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BACE1 modulates filopodia-like protrusions induced by sodium channel β 4 subunit

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Abstract

Processing of APP by BACE1 plays a crucial role in the pathogenesis of Alzheimer disease (AD). Recently, the voltage-gated sodium channel (Na_v) $\beta 4$ subunit ($\beta 4$), an auxiliary subunit of Na_v that is supposed to serve as a cell adhesion molecule, has been identified as a substrate for BACE1. However, the biological consequence of BACE1 processing of $\beta 4$ remains illusive. Here we report the biological effects of $\beta 4$ processing by BACE1. Overexpression of $\beta 4$ in Neuro2a cells promoted neurite extension and increased the number of F-actin rich filopodia-like protrusions. While coexpression of BACE1 together with $\beta 4$ further accelerated neurite extension, the number of filopodia-like protrusions was reduced. Overexpression of C-terminal fragment of $\beta 4$ that was generated by BACE1 ($\beta 4$ -CTF) partially recapitulated the results obtained with BACE1 overexpression. These results suggest that the processing of $\beta 4$ by BACE1 regulates neurite length and filopodia-like protrusion density in neurons.

Introduction

The voltage-gated sodium channels (Na_v) consist of a complex of pore-forming α subunits and one or two β subunits [1]. There are five identified β subunits: $\beta 1$, $\beta 1A$, $\beta 2$, $\beta 3$, and $\beta 4$. β subunits are auxiliary components of the voltage-gated sodium channel and have been known to modulate sodium channel activity [1-3] and cell surface expression of α subunits [4-6].

$\beta 4$ is structurally related to $\beta 2$ and binds to the α subunit by disulfide bond [3]. Coexpression of $\beta 4$ with brain Na_v 1.2a or skeletal muscle Na_v 1.4 subunits in the human embryonic cell line tsA-201 resulted in negative shift in the voltage dependence of channel activity [3]. $\beta 4$ has also been shown to be the endogenous open-channel blocker responsible for resurgent kinetics [7]. Furthermore, $\beta 4$ expression is downregulated in Huntington disease (HD) transgenic mice [8].

Based on structural and amino acid homologies, β subunits belong to the immunoglobulin superfamily of cell adhesion molecules (IgCAMs) [9, 10]. In the nervous system, IgCAMs play important roles in cell-cell interactions, such as axon guidance and migration [11]. Recent reports strongly suggest that β subunits play a functional role in neurite outgrowth as IgCAMs. $\beta 1$ and $\beta 2$ exhibit strong homophilic cell adhesion leading to ankyrin recruitment at sites of cell-cell contact [12]. $\beta 1$ promotes neurite extension in cerebellar granule neurons [13]. Recently we have reported that overexpression of $\beta 4$ induces neurite extension and increases spine density in cultured cells, indicating that $\beta 4$ modulates neurite outgrowth activity [8].

β -site APP cleaving enzyme (BACE1) is a membrane-bound aspartyl protease that cleaves amyloid precursor protein (APP). Sequential processing of APP by BACE1

and γ -secretase leads to the release of amyloid- β peptide ($A\beta$) which accumulates in senile plaques in Alzheimer disease (AD) [14]. Although the role of BACE1 as APP processing enzymes is well established, the physiological consequence of other processing is not clear.

Recently we and others have reported that voltage-gated sodium channel β subunits are new substrates for BACE1 and γ -secretase [15, 16] but the physiological function of this sequential processing has not been determined. We report in this paper that BACE1 modulates β 4-induced neurite morphology in neuroblastoma (Neuro2a) cells.

Materials and methods

Plasmids. Mouse sodium channel β 4 subunit was subcloned as described previously [8]. C-terminal fragment of β 4 (β 4-CTF) (nucleotides 358-687 of full length β 4 cDNA, GenBankTM accession number **BK001031**) was amplified by PCR using the oligonucleotide primers anchored with BamHI (5'-end) and NotI (3'-end) restriction sites. The forward primer was 5'-gactacggatccgacctggagttcagtgacac-3' and the reverse primer was 5'-aaagacgcggccgctcacacttttgtgggtggct-3'. PCR was carried out for 35 cycles with each cycle consisting of 30 sec at 94 °C, 30 sec at 50 °C and 30 sec at 68 °C using KOD plus (Toyobo, Osaka, Japan). The PCR products were then digested with BamHI and NotI and subcloned into the expression vector pSecTag2/HygroB (Invitrogen, Carlsbad, California). Human BACE1 cDNA (nucleotides 408-2034; GenBankTM accession number **NM_012104**) was amplified by PCR using the

oligonucleotide primers anchored with BamHI (5'-end) and XhoI (3'-end) restriction sites. The forward primer was 5'-gactacggatccagggacggacgtgggccagt-3' and the reverse primer was 5'-aaagacctcgagcttgtgaccaaagtgaacca-3'. PCR was carried out for 35 cycles with each cycle consisting of 30 sec at 94 °C, 30 sec at 50 °C and 2 min at 68 °C using KOD plus. The PCR products were then digested with BamHI and XhoI and subcloned into the expression vector pcDNA3 (Invitrogen). The sequences of all the constructs were verified by sequencing.

Antibodies. The antibodies used were as follows: rabbit polyclonal anti- β 4 [8]; mouse monoclonal anti-GFP (Roche Diagnostics, Basel, Switzerland); rat monoclonal anti-tubulin (Chemicon, Temecula, CA); goat anti-mouse IgG (H+L)-Alexa Fluor 488 and goat anti-rabbit IgG (H+L)-Alexa Fluor 647 (Invitrogen) were utilized as secondary antibodies in indirect immunofluorescence.

Cell culture, transient transfection and treatments. Mouse neuroblastoma (Neuro2a) cells were maintained as described previously [8]. Neuro2a cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. After transfection, cells were differentiated with 5 mM dbcAMP [*N*6,2'-*O*-dibutyryladenosine-3':5'-cyclic monophosphate sodium salt (Nacalai Tesque, Kyoto, Japan)].

F-actin staining, immunocytochemistry, image analysis and quantification. Cells were fixed and permeabilized as described previously [8]. Cells were incubated 1 hour at room temperature with Alexa Fluor 546 Phalloidin (Invitrogen) which labeled filamentous actin (F-actin). Immunocytochemistry was carried out as described previously [8]. To identify the neurites and filopodia-like protrusions, Neuro2a cells were transfected with Venus and immunostained by anti-GFP. Image analysis were

performed as previously described with slight modification [17-19]. Protrusions, longer than the soma diameter, were classified as neurite. About 150 cells from 10 random fields were selected and longest neurite was measured with the MacSCOPE program (Mitani, Tokyo, Japan). The number of filopodia-like protrusions was counted on any neurite for 30 μm from at least 20 randomly selected cells.

Statistics. Data were expressed as mean \pm standard error of the mean (SEM). Neurite length and the number of filopodia-like protrusions were compared by Student's *t*-test or ANOVA followed by Fisher's Protected Least Significant Difference (PLSD).

Results

Overexpression of $\beta 4$ extends length of neurites and increases the number of filopodia-like protrusions

We previously reported that overexpression of $\beta 4$ induced neurite extension and increased number of branch point [8]. To investigate other morphological changes in $\beta 4$ expressing cells, Neuro2a cells were transiently transfected with $\beta 4$ or mock and differentiated by 5 mM dbcAMP. To visualize neurites, cells were cotransfected with Venus, a variant of yellow fluorescent protein (YFP) [20]. Neuro2a cells were immunostained with anti- $\beta 4$ antibodies 24 h after transfection. Because Venus signal is not enough to visualize small structures in neurite, Neuro2a cells were also stained by anti-GFP. Quantification of GFP-positive neurites by McSCOPE software showed that neurites were significantly longer in cells expressing $\beta 4$, ($63.9 \mu\text{m} \pm 3.3 \mu\text{m}$) compared

with mock cells ($42.1 \mu\text{m} \pm 1.2 \mu\text{m}$) (Fig. 1A-D, I) confirming our previous observation using ArrayScanTM [8]. In addition to the extension of neurites, overexpression of $\beta 4$ also led to the increased number of small and thin protrusions (2.8 ± 0.2 per $10 \mu\text{m}$ of neurite) compared with mock cells (1.8 ± 0.2 per $10 \mu\text{m}$) (Fig. 1E-H, J). The features of $\beta 4$ -induced protrusions is similar to the thin, small and headless dendritic filopodia that have been previously reported [21].

Dendritic filopodia are highly motile structures that may play important roles in early synaptogenesis, especially in the process of axon-dendrite contact, and they are believed to be a precursor of the spine [21]. During spine morphogenesis, F-actin is observed in dendritic filopodia and it forms cluster when filopodia-to-spines transition occurred [22]. Thus, it is widely accepted that actin cytoskeleton dynamics is involved in spine formation [21, 22]. In addition to actin assembly, microtubules transport has also been reported to be one of the underlying molecular mechanisms of neurite formation [23, 24]. To examine whether $\beta 4$ -induced neurites and protrusions contain F-actin or microtubules, Neuro2a cells were transfected with $\beta 4$ and stained by phalloidin (a high-affinity probe for F-actin) and anti-tubulin antibody. Immunocytochemical analysis showed that $\beta 4$ -induced neurites were stained by anti-tubulin (Fig. 2A). In contrast, $\beta 4$ -induced protrusions were stained by phalloidin (Fig. 2B). These results indicate that $\beta 4$ -induced protrusions contain F-actin and are similar to dendritic filopodia. Thus, we called $\beta 4$ -induced protrusions “filopodia-like protrusions” in this paper.

Overexpression of BACE1 accelerates neurite extension and reduces the number of filopodia-like protrusions induced by $\beta 4$

We have previously shown that $\beta 4$ is a novel substrate of BACE1 but the physiological role of the cleavage is unknown [15]. BACE1 generates an N-terminal fragment ($\beta 4$ -NTF) and a C-terminal fragment ($\beta 4$ -CTF) as shown in Fig. 3A. And indeed, 15-kDa C-terminal fragment of $\beta 2$ of Na_v ($\beta 2$ -CTF) inhibits $\beta 2$ -mediated cell migration [16]. As $\beta 4$ is structurally similar to $\beta 2$ [3] and can also be cleaved by BACE1, we wondered whether BACE1 cleavage of $\beta 4$ would modulate neurite extension and filopodia-like protrusions.

To make this examination possible, Neuro2a cells were cotransfected with $\beta 4$ and BACE1. $\beta 4$ and mock cotransfected cells were used as control. Quantification of GFP-positive neurites revealed that overexpression of BACE1 accelerated neurite extension induced by $\beta 4$ ($70.9 \mu\text{m} \pm 2.5 \mu\text{m}$) compared with control ($60.5 \mu\text{m} \pm 3.0 \mu\text{m}$) (Fig. 3B, C, H). In contrast, no difference was observed in the neurite length between protease-inactive BACE1 (D93G) [25] expressing cells ($60.4 \mu\text{m} \pm 2.5 \mu\text{m}$) and control (Fig. 3C, D, H). On the other hand, overexpression of BACE1 markedly decreased the number of filopodia-like protrusions induced by $\beta 4$ (0.9 ± 0.2 per $10 \mu\text{m}$) when compared with control (3.0 ± 0.2 per $10 \mu\text{m}$) (Fig. 3E, F, I) and inactive BACE1 (D93G) did not affect the number of filopodia-like protrusions (2.7 ± 0.2 per $10 \mu\text{m}$) (Fig. 3F, G, I). BACE1 transfection without $\beta 4$ had no effect on neurite length and the number of filopodia-like protrusions (data not shown). These results indicate that BACE1 regulates neurite extension and the number of filopodia-like protrusions induced by $\beta 4$.

To further examine whether $\beta 4$ -NTF or $\beta 4$ -CTF was responsible for the modulation of neurite length and protrusions density, we transiently expressed $\beta 4$ -CTF

in Neuro2a cells. β 4-CTF localized in membrane and their distribution (Fig. 4C) was similar to that of β 4 (Fig. 4B). Quantification of GFP-positive neurites showed that overexpression of β 4-CTF promoted neurite extension ($60.3 \mu\text{m} \pm 3.0 \mu\text{m}$) that was comparable to that of β 4 ($57.0 \mu\text{m} \pm 3.0 \mu\text{m}$) when compared with mock ($36.8 \mu\text{m} \pm 1.4 \mu\text{m}$) (Fig. 4D, E, H). These data indicate that it is likely that β 4-CTF promotes neurite extension. In contrast, overexpression of β 4-CTF did not increase the number of filopodia-like protrusions (1.6 ± 0.2 per $10 \mu\text{m}$) that was comparable to that of mock (1.4 ± 0.2 per $10 \mu\text{m}$) when compared with β 4 (2.8 ± 0.2 per $10 \mu\text{m}$) (Fig. 4F, G, I), indicating that β 4-CTF significantly attenuates the formation of filopodia-like protrusions. These results corresponded with the results of overexpression of β 4 and BACE1. Taken together, our data suggest that the cleavage of β 4 by BACE1 modulates the number of filopodia-like protrusions induced by β 4.

Discussion

In this study, we report the biological role of BACE1 cleavage of β 4. First, overexpression of β 4 promoted neurite extension and increased the number of F-actin rich protrusions which were small, thin, and similar to dendritic filopodia in Neuro2a cells (Fig. 1, 2). Second, overexpression of BACE1 caused further neurite extension and significantly decreased the density of filopodia-like protrusions induced by β 4 (Fig. 3). Finally, overexpression of β 4-CTF extended neurite but not induce increasing the number of filopodia-like protrusions (Fig. 4). These results suggest that BACE1 regulates neurite morphology induced by β 4 in Neuro2a cells.

Overexpression of $\beta 4$ caused neurite extension and increased the number of filopodia-like protrusions, suggesting that $\beta 4$ can function as a cell adhesion molecule and may be involved in neurite morphological changes. Feature and structure of $\beta 4$ -induced protrusions were similar to dendritic filopodia, which are small, thin and F-actin rich protrusions [21, 22]. It has been proposed that filopodia play important roles in synaptogenesis [21]. Recently we reported that overexpression of $\beta 4$ increased spine density in hippocampal primary neuron [8]. Our results suggest that $\beta 4$ may be involved in spine morphogenesis.

We found that BACE1 markedly decreased the number of filopodia-like protrusions induced by $\beta 4$. We also found that $\beta 4$ -CTF did not increase the number of filopodia-like protrusions. The results suggest that extracellular domain of $\beta 4$ may play important roles in the formation of filopodia-like protrusions. Voltage-gated sodium channel β subunits interact with other cell adhesion molecules and extracellular matrix through their extracellular region. For example, $\beta 1$ interacts with neurofascin, N-cadherin, connexin and contactin [5, 6, 26]. Both $\beta 1$ and $\beta 2$ interact with tenascin-C and tenascin-R [27, 28]. $\beta 4$ -NTF may interact with cell adhesion molecules or extracellular matrix and these interactions may be important for the formation of filopodia-like protrusions.

BACE1 are preferentially associated with lipid rafts and BACE1 cleavage occurs predominantly in lipid raft [29, 30]. Recently, we reported that $\beta 4$ is also associated with lipid raft [15]. These results indicate that lipid raft may play important roles in $\beta 4$ processing by BACE1. Not only $\beta 4$ but also other β subunits are cleaved by BACE1 [15]. It is possible that physiological functions of $\beta 1$, $\beta 2$ and $\beta 3$ subunits are also regulated by BACE1 in lipid raft.

Overexpression of $\beta 4$ in mouse hippocampal primary neurons increased the number of spines [8] and $\beta 4$ -induced F-actin rich protrusions in Neuro2a cells are similar to dendritic filopodia. These results suggest that $\beta 4$ may be related to spine morphogenesis and maintenance. Synapse and dendritic spine loss are common pathological features in neurodegenerative disease [31]. During the course of AD and aging, increases of BACE1 protein and its activity levels have been reported [32, 33]. These reports suggest that aberrant $\beta 4$ and processing of other substrates by BACE1 may lead to neuritic degeneration. Our findings may provide new insight into both understanding the function of BACE1 and pathogenic mechanism of neurodegenerative disorders.

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Figure legends

Fig. 1. Overexpression of $\beta 4$ induces neurite extension and increases formation of filopodia-like protrusions in Neuro2a cells. (A-D) Low magnification images of mock (A, B) and $\beta 4$ (C, D) overexpressing Neuro2a cells immunostained by anti-GFP (green) and anti- $\beta 4$ (red). Arrows indicate neurite (A, C). Scale bar, 50 μm . (E-H) High magnification images of mock (E, F) and $\beta 4$ (G, H) overexpressing Neuro2a cells. Arrowheads indicate protrusions (E, G). Scale bar, 5 μm . (I) Quantification of the neurite length. $\beta 4$ -positive cells extended neurite length compared with mock cells.

Mock: $n = 145$; $\beta 4$: $n = 123$ (** $p < 0.0001$, Student's t -test). (J) Quantification of the number of filopodia-like protrusions. $\beta 4$ expressing cells significantly increased the number of filopodia-like protrusions. Mock: $n = 20$; $\beta 4$: $n = 20$ (* $p < 0.005$, Student's t -test). Error bars indicate SEM.

Fig. 2. $\beta 4$ -induced neurite contain microtubules and protrusions are rich in F-actin. (A) Low magnification image (B) and high magnification image of $\beta 4$ overexpressing Neuro2a cells were stained by anti-tubulin (green) and phalloidin (red). Neurites were mainly immunostained by anti-tubulin (arrow) (A). Scale bar, 20 μm . $\beta 4$ -induced protrusions were stained by phalloidin (arrowheads) (B). Scale bar, 5 μm .

Fig. 3. Overexpression of BACE1 reduces the number of filopodia-like protrusions. (A) Proteolytic processing of $\beta 4$ by BACE1. BACE1 cleavage generates $\beta 4$ -NTF and $\beta 4$ -CTF. NTF, N-terminal fragment; CTF, C-terminal fragment. The number indicates amino acid residue of full length $\beta 4$. (B-D) Low magnification images of Neuro2a cells transfected with $\beta 4$ together with mock (B), BACE1 (WT) (C) or protease-inactive BACE1 (D93G) (D). Neuro2a cells were immunostained by anti-GFP. Arrows indicates neurite (B-D). Scale bar, 50 μm . (E-G) High magnification images of Neuro2a cells transfected with $\beta 4$ together with mock (E), BACE1 (WT) (F) or BACE1 (D93G) (G). $\beta 4$ and mock transfected cells were used as control. Arrowheads indicate filopodia-like protrusions (E-G). Scale bar, 5 μm . (H) Quantification of the neurite length. Overexpression of BACE1 (WT) accelerated neurite extension in $\beta 4$ expressing Neuro2a cells. In contrast, overexpression of BACE1 (D93G) did not accelerate neurite extension. $\beta 4$ /Mock: $n = 122$; $\beta 4$ /BACE1 (WT): $n = 125$; $\beta 4$ /BACE1 (D93G): $n = 118$

(* $p < 0.003$, ANOVA followed by Fisher's PLSD). (I) Quantification of the number of filopodia-like protrusions. BACE1 (WT) markedly decreased the number of filopodia-like protrusions when compared with control. In contrast, there are no differences between BACE1 (D93G) expressing cells and control cells. $\beta 4$ /Mock: $n = 20$; $\beta 4$ /BACE1 (WT): $n = 20$; $\beta 4$ /BACE1 (D93G): $n = 20$ (** $p < 0.0001$, ANOVA followed by Fisher's PLSD). Error bars indicate SEM.

Fig. 4. Overexpression of $\beta 4$ -CTF extends neurite but not increase the number of filopodia-like protrusions. (A) A schematic diagram of construction of $\beta 4$ -CTF cloned into pSecTag2B. CTF, C-terminal fragment. (B, C) Subcellular localization of $\beta 4$ (B) and $\beta 4$ -CTF (C) expressed in Neuro2a cells immunolabeled by anti- $\beta 4$. (D, E) Low magnification images of $\beta 4$ (D) and $\beta 4$ -CTF (E) overexpressing Neuro2a cells immunostained by anti-GFP. Arrows indicate neurite. Scale bar, 50 μm . (F, G) High magnification images of $\beta 4$ (F) and $\beta 4$ -CTF (G) overexpressing Neuro2a cells. Arrowheads indicate filopodia-like protrusions. Scale bar, 5 μm . (H) Quantification of the neurite length. Overexpression of $\beta 4$ -CTF induced neurite extension almost same length as $\beta 4$ expressing cells. Mock: $n = 109$; $\beta 4$: $n = 120$; $\beta 4$ -CTF: $n = 120$. (* $p < 0.0001$, ANOVA followed by Fisher's PLSD) (I) Quantification of the number of filopodia-like protrusions. The number of filopodia-like protrusions did not increase in $\beta 4$ -CTF expressing cells compared with $\beta 4$ expressing cells. Mock: $n = 20$; $\beta 4$: $n = 20$; $\beta 4$ -CTF: $n = 20$ (* $p < 0.0001$, ANOVA followed by Fisher's PLSD). Error bars indicate SEM.