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Identification of BACE2 as an avid β -amyloid-degrading protease

Samer O Abdul-Hay, Tomoko Sahara, Melinda McBride, Dongcheul Kang and Malcolm A Leissring*

Abstract

Background: Proteases that degrade the amyloid β -protein ($A\beta$) have emerged as key players in the etiology and potential treatment of Alzheimer's disease (AD), but it is unlikely that all such proteases have been identified. To discover new $A\beta$ -degrading proteases ($A\beta$ DPs), we conducted an unbiased, genome-scale, functional cDNA screen designed to identify proteases capable of lowering net $A\beta$ levels produced by cells, which were subsequently characterized for $A\beta$ -degrading activity using an array of downstream assays.

Results: The top hit emerging from the screen was β -site amyloid precursor protein-cleaving enzyme 2 (BACE2), a rather unexpected finding given the well-established role of its close homolog, BACE1, in the production of $A\beta$. BACE2 is known to be capable of lowering $A\beta$ levels via non-amyloidogenic processing of APP. However, *in vitro*, BACE2 was also found to be a particularly avid $A\beta$ DP, with a catalytic efficiency exceeding all known $A\beta$ DPs except insulin-degrading enzyme (IDE). BACE1 was also found to degrade $A\beta$, albeit ~150-fold less efficiently than BACE2. $A\beta$ is cleaved by BACE2 at three peptide bonds—Phe19-Phe20, Phe20-Ala21, and Leu34-Met35—with the latter cleavage site being the initial and principal one. BACE2 overexpression in cultured cells was found to lower net $A\beta$ levels to a greater extent than multiple, well-established $A\beta$ DPs, including neprilysin (NEP) and endothelin-converting enzyme-1 (ECE1), while showing comparable effectiveness to IDE.

Conclusions: This study identifies a new functional role for BACE2 as a potent $A\beta$ DP. Based on its high catalytic efficiency, its ability to degrade $A\beta$ intracellularly, and other characteristics, BACE2 represents a particularly strong therapeutic candidate for the treatment or prevention of AD.

Keywords: Amyloid- β -protein, Alzheimer disease, β -site APP-cleaving enzyme-1, β -site APP-cleaving enzyme-2, Functional screen, Gene therapy, Protease, Proteolytic degradation

Background

Alzheimer disease (AD) is a progressive and presently incurable neurodegenerative disorder characterized by abnormal accumulation of the amyloid β -protein ($A\beta$) in brain regions important for mnemonic and cognitive functions. $A\beta$ is a heterogeneous mixture of peptides ranging from 37 to 43 amino acids in length [1] produced via sequential cleavage of the amyloid precursor protein (APP) by BACE1 and the presenilin/ γ -secretase complex [2-4]. Autosomal-dominant mutations in 3 genes—APP and presenilin-1 and -2—are known to cause rare, familial forms of AD either by increasing the production of all forms of $A\beta$ or by increasing the relative production of longer, more amyloidogenic forms,

such as $A\beta$ 42 [5]. Nevertheless, the precise mechanisms underlying sporadic AD, which makes up the vast majority of cases, remain to be elucidated.

$A\beta$ -degrading proteases ($A\beta$ DPs) are potent regulators of cerebral $A\beta$ levels and, as such, represent important players in the etiology and potential treatment of AD [6]. Amyloidogenesis and downstream cytopathology can be attenuated and even completely prevented by enhancing the activity of any of several $A\beta$ DPs, while, conversely, genetic deletion of one or more $A\beta$ DPs leads to significant elevations in cerebral $A\beta$ [7]. Significantly, patients with sporadic AD were recently shown to exhibit defects in the clearance of $A\beta$ (rather than increases in its production) [8] and, in light of the large body of evidence implicating $A\beta$ DPs in the regulation of cerebral $A\beta$ levels [7], it is reasonable to infer that defects in one or more $A\beta$ DPs could contribute to

* Correspondence: leissring@mayo.edu
Department of Neuroscience, Mayo Clinic, 4500 San Pablo Road, Birdsall Bldg., Rm. 117, Jacksonville, FL 32224, USA

impaired A β clearance. While more than twenty proteases are now known to degrade A β [7], these were not identified through any systematic approach, but instead emerged haphazardly from a disconnected set of largely serendipitous discoveries. Nevertheless, essentially all A β DPs now known to regulate A β in vivo were originally identified through exclusively in vitro or cell-based approaches [9].

To discover new A β DPs more systematically, we conducted an unbiased, cell-based, functional screen of 352 proteases in the human genome. The top A β -lowering protease emerging from this screen was β -site APP-cleaving enzyme-2 (BACE2) [10]. Previous studies have shown that BACE2 can lower A β levels via α -secretase-like cleavage of APP within the A β sequence [11-16], an activity that has been dubbed “ θ -secretase” [17]. However, we found that BACE2 is also a remarkably avid A β DP, with a catalytic efficiency exceeding all other known A β DPs except insulin-degrading enzyme (IDE).

Results and discussion

Functional screen for novel A β DPs

To identify novel A β DPs, we performed a cell-based functional screen using a commercial library consisting of 352 full-length, sequence-verified, human cDNAs encoding diverse members of all protease classes. We experimented with several approaches before settling on a final configuration for the primary screen. Assays designed to monitor degradation of exogenous A β were found to be confounded by the highly dominant effect of IDE, which mediates the vast majority of extracellular A β degradation in cultured cells [18-20]. Transient transfection of cDNAs into cell lines stably expressing APP was also tried, but this approach suffered from incomplete transfection efficiency, which attenuated the effect on net extracellular A β levels. We therefore elected to conduct the screen by co-transfecting protease-encoding cDNAs, together with positive and negative controls, into a rodent cell line (CHO cells) together with a plasmid encoding wild-type human APP fused to alkaline phosphatase (AP) (see Figure 1A; *Methods*). Use of the APP-AP construct ensured that human A β production was limited to cells also expressing candidate A β DPs, while also providing an internal control for transfection efficiency (via AP activity). Importantly, the co-transfection strategy also increased the likelihood of detecting A β DPs that degrade A β intracellularly, prior to its secretion, in addition to those that act exclusively extracellularly. Cytotoxicity was also quantified via an MTT conversion assay, but no significant cell death was detected so these data were not incorporated into subsequent analyses. The screen was performed in quadruplicate and, for each well, the ratio of A β 40 concentration

to AP activity was calculated, then normalized to appropriate intra-plate controls (Figure 1B).

From among the 352 proteases examined, by far the largest decrease in normalized A β levels ($97 \pm 1.2\%$) was induced by BACE2, which was in fact the only protease to lower A β levels more than 75%, our pre-determined cut-off for viable hits (Figure 1B).

BACE2 transfection lowers A β levels

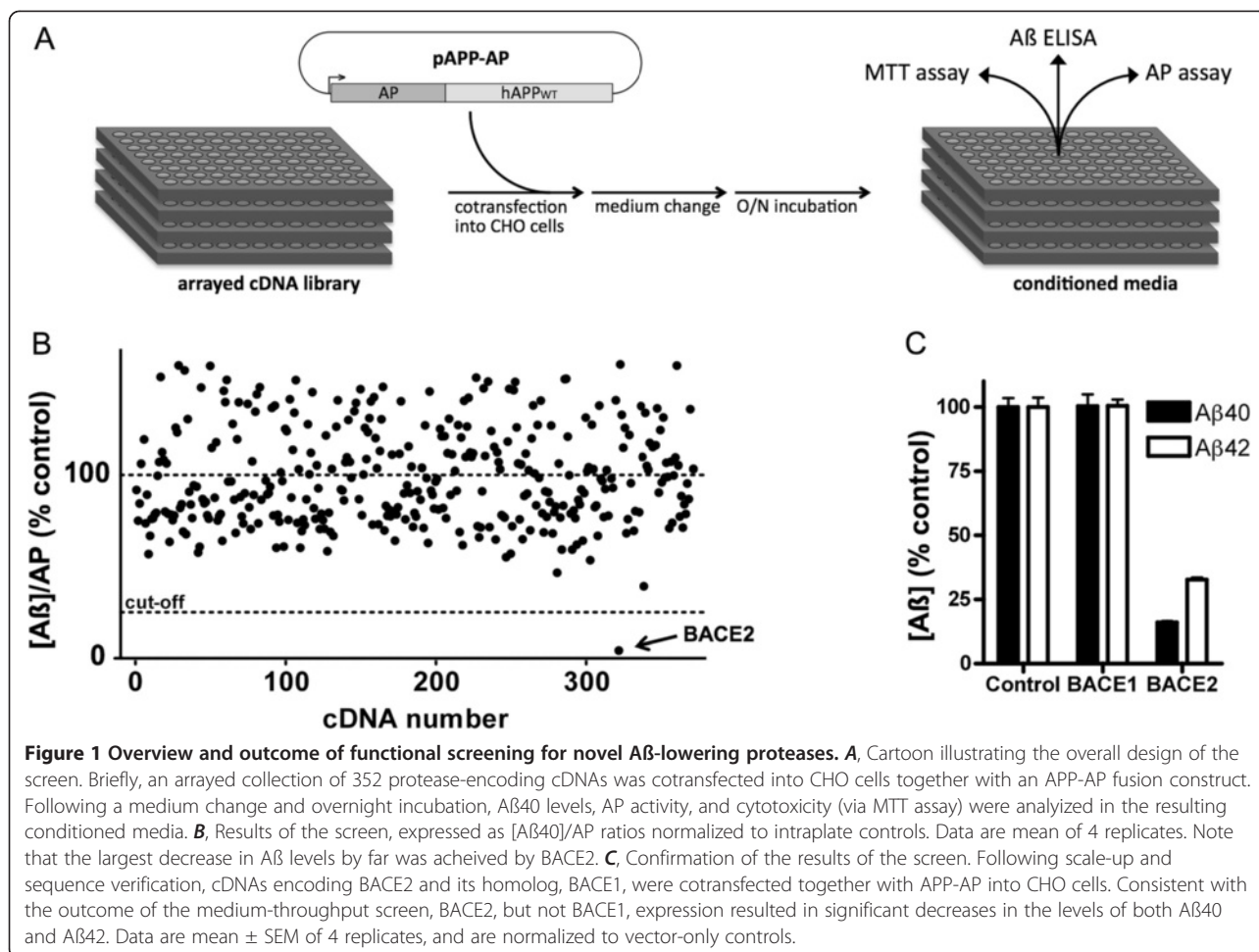
To confirm and extend the results obtained in the cDNA screen, we compared the degree to which overexpression of BACE2 and its homolog BACE1 [21] affected the net production of different A β species. Consistent with the results of the primary screen, BACE2 transfection in CHO cells decreased the levels of both A β 40 and A β 42 (Figure 1C). Overexpression of BACE1 in this cell type, by contrast, had no effect on net A β levels (Figure 1C). We note that BACE1 overexpression would not be expected to increase A β production in CHO cells, since previous studies have established that γ -secretase, rather than β -secretase, is the rate-limiting step in A β production in this cell type [22].

BACE2 and BACE1 degrade A β in vitro

Expression of BACE2 in cells could lower A β levels either directly, via proteolytic degradation, or indirectly, via alternative mechanisms such as hydrolysis of APP or APP C-terminal fragments (CTFs) [11-16]. To distinguish these possibilities, we tested the ability of recombinant BACE2 to hydrolyze synthetic A β in vitro, using a well-established fluorescence polarization-based A β degradation assay [23]. Recombinant BACE2 was found to avidly degrade A β in this paradigm, confirming that BACE2 is indeed a bona fide A β DP (Figure 2A). Recombinant BACE1 also hydrolyzed A β , indicating that it too is an A β DP (Figure 2B). However, BACE1 was much less efficient than BACE2, requiring 24 h to degrade A β to a similar extent as was achieved following a 10-min incubation with BACE2 (Figure 2B). Based on these results, the efficiency of BACE1 would appear to be ~ 150 -fold lower than that of BACE2.

BACE2-mediated A β degradation is pH-dependent

As an aspartyl protease, the catalytic efficiency of BACE2 is expected to be pH-dependent. To confirm this, we compared the rate of hydrolysis of A β 40 across a range of pH values. Consistent with expectations, BACE2 was found to be maximally effective at pH 3.5 (Figure 2C), and decreasingly effective at higher pH values. These findings strongly suggest that BACE2 would not be operative at the cell surface or within the extracellular space.



BACE2 does not degrade fibrillar A β

Individual A β DPs can be categorized in terms of their ability or inability to degrade fibrillar forms of A β . Many well-established A β DPs, such as IDE and NEP, avidly degrade monomeric A β but cannot degrade fibrillar forms and are therefore categorized as pure peptidases. Others, such as plasmin, degrade A β fibrils and thus can also be categorized as fibrilases [7]. To determine to which category BACE2 belongs, we incubated recombinant BACE2 with pre-formed fibrils of A β 42 and quantified the degree of aggregation by thioflavin T fluorescence. No significant reduction in aggregation was observed, even following incubation at 37°C for up to 3 d (Figure 2D). These results suggest that, as is true for the majority of A β DPs [7], BACE2 does not degrade A β fibrils.

BACE2 cleaves A β at 3 sites

We next investigated which peptide bond(s) within A β are hydrolyzed by BACE2 and BACE1. To that end, we co-incubated N-terminally biotinylated A β 40 or A β 42 (300nM) with BACE2 (5 nM) and analyzed the products

by immunoprecipitation/mass spectrometry (IP/MS) (see *Methods*). Within 1 h, BACE2 almost completely hydrolyzed both A β species, generating the shorter fragment, A β 34, in both cases (Figure 3A-D). To test whether any additional cleavages can occur, we incubated N-terminally biotinylated A β 40 (300 nM) with a larger amount of BACE2 (25 nM) for 1 and 24 h. At these higher concentrations and longer incubation times, A β 19 and A β 20 were the principal N-terminal fragments remaining at the end of the reaction (Figure 3E-F). Collectively, these *in vitro* results suggest that BACE2 cleaves A β at three different positions: Phe19-Phe20, Phe20-Ala21, and Leu34-Met35, with the latter cleavage site being the initial and principal one, as is consistent with previous observations [13,14,24].

To confirm whether BACE2 cleaves A β at the same sites in a more physiological setting, we analyzed A β species in the conditioned media of cells expressing APP-AP either alone or together with BACE2 by IP/MS (see *Methods*). As expected for cells expressing APP-AP alone, the medium from these cells contained A β 42, A β 40, A β 39, A β 38, and A β 37 (Figure 4A). BACE2

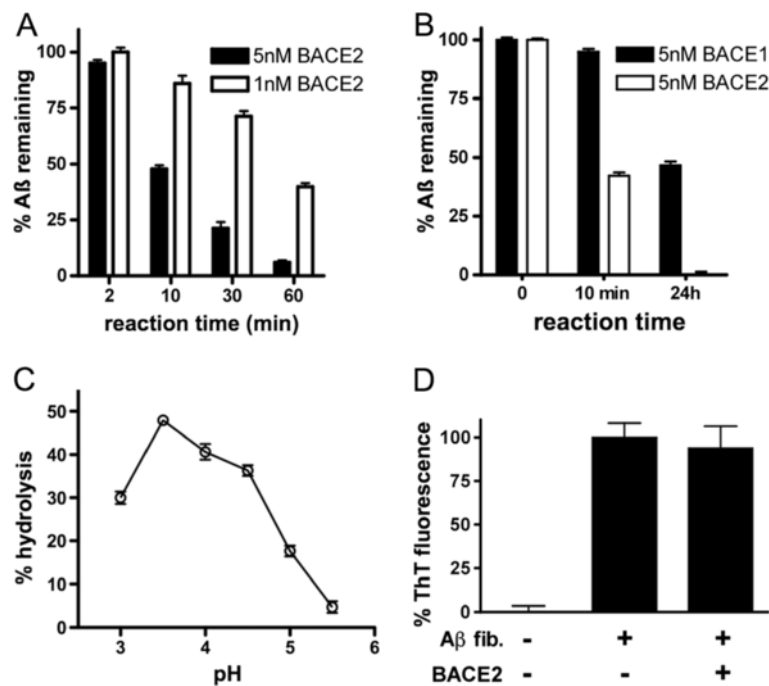


Figure 2 BACE2 degrades A β in vitro. **A**, Percent A β remaining following incubation with different concentrations of recombinant BACE2 for various lengths of time. Data are mean \pm SEM of 4 replicates, normalized to protease-free controls. **B**, Comparison the relative A β -degrading ability of recombinant BACE2 vs. BACE1. Note that 24 h incubation with BACE1 was required to achieve approximately the same extent of degradation as effected by BACE2 in 10 min. Data are mean \pm SEM of 3 replicates, normalized to protease-free controls. **C**, BACE2 activity is pH dependent. Percent A β degradation catalyzed by equivalent amounts of BACE2 at different pH values. Data are mean \pm SEM of 4 replicates. **D**, BACE2 does not degrade fibrillar A β . Lack of effect of BACE2 (10 nM) on preformed A β 42 fibrils following incubation at 37°C for 5 d, as determined by thioflavin T fluorescence. Data are mean \pm SEM of 3 replicates.

expression suppressed the signal of all of these species, and new peaks corresponding to A β 19, A β 20, and A β 34 emerged (Figure 4B), confirming that the cleavage sites mediated by BACE2 in vitro are also hydrolyzed in intact cells. The appearance of A β 34 is particularly notable, because cleavage at position 34 can only occur after production of full-length A β , as this peptide bond is positioned within the transmembrane domain of APP, as has been shown previously [24]. Although this result clearly indicates that BACE2 does indeed degrade A β after it is produced, it is not possible to quantify the extent to which the A β 19 and A β 20 peaks are the result of θ -secretase activity or subsequent degradation of the A β 34 fragment (or full-length A β). As a consequence, it is difficult to estimate the exact extent to which the A β -lowering effect of BACE2 can be assigned to non-amyloidogenic processing versus A β degradation per se in experimental paradigms of this type.

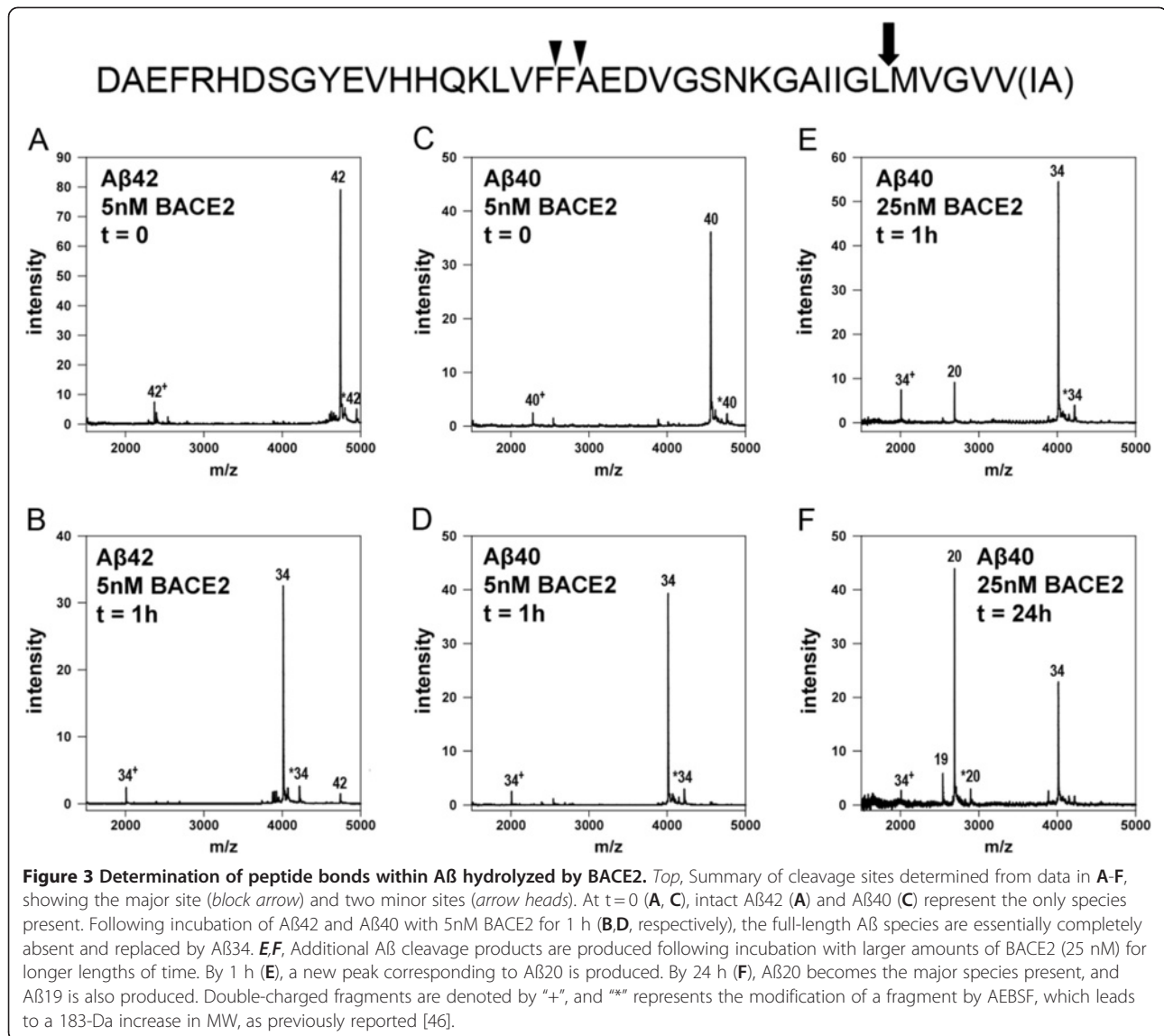
BACE2 degrades A β more efficiently than well-established A β DPs

Having established BACE2 as an A β DP, we next investigated how BACE2 compares to other known A β DPs in terms the ability to degrade A β in vitro and to lower net

A β levels in cells. To compare the relative efficiency of BACE2 in vitro, we monitored the degradation of a fixed amount of A β (200 nM) by recombinant BACE2 (5 nM) as compared to equal quantities of several well-established A β DPs, including IDE, NEP and plasmin. Under these conditions, BACE2 hydrolyzed A β more efficiently than all other A β DPs except IDE (Figure 5A). We note that the concentration of A β used in this experiment was considerably lower than the K_M for each of the proteases tested (see [23] and below), making the initial velocity of this reaction a good index of the relative catalytic efficiency.

Kinetics of A β degradation by BACE2

To investigate the catalytic efficiency of BACE2 more quantitatively, we determined the kinetics of degradation of both A β 40 and A β 42 by BACE2 (see *Methods*). For this analysis, we were careful to use freshly prepared batches of monomeric human A β 40 and A β 42 peptides, which we routinely prepare by size-exclusion chromatography and which have been extensively characterized [25,26]. BACE2 cleaved both A β species with similar kinetics, exhibiting apparent K_M values in the low micromolar range and albeit with apparent k_{cat} values slightly



higher for A β 40 relative to A β 42 ($0.135 \pm 0.016 \text{ min}^{-1}$ and $0.025 \pm 0.005 \text{ min}^{-1}$, respectively; Table 1). In terms of catalytic efficiency (k_{cat}/K_M), BACE2 degrades A β 40 approximately 4-fold more efficiently than A β 42 (Table 1). These parameters exceed the published values for most other well-characterized A β DPs, including NEP [23], ECE1 [27], and plasmin [23], while being comparable to those of IDE [23,28]. Consequently, these values are in good agreement with the side-by-side comparison of A β degradation in vitro discussed above (Figure 5A).

To investigate the relative ability of BACE2 to lower A β levels under more physiological conditions, we co-transfected CHO cells with APP together with BACE2 or several other A β DPs, then quantified net A β 40 and A β 42 levels in the conditioned medium by ELISA. We emphasize that this approach cannot control for intrinsic differences in transcription or translation efficiency, and,

in the case of BACE 2, the A β -lowering effect can also be mediated to an undetermined degree by BACE2-mediated θ -secretase activity. Nevertheless, the results were in good agreement with the in vitro findings: BACE2 lowered net A β 40 and A β 42 levels to a comparable extent as IDE, with both of the latter being significantly more effective than NEP or plasmin (Figure 5B, C).

BACE2 colocalizes with A β intracellularly

Having determined that BACE2 is functionally among the most efficient A β DPs yet discovered, we subsequently investigated the subcellular localization of BACE2, focusing in particular on the extent to which it colocalizes with A β in acidic compartments, where BACE2 is expected to be operative. In agreement with other published findings [29], application of fluorescently tagged A β to live cells resulted in its

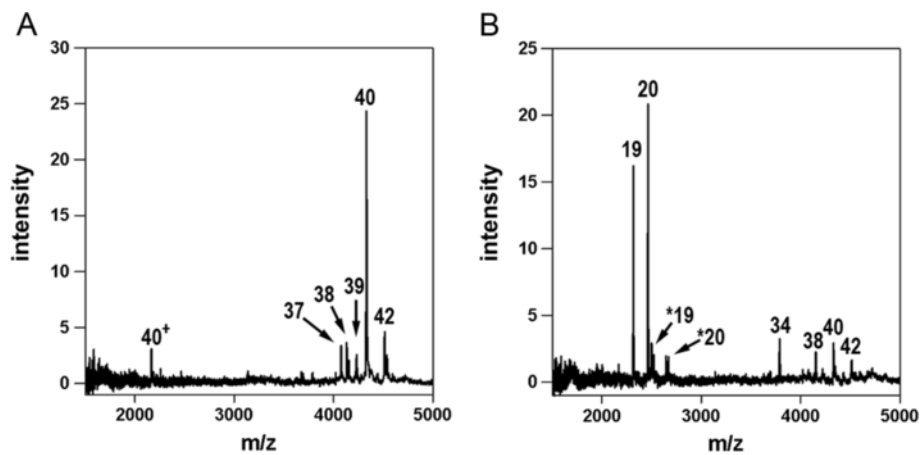


Figure 4 Overexpression of BACE2 in cells yields A β fragments identical to those produced *in vitro*. **A,B**, Spectra of A β fragments determined by IP/MS analysis of the conditioned media of CHO cells transfected with APP and empty vector (**A**) or APP and BACE2 (**B**) (see *Methods*). **A**, APP expression alone produces peaks corresponding to A β 42, A β 40, A β 39, A β 38 and A β 37. **B**, Co-expression of APP and BACE2 results in decreases in the relative abundance of the aforementioned A β species and the appearance of three new fragments: A β 34, A β 20 and A β 19. Double-charged fragments are denoted by "+", and "*" represents the modification of a fragment by AEBSF, which leads to a 183-Da increase in MW, as previously reported [46].

accumulation at intracellular sites largely overlapping with lysosomes (Figure 6A). To test whether BACE2 is also localized to lysosomes and/or other compartments containing A β , we analyzed CHO cells expressing BACE2 tagged at its N-terminus with green fluorescent protein (BACE2-GFP). As determined by confocal microscopy, BACE2-GFP was found to be present in lysosomes (Figure 6B) and also to overlap significantly with fluorescently labeled A β (Figure 6C).

BACE2 degrades A β at intracellular sites

To directly assess whether BACE2 degrades A β at intracellular sites, we tested the ability of BACE2-expressing cells to degrade exogenously applied A β by multiple methods. Cells overexpressing BACE2-GFP and loaded with fluorescently tagged A β 40 showed significantly reduced intracellular A β 1 h after washing, but this was not the case for cells overexpressing GFP alone (Figure 7A). Consistent with this, levels of intracellular A β , both fluorescently tagged and unmodified, were found to be consistently lower in cells overexpressing (untagged) BACE2 relative to vector-transfected controls (Figure 7B,C). Notably, significantly lower levels of intracellular A β were observed both 5 min and 2 h after washing in multiple paradigms. Collectively, these results strongly suggest that BACE2 is a bona fide A β DP that avidly degrades A β within acidic compartments.

Conclusions

One of the most fruitful outcomes of the genomic revolution is the emergence of genome-scale collections of full-length, sequence verified cDNAs. Combined with

appropriate functional assays, cDNA libraries have catalyzed significant advances in our understanding of AD pathogenesis, including the seminal discovery that β -secretase activity, the first step in the production of A β , is mediated by BACE1 [21]. Here, we utilized a similar approach to discover new candidate A β DPs, using a functional assay sensitive to both extracellular and intracellular A β degradation (as well as other potential A β -lowering mechanisms). Rather unexpectedly, the top hit emerging from a screen of 352 proteases was BACE2, a close homolog of BACE1. Subsequent characterization confirmed that, in addition to BACE2's established ability to lower A β production via θ -secretase-mediated processing of APP [11-16], BACE2 also avidly degrades A β with a catalytic efficiency exceeding almost all well-established A β DPs.

The finding that BACE2 is an avid A β DP suggests a novel and unexpected role for this protease in the pathogenesis of AD. Indeed, given its close homology with BACE1, it was initially hypothesized that BACE2 might mediate the *production* of A β , via β -secretase cleavage of APP, instead [15,16]. However, most evidence now suggests that BACE2 does not contribute appreciably to A β production *in vivo* [3]. For instance, cultured neurons from BACE2 knockout mice did not show reductions in A β following transfection with APP [30] and conversely, overexpression of BACE2 in APP transgenic mice failed to increase cerebral A β levels, as would be expected if BACE2 possessed β -secretase-like activity.

In addition to its potent ability to degrade A β , BACE2 also possesses a second A β -lowering function for

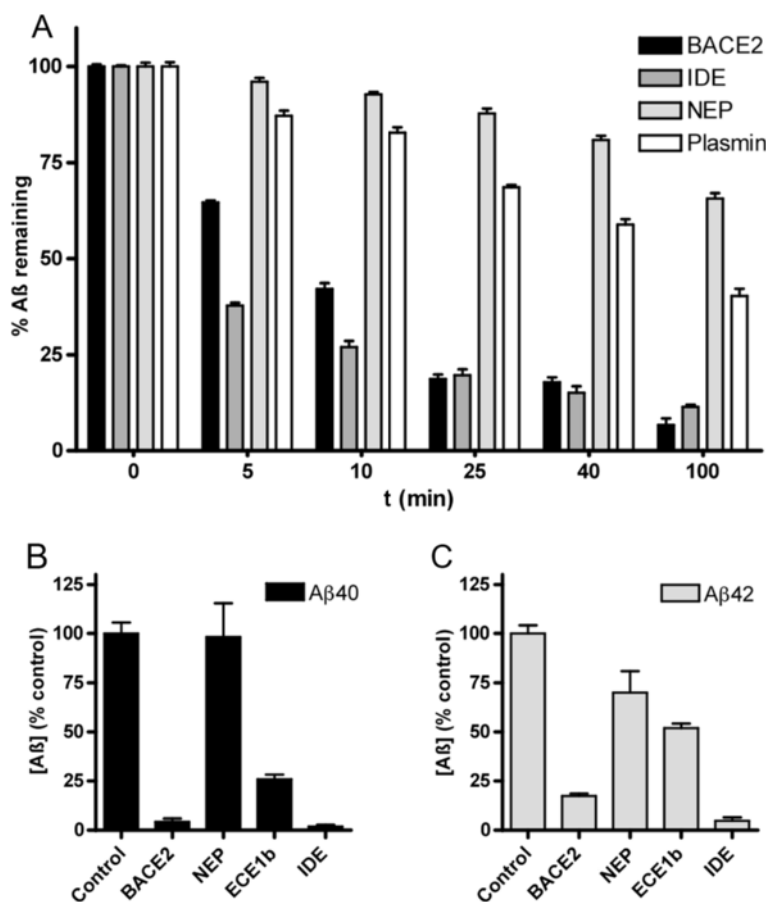


Figure 5 Comparison of the efficacy of BACE2 relative to other well-established AβDPs in vitro and in cultured cells. **A**, Degradation of Aβ in vitro by equivalent nominal concentrations (5 nM) of recombinant BACE2, IDE, NEP and plasmin. Note that BACE2 degrades Aβ at a faster rate than NEP and plasmin, but not IDE. **B,C**, Effects on Aβ40 (**A**) and Aβ42 (**C**) levels following cotransfection of CHO cells with APP together with equivalent quantities of cDNAs encoding BACE2, ECE1b and IDE. In good agreement with the results in vitro (**A**), BACE2 lowers the levels of both Aβ species to an extent exceeding NEP and ECE1b, but comparable to IDE. Data are mean ± SEM of 4 replications, normalized to controls cotransfected with empty vector (V₀).

BACE2, one that is quite independent of Aβ degradation. Specifically, BACE2 has been shown to cleave APP and the β-secretase-derived APP-CTF within the Aβ sequence, in a manner analogous to α-secretase [11-16]. This activity, dubbed θ-secretase [17], occurs at positions 19 and 20 within the Aβ sequence, precisely the same cleavage sites identified in the present study [13,14]. As is true for α-secretase, θ-secretase activity lowers Aβ levels by shuttling APP away from the amyloidogenic processing pathway [11-16].

Table 1 Kinetic parameters of Aβ40 and Aβ42 degradation by BACE2

	Aβ40	Aβ42
K_M (μM)	2.8 ± 0.7	2.3 ± 0.6
V_{max} (μM min ⁻¹)	0.68 ± .083	0.12 ± 0.025
k_{cat} (min ⁻¹)	0.135 ± 0.016	0.025 ± 0.005
k_{cat}/K_M (M ⁻¹ min ⁻¹)	4.82 × 10 ⁷	1.07 × 10 ⁷

As confirmed by previous work [24], we found that BACE2 also cleaves Aβ at the Leu34-Met35 peptide bond, which was in fact the initial and principal site of cleavage. Notably, cleavage at this position can only occur after production of full length Aβ by β- and γ-secretase, because this peptide bond in APP or in APP CTFs is normally embedded within the cell membrane [24]. This fact, together with the finding that Aβ34 is produced in cells overexpressing of BACE2 and APP, provides clear evidence that the Aβ-degrading activity of BACE2 contributes significantly to the overall Aβ-lowering effect of BACE2 overexpression, even in the context of concurrent θ-secretase activity.

Given that BACE2 can lower Aβ both by decreasing its production and by mediating its degradation, which of these mechanisms are relevant to the pathogenesis or the potential treatment of AD? The answer depends critically on precisely where and to what extent BACE2 is expressed in vivo. Although BACE2 protein is readily

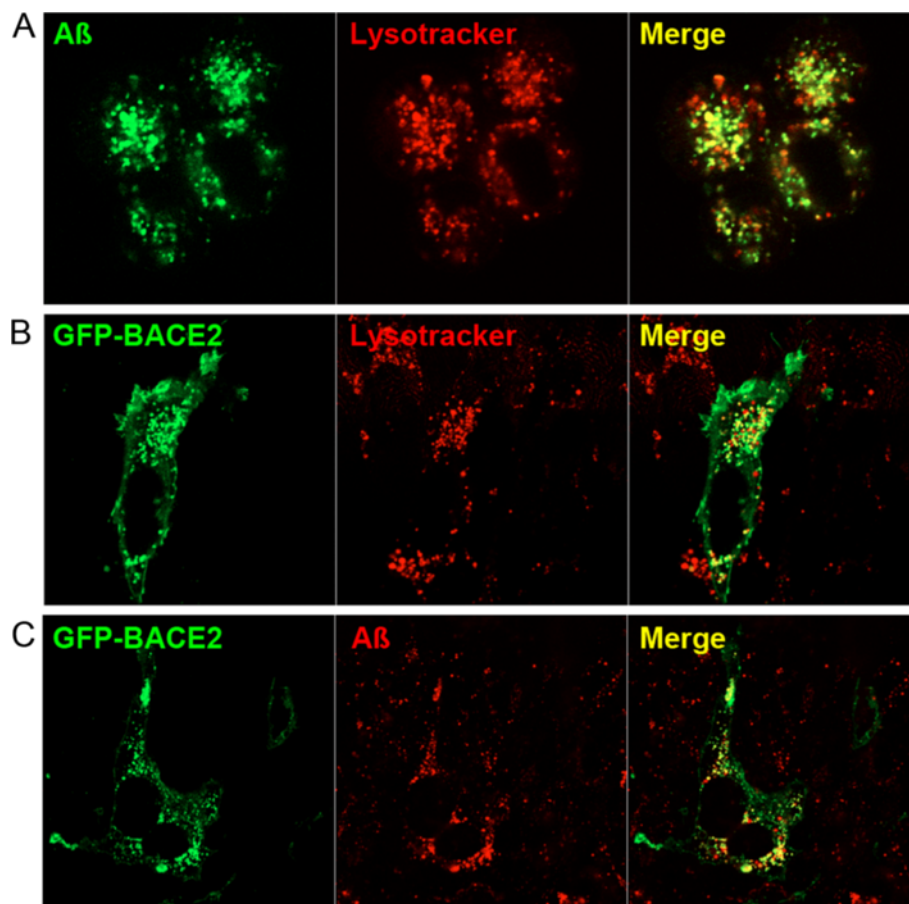


Figure 6 BACE2 is localized to intracellular compartments relevant to A β degradation. **A**, Exogenous administration of fluorescently labeled A β 40 (green) to CHO cells results in accumulation at intracellular sites overlapping with lysosomes, as labelled by Lysotracker Red (red) and visualized by confocal microscopy. **B**, BACE2 is expressed in multiple intracellular compartments, including lysosomes. Distribution of GFP-tagged BACE2 (green) in cells labeled with Lysotracker Red (red) shows significant localization within lysosomes (yellow). **C**, BACE2 colocalizes with exogenously administered A β . Confocal images showing significant overlap (yellow) between BACE2 (green) and fluorescently labeled A β (red). For these experiments, cells were imaged within 5 minutes of washing in cold PBS to remove medium containing excess fluorescently labeled A β . Note that the majority of BACE2-GFP-expressing cells contained very low levels of fluorescent A β (see Figure 7), and the particular cell shown exhibited relatively high levels of internalized A β , allowing us to highlight the overlap with BACE2.

detected in brain extracts [15,30-36], and its activity has even been shown to be comparable to that of BACE1 in post-mortem brain [31,33], there is conflicting evidence about which cell types express BACE2. Studies in mice, on the one hand, suggest that the protease is expressed abundantly in glia but only minimally in neurons [30]. To the extent that these findings apply to humans, θ -secretase cleavage of APP by BACE2 would be unlikely to play any significant pathophysiological role in AD, given that APP itself is expressed predominantly in neurons, with only modest expression levels in non-neuronal brain cells [31]. On the other hand, multiple studies in post-mortem human brain tissue have reported detectable BACE2 expression not only in astrocytes, but also in neurons [15,33], suggesting that the θ -secretase activity of BACE2 may, to some extent, contribute to the overall economy of brain A β . The

pathophysiological relevance of BACE2's function as an A β DP is similarly difficult to predict and likewise dependent on the extent to which the protease is expressed in neurons. Astrocytes are known mediate the clearance of A β [37], but the contribution of intra-astrocytic A β degradation relative to intraneuronal or extracellular degradation in vivo remains to be established. As was true for other A β DPs first identified in cells [9], the answer to these questions will require further study in relevant animal models.

Notwithstanding uncertainty about its role in AD pathogenesis, a number of considerations suggest that BACE2 represents an especially strong therapeutic candidate, particularly for gene therapy-based approaches. BACE2 can lower A β catalytically via two independent mechanisms, and its A β -degrading ability alone exceeds that of most other A β DPs, some of which are being

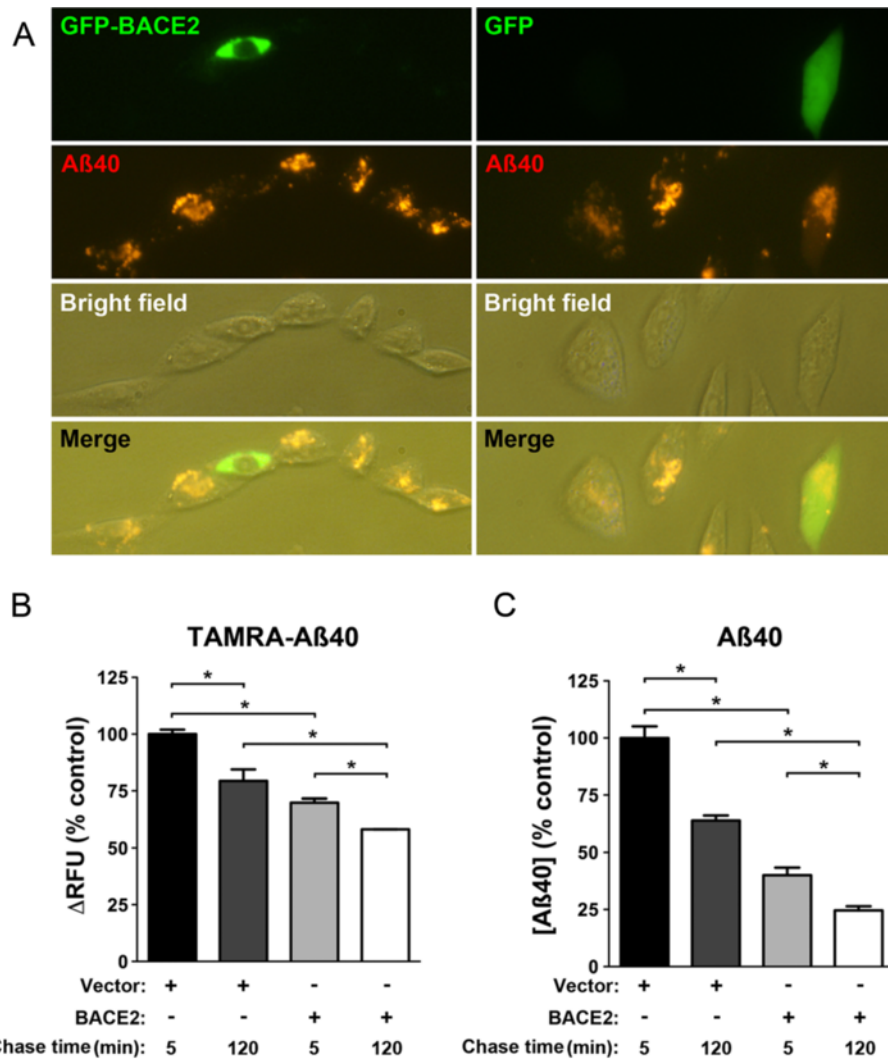


Figure 7 BACE2 degrades Aβ at intracellular sites. **A**, CHO cells expressing GFP-BACE2 (green, left), but not those expressing GFP alone (green, right), exhibit marked reductions in intracellular Aβ (red). For these experiments, cells were loaded for 6 h with 400 nM fluorescently labeled Aβ40, washed, then incubated at 37°C for 1 h prior to imaging by conventional fluorescence microscopy. **B,C**, BACE2 overexpression significantly lowers intracellular Aβ. **B**, Quantification of intracellular pools of fluorescently labeled Aβ40 in CHO cells 0 and 2 hours after loading. **C**, Relative levels of intracellular (unmodified) Aβ40 in CHO cells 0 and 2 hours after loading, as quantified by ELISA. Data are mean ± SEM of 3 replicates, normalized to vector-only controls. **P* < 0.05 by Tukey's multiple comparisons test.

considered for gene therapy clinical trials [38]. Moreover, as an aspartyl protease, BACE2 possesses distinct advantages relative to other AβDPs. First, it is operative with subcellular compartments most relevant to Aβ production—i.e., those containing active β- and γ-secretase, which are both aspartyl proteases—thus allowing it to impact Aβ levels prior to secretion. In this connection, there is growing evidence that intracellular Aβ may represent an especially pathogenic role in AD [39], so modulation of this pool may be particularly appropriate therapeutically. Second, because BACE2 is operative exclusively at intracellular sites, its expression could be readily restricted to the site of administration. This is in

contrast to many other AβDPs which are secreted and/or active extracellularly [19,40,41] and thus less capable of being confined to specific regions.

In conclusion, this study identifies BACE2 as a novel and highly efficient AβDP. This newly identified function of BACE2, together with its established ability to also lower Aβ production via θ-secretase activity, suggests that BACE2 may play a significant role in AD pathogenesis. Moreover, even if BACE2 plays no role in the etiology of AD, BACE2 nevertheless represents a particularly attractive candidate for gene therapeutic approaches to the treatment of prevention of this presently incurable disease.

Methods

cDNA screening

A library of 352 full-length, sequence verified, human cDNAs encoding diverse members of all protease classes was purchased from a commercial source (OriGene Technologies, Inc.) in 96-well format (100 ng/well). For negative and positive controls, a subset of blank wells on each plate were supplemented with empty vector or a construct expressing a well-established A β DP, human ECE1b [27], respectively (100 ng/well). As a source of human A β and also as a transfection control, each well was cotransfected with a hybrid construct, APP-AP (60 ng/well), comprised of a vector expressing wild-type human APP fused at its N-terminus with alkaline phosphatase (AP) [42]. Additional blank wells were left untreated for cell-free background controls. CHO cells (4.8×10^4 /well) suspended in DMEM/Opti-MEM supplemented with 5%FBS were then co-transfected with APP-AP and protease-encoding cDNAs using Fugene 6.0, according to manufacturer's recommendations (Promega Corp.). Transfected cells were allowed to grow overnight under standard cell culture conditions (5% CO₂; 37°C; 95% humidity) then the medium was exchanged. 24 h later, the conditioned media were collected for downstream analysis (see below). All experiments were conducted in compliance with and with approval by the Mayo Clinic Institutional Review Board.

AP activity

Following heat treatment to inactivate endogenous phosphatases (65°C for 15 min) present in the media, conditioned media (30 μ L/well) was added to 96-well plates containing AP substrate, 4-nitrophenylphosphate (170 μ L/well, 2 mg/mL), dissolved in AP buffer (1 M diethanolamine, 0.5 mM MgCl₂, 10 mM L-homoarginine, pH 9.8). Plates were incubated for 30 min and AP activity was determined from absorbance (OD₄₀₅) using a SpectraMax[®] M5^e multilabel plate reader (Molecular Devices).

A β ELISA

A β levels were quantified using a sandwich ELISA system based on antibody pairs 33.1.1/13.1.1 for A β 40 and 2.1.3/4 G8 for A β 42 as described previously [43]. Conditioned media were supplemented with Complete[™] Protease Inhibitor Cocktail (Roche) just after collection and analyzed immediately. For experiments quantifying intracellular A β , cells were plated in 96-well plates (2×10^4 cells per well) and transfected with BACE2-encoding cDNA or empty vector, washed, then incubated with 400 nM synthetic A β for 6 h. After washing with PBS, intracellular A β was extracted with 5 M guanidinium isothiocyanate and quantified using a commercially available ELISA (Wako Chemicals USA, Inc.)

after 10-fold dilution in the manufacturer-provided dilution buffer.

Mass spectrometry

The cleavage sites within A β 40 and A β 42 hydrolyzed by BACE2 and BACE1 were determined essentially as described [44] with minor modifications. Briefly, A β peptides or biotinylated A β peptides were incubated for various lengths of time with recombinant BACE2 enzyme in Assay Buffer (25 mM acetate buffer, pH 4.0, supplemented with 0.1% BSA). The reaction was stopped by addition of protease inhibitor cocktail and pH adjustment. A β fragments were immediately precipitated by magnetic beads coated with streptavidin (for biotinylated A β) or magnetic beads coated with Ab9 antibody [45] (for unmodified A β). Beads were washed with 10 mM NH₄CO₃, pH 8.0, and peptide fragments were eluted using 0.5% trifluoroacetic acid in 75% acetonitrile in water, followed by the addition of an equal volume of a saturated sinapic acid solution dissolved in 0.5% trifluoroacetic acid in 50% acetonitrile and water. Digested products were spotted onto a gold chip, dried, and analyzed using a Ciphergen ProteinChip SELDI time-of-flight system (Bio-Rad). Mass spectra were acquired automatically in a linear positive mode at 1350 shots per spectrum. Peptides containing a 183-Da increase in MW were identified as being modified by AEBSE, as previously reported [46]. Same procedure was applied to detect the endogenous A β fragments produced by CHO cells transfected with APP and BACE2 (using Ab9 as a capture antibody).

In vitro analyses of A β degradation by BACE2

The kinetics of A β 40 and A β 42 degradation by BACE2 were determined using freshly prepared, monomeric A β peptides separated from aggregated species by size-exclusion chromatography and characterized as described [25,26]. A β peptides were diluted in neutral Dilution Buffer (20 mM Tris, pH 8.0 supplemented with 0.1% BSA) and reactions were initiated by transfer into 20 times more volume of Assay Buffer supplemented with purified recombinant BACE2 (R&D Systems, nominal concentration 1 or 5 nM) or, as a control for non-specific loss of A β , the latter buffer lacking BACE2. Where required, reactions were terminated by supplementation with protease inhibitor cocktail and adjustment to neutral pH. For ELISA-based experiments, A β 42 and A β 40 were quantified using the sandwich ELISAs described above. For determination of kinetic parameters, ELISAs were used to quantify the initial velocities of degradation of a range of different concentrations of A β 40 (0.2 to 16 μ M) or A β 42 (0.6 to 16 μ M) by a fixed amount of recombinant BACE2 (5 nM) in Assay Buffer, and K_M and v_{max} values

were determined in triplicate by fitting a hyperbolic curve to these data in Prism 5.0 (GraphPad Software, Inc.). For determination of the pH dependence of A β degradation, experiments were carried same as described above, using Assay Buffer at different pH values (3.0, 3.5, 4.0, 4.5, 5.0, 5.5). The reactions were stopped at 10 min and the remaining 200nM of A β was determined using a well-characterized fluorescence polarization-based activity A β degradation assay, as described [23]. For comparison of the rate of degradation of A β by different proteases, we incubated 200 nM of A β fluorescent substrate (FA β B) with 5nM of different protease in their corresponding buffers: BACE1 and BACE2 using Assay Buffer and IDE, NEP, and plasmin in PBS, pH 7.4 supplemented with 0.1% BSA. The reactions were stopped by addition of protease inhibitor cocktail, 500nM streptavidin, and adjustment to neutral pH. The degree of A β hydrolysis was immediately determined using a polarization-based A β degradation assay [23]. Recombinant BACE2 (R&D Systems) and plasmin (EMD Biosciences) were purchased from commercial sources, while recombinant IDE and secreted NEP (i.e., lacking the transmembrane domain) were generated and purified as described [23]. All reactions were performed at 37°C.

Fluorescence microscopy

CHO cells (10^6 cells/cm²) were plated onto 8-well poly-D-lysine-coated, glass-bottom chambers (MatTek Corp.) in culture medium (DMEM/Opti-MEM supplemented with 5%FBS). For BACE2 transfections, cell were transfected with a construct encoding BACE2 tagged at its C-terminus with GFP (OriGene Technologies, Inc. Cat. No. RG04860) using Fugene 6.0 transfection reagent according to manufacturer's recommendations (Promega Corp.). For A β colocalization experiments, cells were washed twice in fresh culture medium, then incubated in the latter medium supplemented with either A β 40 (500 nM) labeled at the N-terminus with HiLight FluorTM 488 or HiLight FluorTM 555 (AnaSpec, Inc.). For lysosomal staining, cells were incubated with LysoTracker Red according to manufacturer's recommendations (Invitrogen Corp.), then washed 2 times with fresh culture medium prior to imaging. For confocal microscopy, cells were washed with fresh medium then imaged immediately using the 488-nm and 543-nm laser lines on a Zeiss LCM 510 META confocal microscope (Carl Zeiss, Inc.). Images were processed and analyzed using MetaMorph software according to manufacturer's recommendations (Molecular Devices, Inc.). For conventional fluorescence microscopy of intracellular A β , cells were washed with fresh medium, then incubated at 37°C for 1 h prior to imaging using a Nikon Labophot 2 fluorescent microscope (Nikon Inc.).

Abbreviations

A β : Amyloid β -protein; ABDP: A β -degrading protease; AD: Alzheimer disease; AP: Alkaline phosphatase; APP: Amyloid precursor protein; BACE1: β -site APP-cleaving enzyme-1; BACE2: β -site APP-cleaving enzyme-2; ECE1: Endothelin-converting enzyme-1; IDE: Insulin-degrading enzyme; NEP: Nephilysin.

Competing interests

The authors declare they have no competing interests.

Authors' contributions

SA-H contributed to the design of experiments, executed the screen and all follow up experiments, analyzed data, and drafted the manuscript. TS assisted with the execution of the primary screen. MM and DK assisted with the maintenance of cell cultures. ML conceived of the experimental approach, designed experiments, analyzed data and wrote the manuscript. All authors read and approved the final manuscript.

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