in fluorescence at 590 nm (excitation at 530 nm) was measured in 96-well plates (Nunc) on a Fluoroskan Ascent FL Fluorescence plate reader (Labsystems) while gentle shaking of the samples was applied during the intervals between measurements. Initial velocities were determined for the first 20 min of the progression curve via linear regression. Initial velocities transformed to μmol/s × μmol of enzyme were plotted against increasing substrate concentrations (1–27 μM). Kₘ and kₐ were calculated from a nonlinear least squares best fit to the Michaelis-Menten equation. For the commercial BACE-NT, purchased from Oncogene, the second order rate constant kₐ/Kₘ was calculated from a quasilinear plot of velocity as a function of substrate concentration (see Table I). To cleave off the pro-domain, purified BACE-NT was treated overnight with furin (New England Biolabs) in 100 mM HEPES, pH 7.5, 0.5% Triton X-100, 5 mM CaCl₂, whereas a control was incubated in buffer only. The absence of the pro-domain was controlled by immunoblotting with the pro-peptide antibody GM190. BACE-NT with and without pro-peptide was subjected to the enzyme activity assay.

RESULTS

Endogenous BACE Occurs as a Dimer—To address the question of whether BACE forms a complex, we studied endogenous BACE in murine brain and human cell lines under native and denaturing conditions. Murine BACE is prominently expressed in neuronal tissue (4). We compared BACE expression in the neocortex of 5-day-old (P5) mouse brains and adult mice in Triton X-100-solubilized membrane preparations by immunoblotting using BN-PAGE (Fig. 1A) and denaturing SDS-PAGE analysis (Fig. 1B). In a transgenic mouse (Thy-1-BACE; TB) with neuronal human BACE overexpression under control of the Thy-1 cassette, we identified a robust BACE signal, which was detectable in the range of 140 kDa on a BN-PAGE, whereas no band was seen at the molecular size expected for monomeric BACE (70 kDa; Fig. 1A). A single band at 140 kDa of endogenous BACE was detected in lysates of brains from 5-day-old nontransgenic mice. A much lower signal of identical size was observed in adult brain. The specificity of the signal for BACE was demonstrated by the absence of a signal at 140 kDa in brains of P5 BACE −/− mice (Fig. 1A; long exposure).

These findings were confirmed in two established cell lines. Untransfected HEK 293 cells and the glioblastoma cell line H4 contain endogenous BACE migrating to 140 kDa under native conditions (Fig. 1A; long exposure). The intensity of the 70-kDa band in the SDS-PAGE analysis (Fig. 1B) corresponds to the signal detected at the molecular mass of 140 kDa in the BN-PAGE for the same samples (Fig. 1A). Nonneuronal BACE migrated at a slightly higher molecular weight than neuronal BACE under denaturing conditions (Fig. 1B). The apparent molecular mass of 140 kDa of BACE under native conditions suggests that BACE is a dimer.

Co-immunoprecipitation of Differentially Tagged BACE—To investigate the putative dimerization of BACE, we tagged full-length BACE (BACE-FL) with a HA epitope tag (BACE-FL-HA) or an Myc epitope tag (BACE-FL-Myc) at the C terminus of the protein (Fig. 2). A C-terminal epitope tag does not affect the activity of BACE (9). Immunoprecipitation was performed in total cell lysates of HEK 293 cells stably expressing BACE-FL-HA or BACE-FL-Myc alone and in cell lines that co-express both BACE-FL-HA and BACE-FL-Myc. Immunoprecipitation was performed by capturing either the HA tag or the Myc tag followed by immunoblotting. Co-immunoprecipitation of the mature glycosylated species demonstrated the existence of BACE dimers (as indicated by the arrows in Fig. 2). There was also cross-pull-down of immature pro-peptide containing BACE dimers. In addition, we controlled the experiment by mixing the lysates of the single expressing clones to exclude false positive co-immunoprecipitation due to artificial post-lysis aggregation. Under these conditions no co-immunoprecipitation was observed (Fig. 2).

The Non-membrane-bound BACE Ectodomain Occurs as a Monomer—To identify essential domains for homodimerization of BACE, we performed a domain deletion analysis. It has previously been shown by crystallography that the inhibitor-complexed BACE ectodomain without a transmembrane an-
As well as all mutant BACE variants, displayed a clear increase (56). Overexpressed monomeric BACE-NT, which produces a selectively Myc-positive. The same EE-17-positive and Myc-positive Myc antibody (arrow) on right panel). A cell line co-expressing untagged BACE-FL together with a Myc-tagged BACE-NT shows an EE-17-positive band at 140 kDa (arrow on left panel) that is not detected by the Myc antibody (arrow on right panel) and a 70 kDa band, which is selectively Myc-positive. The same EE-17-positive and Myc-positive monomeric band is found in the BACE-NT-Myc sample.

The ectodomain of a monomer (35, 55). We, therefore, initially analyzed BACE with a deletion of its membrane domain and the cytosolic domain (BACE-NT). In contrast to the 140-kDa membrane-bound BACE, BACE-NT migrated at the monomeric size of ~70 kDa on BN-PAGE (Fig. 3). Furthermore, a cell line co-expressing untagged BACE-FL (FL) together with an Myc-tagged BACE-NT (NT-Myc) showed a 70-kDa band detected with the N-terminal antibody (left panel, Fig. 3) in addition to the 140-kDa band (arrow in left panel). The Myc antibody, however, did not detect a dimeric band (arrow in right panel) but solely a band at 70 kDa. Therefore, this species was identified as monomeric BACE-NT-Myc (Fig. 3). In accordance with the above finding, a co-immunoprecipitation experiment using a cell line co-expressing BACE-FL-HA together with a soluble BACE-NT-Myc did not result in any cross-pull-down (data not shown). Taken together, these experiments demonstrate that BACE-FL associates to a homodimer, whereas its ectodomain occurs exclusively as a strict monomer even in the presence of excess BACE-FL.

Membrane Attachment of the BACE Ectodomain Is Required for Dimerization—To further investigate why BACE-NT failed to dimerize, we generated several deletion constructs (schematically shown in Fig. 4A). Membrane attachment of the individual BACE derivatives was confirmed by cell surface immunostainings with an N-terminal antibody detecting the extracellular domain of mature BACE (Fig. 4B). The stainings showed the expected cell surface expression of BACE-FL, BACE-ΔC, and BACE-pA. BACE-NT is not detectable at the plasma membrane, whereas BACE-GPI is expressed on the plasma membrane, in accordance with a recent study (16). BACE-KXXX, like BACE-NT, can only be detected in permeabilized cells (insets, Fig. 4B).

To test the functionality of the constructs, we measured the cellular activity by specific detection of the N-terminal cleavage product APPβ from supernatants of Swedish APP-expressing cell lines using the neo-epitope-specific antibody sw192 (Fig. 4C (56)). Overexpressed monomeric BACE-NT, which produces a relatively fainter signal in the blot due to continuous secretion as well as all mutant BACE variants, displayed a clear increase in APPβ accumulation over 12 h in the supernatant in comparison to control cells with endogenous BACE levels (Fig. 4C). Intracellular APP levels were roughly equal, and the production of membrane-retained APP stubs correlated with APPβ levels (data not shown). These results show that all investigated BACE variants were proteolytically active. Kinetic differences are not detected by this analysis due to the saturation of the signal from the accumulated product APPβ.

After demonstrating the proteolytic activity, the recombinant BACE species were analyzed by BN-PAGE. BACE-FL occurred as a dimer (Fig. 4D), whereas BACE-NT obtained from the supernatant of HEK 293 cells migrated to monomeric size (Fig. 4D), consistent with intracellular BACE-NT (Fig. 3). BACE lacking the complete cytoplasmic domain (BACE ΔC) shows a dimeric band as well as a BACE derivative, which had the transmembrane domain replaced by a stretch of 23 alanine residues (BACE-pA; Fig. 4D). This suggests that neither the cytoplasmic tail nor the authentic transmembrane domain is essential for dimerization. To test whether the membrane association of BACE was necessary for dimerization, we analyzed a GPI-anchored version of BACE-NT (BACE-GPI). The BACE-GPI variant also migrates to dimeric size, indicating that the ectodomain of BACE is sufficient for homodimerization as long as it is bound to the membrane (Fig. 4D). To determine the cellular compartment where dimerization occurs, we attached a C-terminal KXXX-ER retention motif to BACE-FL (57). The KXXX motif retained BACE in the ER as demonstrated by the presence of the pro-peptide, which is cleaved off in later compartments (data not shown; see also Fig. 5C). ER/cis-Golgi resident BACE-KXXX still shows the size of a dimer (Fig. 4D). The additional higher molecular weight band with a size of about 220 kDa occasionally seen (Fig. 4D) may be due to the association of an ER resident protein, probably a chaperone, which was described for the inactive and instable pancreatic splice variant BACE 457 (58, 59). Slight shifts in molecular weight on the SDS-PAGE (lower panel) correspond to the size reduction upon deletion (ΔC, GPI) or the incomplete maturation caused by the ER retention motif (KXXX, Fig. 4D). Taken together, these experiments demonstrate the existence of proteolytically active 140-kDa native BACE dimers and provide evidence for the existence of strong protein-protein interactions between the ectodomains of BACE.

**Purified Membrane-bound BACE Is a More Potent Enzyme than the Monomer**—BACE-NT has widely been used in enzymatic assays for the determination of kinetic parameters and inhibitor studies (8, 37, 39, 41, 47, 60–63). We were, thus, interested in clarifying whether the native BACE dimer shows different kinetic parameters with respect to those published for the monomeric BACE ectodomain. To study the in vitro kinetics of monomeric and dimeric BACE, we purified BACE-FL from membrane preparations and BACE-NT from supernatant via nickel nitritotriacetic acid-Sepharose. The Coomassiestained SDS-PAGE (Fig. 5A) demonstrates the purification of BACE-NT and BACE-FL to almost homogeneity. BN-PAGE analysis confirmed that purified BACE-FL was present as a dimer, whereas BACE-NT was a monomer (Fig. 5B). Commercial BACE-NT (BACE-NT-onco), which was used as an independent control for the kinetic parameters, showed the same size and purity as purified BACE-NT (Fig. 5A). On the corresponding Western blot, the pro-domain-specific antibody GM190 (9) demonstrated that BACE-NT-onco and BACE-NT produced in our laboratory still carried the pro-domain (Fig. 5A), which is in agreement with earlier reports (9, 11, 14, 60). The Michaelis-Menten kinetics obtained for the quenched fluorogenic substrate harboring the Swedish APP cleavage site (62) revealed a higher affinity and catalytic activity for dimeric BACE-FL compared with monomeric BACE-NT and the commercial BACE-NT-onco (Fig. 5C and Table I). Because under the experimental conditions used, no saturation of BACE-NT with the substrate could be reached, we determined kcat at 10 μM, which shows that BACE-FL is about 30 times more cata-
lytically active than BACE-NT at this substrate concentration (Table I). Due to a nearly linear Michaelis-Menten curve within the range of substrate concentrations applied, \( k_{\text{cat}} \) and \( K_m \) for the commercial BACE-NT could not be estimated by a nonlinear fit. We, therefore, calculated the second order rate constant \( k_{\text{cat}}/K_m \) from a quasilinear plot of velocity as a function of substrate concentration. The obtained value is very similar to the one for the BACE-NT produced in our laboratory, as is the \( k_{\text{cat}} \) at 10 \( \mu \)M (Table I). We confirmed by BN-PAGE analysis that the assay conditions (pH 4.5, 26 °C) BACE-FL was in a dimeric conformation, whereas BACE-NT was a monomer (data not shown). To further exclude the possibility that the presence of the pro-domain in BACE-NT was responsible for the difference in kinetics, we removed the pro-domain by incubation of the purified BACE-NT with furin and compared the kinetics of the pro-domain free BACE-NT (nopro BACE-NT) to untreated BACE-NT (Fig. 5D). Both versions of the enzyme showed a similarly low affinity to the substrate with an almost linear Michaelis-Menten plot within the range of substrate concentrations utilized. In accordance with Shi et al. (14), we thus suggest that the pro-domain of BACE has little effect on the BACE active site. The kinetic data rather propose that the relatively poor kinetic parameters of BACE-NT are due to its inability to dimerize.

**DISCUSSION**

In this study, we characterized endogenous and ectopically expressed BACE under native conditions and found that it exists as a homodimer in murine brain as well as in two independent cell lines. Membrane attachment was shown to be required for dimerization. Retention of BACE-KKXX showed that dimerization occurred as early as in the ER/cis-Golgi. In contrast to previous studies (64, 65), we detected endogenous native BACE exclusively at a size of 140 kDa on BN-PAGE, which corresponds to a stable BACE homodimer as shown via co-immunoprecipitation experiments. Sidera et al. (64) analyzed the molecular weight of purified full-length BACE on SDS-PAGE gels and detected a larger band at around 140 kDa in addition to the monomeric size. They hypothesized that this might represent a putative dimer (64). This was in contrast to the findings by Huse et al. (10) who found only monomeric BACE on velocity gradients. In another study, activity of BACE extracted from guinea pig brain was demonstrated to be associated with a high molecular weight complex with a size ranging from 140 to 600 kDa (65). A recent study by Sidera et al. (66) provides further evidence for the existence of BACE dimers in cultured cells and in human brain. These authors demonstrated SDS stability of a fraction of BACE in human brain. We also observed SDS-resistant BACE dimers in cultured cells.
upon overexpression (data not shown). However, because SDS extraction also causes aggregation, we preferred to use mild extraction conditions with Triton X-100 in combination with the BN-PAGE technique.

All membrane-attached BACE variants like BACE-FL, BACE-ΔC and BACE-pA were capable of dimerizing, suggesting that the authentic transmembrane domain and the C terminus of BACE were dispensable for dimerization. Attachment

TABLE I

| Enzyme          | $K_M$ (μM) | $k_{cat}$ (S$^{-1}$) | $k_{cat}/K_M$ (μM$^{-1}$S$^{-1}$) |
|-----------------|------------|---------------------|----------------------------------|
| BACE-FL         | 5.9 ± 0.5  | 1.59 ± 0.08         | 0.269 ± 0.017                    |
| BACE-NT         | 50.5 ± 5.2 | 0.21 ± 0.02         | 0.0042 ± 0.0001                  |
| BACE-NT-onco    | ND         | 0.0042 ± 0.0007     | 0.00006 ± 0.00005                 |

Note that for commercial BACE-NT-onco the second order rate constant $k_{cat}/K_M$ was calculated from a quasilinear plot of velocity as a function of substrate concentration.
of the BACE ectodomain via a GPI anchor to the membrane also resulted in dimerization. From these experiments we conclude that the ectodomain is sufficient for dimerization as long as it is bound to the membrane.

Although soluble and membrane-retained BACE species were catalytically active, dimeric and monomeric BACE displayed significant differences in their kinetic profiles. The published kinetic parameters for BACE-NT in vitro using Swedish APP-like substrates (37, 41, 60) similar to the one used in this study are very variable. Methodologically, fluorogenic substrates as well as matrix-assisted laser desorption ionization time-of-flight and HPLC were applied to investigate cleavage. From these analyses $K_m$ values from 4.5 $\mu M$ to 1 mM were estimated (37, 41, 47, 60, 63). To our knowledge, kinetic parameters for BACE-FL have only been published by Kopcho et al. (63) so far. They compared the kinetics of BACE-FL enriched in unpurified membrane preparations of Chinese hamster ovary cells with purified versions of BACE-NT and detected no significant kinetic differences using a Swedish APP-like substrate. However, they did not analyze the native size of BACE-FL in their preparation, and a direct comparison of both species is impossible since BACE-FL was not purified (63). Another report states that they observed an almost identical catalytic activity for the purified ectodomain of BACE and a full-length version of the enzyme. However, no kinetic parameters were published (41). In our in vitro study, we used BACE-FL purified from membrane preparations and ensured stability of the native dimer under assay conditions. By applying these criteria, we found that in direct comparison, dimeric BACE-FL displays a higher affinity and turnover rate toward a Swedish APP-like substrate than monomeric BACE-NT. We also found almost no influence of the pro-domain on the observed kinetic differences. This is in agreement with a series of reports, which show that BACE is not a true zymogen (11–14).

Our in vitro data suggest that it would be advisable to validate inhibition constants for competitive inhibitors obtained with the artificially monomeric BACE-NT by using a native BACE-FL preparation. Additionally, inhibitors that interfere with BACE dimerization leading to an enrichment of monomers could result in a reduction of the activity of this rate-limiting enzyme for amyloid pathology, even if such BACE monomers were stable. Therapeutic disruption of dimers was already successfully shown for the human immunodeficiency virus 1 protease, which acts as a homodimer, each unit contributing one catalytic aspartate to the active site dyad (67, 68).

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