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Extracellular release of BACE1 holoproteins from human neuronal cells

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Abstract

BACE1 is a membrane-bound aspartyl protease involved in production of the Alzheimer's amyloid β-protein. The BACE1 ectodomain is partially cleaved to generate soluble BACE1, but the physiological significance of this event is unclear. During our characterization of BACE1 shedding from human neuroblastoma SH-SY5Y cells stably expressing BACE1, we unexpectedly found that detectable amounts of BACE1 holoproteins were released extracellularly along with soluble BACE1. Treatment with the metalloprotease inhibitor, TAPI-1, inhibited BACE1 shedding but increased BACE1 holoprotein release. Soluble and full-length BACE1 were released in parallel, at least partly originating from the plasma membrane. Furthermore, the release of soluble BACE1, but not full-length BACE1, was increased by deletion of the C-terminal dileucine motif, indicating that dysregulated BACE1 sorting affects BACE1 shedding. These findings suggest that the release of BACE1 holoproteins may be a physiologically relevant cellular process.

Keywords: Alzheimer's disease; BACE1; β-Secretase; Metalloprotease; Shedding

Cerebral accumulation of amyloid β-protein (Aβ) is the main pathological feature of Alzheimer's disease (AD). Aβ is generated through serial cleavages of the amyloid precursor protein (APP) by β- and γ-secretases. APP is alternatively processed by α-secretase in a step that precludes Aβ production [1]. An aspartyl protease called BACE1 (β-site APP cleaving enzyme) was recently identified as β-secretase [2–5].

BACE1 is predominantly expressed by neurons in the brain, while its homolog BACE2 is expressed ubiquitously [6–8]. BACE1 is a type 1 integral membrane protein with N-linked glycosylation and has two active site motifs of aspartyl proteases in the lumenal domain. BACE1 is predominantly localized in endosomes and in the Golgi/TGN (*trans*-Golgi network), but is also present at the plasma membrane [2,9–12]. The cytoplasmic domain of BACE1 harbors a dileucine-based consensus motif (DxxLL) that is thought to mediate its cellular trafficking [10,13,14]. Recent evidence has indicated that mature BACE1 is partly cleaved within its extracellular domain to generate soluble BACE1 for secretion [15,16]. However, the precise cleavage site and protease(s) responsible for this ectodomain shedding are not yet known. In addition, it is unclear whether BACE1 shedding occurs at the plasma membrane and what physiological role this process might play.

The importance of BACE1 in AD pathogenesis has been highlighted by recent reports that the expression and activity levels of BACE1 are increased in the brains of sporadic AD patients [17–19]. Furthermore, BACE1 knockout abolished Aβ production and BACE1-deficient mice did not display overt abnormalities [20–22], suggesting that BACE1 could be a feasible therapeutic target for AD [23]. Therefore, it is important to fully elucidate the mechanisms of BACE1 protein metabolism and cellular transport, with an eye towards developing new insights into AD pathogenesis and therapeutics.

We used human neuronal cells expressing BACE1 as a model for characterizing the shedding of BACE1. During the course of this analysis, we unexpectedly observed that

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full-length BACE1 (FL-BACE1) is released extracellularly along with soluble BACE1. The results of our extensive study on this novel and interesting observation suggest that the release of BACE1 holoproteins may be a physiologically relevant cellular process.

Materials and methods

cDNA constructs and transfection. A human BACE1 cDNA fused with a C-terminal rhodopsin tag was subcloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA); this plasmid was generously provided by Dr. Michael Farzan [24]. The plasmid was transfected into human neuroblastoma SH-SY5Y cells by the calcium phosphate method, and stable transformants were selected with 400 μg/ml G418. A mutant BACE1 cDNA lacking Leu499Leu500 in the cytoplasmic tail (BACE1DelLL) was generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's instructions. The utilized mutagenesis primers were 5′-GCTGATGACATCTCCAA GGGCACCGAGACC-3′ and 5′-GGTCTCGGTGCCCTTGGAGATGT CATCAGC-3′. The resultant cDNA was sequenced, and stable transfectants were generated as described above. The SH-SY5Y cells stably expressing APP were as previously described [25].

Antibodies and chemicals. Rabbit polyclonal anti-BACE1 antibody (NBA) was raised against amino acid residues 102–127 of BACE1 and purified with a column (HiTrap NHS-activated, Amersham Biosciences, Piscataway, NJ, USA) coupled with the peptide used for immunization. We also used two commercial BACE1 antibodies: mouse monoclonal anti-BACE1 ectodomain antibody (MAB9311, R&D Systems, Minneapolis, MN, USA) and rabbit polyclonal anti-BACE1 C-terminal antibody (M-83, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The mouse monoclonal 1D4 antibody to the rhodopsin tag [26] was obtained from the University of British Columbia. The goat polyclonal anti-APP ectodomain antibody (207) [27] was provided by Dr. Steven G. Younkin, and the mouse monoclonal anti-APP antibody (22C11) was purchased from Chemicon (Temecula, CA, USA). TAPI-1 was obtained from Calbiochem (San Diego, CA, USA).

Amino acid sequence analysis of the BACE1 C-terminal fragment (CTF). Cells were collected from twelve 15-cm dishes and homogenized in RSB buffer (10 mM Tris, pH 7.5, 20 mM KCl, and 1.5 mM $MgAc_2$), and the post-nuclear fraction was ultracentrifuged at 100 000*g* for 1 h. Membrane proteins were extracted from the pellet with lysis buffer (20 mM Hepes, pH 7.2, 0.1 M KCl, 2 mM EDTA, and 2 mM EGTA) containing 0.5% NP-40 and protease inhibitors, and subjected to a 5–25% glycerol gradient containing 0.1% NP-40 in the same buffer. After centrifugation at 40,000 rpm for 16 h at 4 °C in a SW41 Ti rotor (Beckman, Fullerton, CA, USA), 12 fractions (1 ml each) were collected and examined for FL-BACE1 and BACE1 CTF by immunoblotting with the 1D4 antibody. The BACE1 CTF was then immunoprecipitated with 1D4, separated by Tris–Tricine SDS–PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, and visualized with Coomassie brilliant blue staining. The amino acid sequence was subsequently analyzed using a protein sequencer (Procise Model 492, Applied Biosystems, Foster City, CA, USA).

Immunoblotting. Immunoblot analyses were performed as described previously [25,28]. Cells were lysed in RIPA buffer containing protease inhibitors. Proteins were separated on 8 or 10% polyacrylamide gels and blotted onto PVDF membranes. Blots were blocked in phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 5% non-fat dried milk, and probed with anti-BACE1 antibodies. Membranes were subsequently incubated with a secondary peroxidase-labeled anti-rabbit IgG, and protein expression was detected with chemiluminescence reagents (Perkin-Elmer, Boston, MA, USA). The protein bands were quantified with an image analyzer LAS-1000 (Fuji Film, Tokyo, Japan).

Immunoprecipitation. Cells were cultured on 6-cm dishes and grown overnight in serum-free DMEM/F12 containing N2 supplements (Invitrogen). Conditioned media were harvested, mixed with NP-40 (0.1%), Tris, pH 8 (10 mM), NaCl (150 mM), and protease inhibitors, and then incubated overnight at 4 °C with anti-BACE1 ectodomain antibody (MAB9311) and protein G–agarose. Immunoprecipitated materials were subjected to immunoblot analysis with BACE1 N-terminal (NBA) or C-terminal (M-83) antibodies. The anti-APP 207 and 22C11 antibodies were used for analysis of APP secretion from APP-expressing cells [29].

Metabolic labeling and pulse-chase experiments. Cells were plated on 6-cm dishes at a density of 2×10^6 cells/dish. After preincubation in methionine/cystine-free DMEM containing N2 supplements for 1 h, cells were labeled with 100 μCi/ml EXPRE^{35}S ^{35}S Protein Labeling Mix (Perkin-Elmer) for 1 h in the same medium. Cells were rinsed with DMEM and chased for an appropriate time in DMEM containing N2 supplements. Cells were lysed in RIPA buffer as above, and media were collected and mixed with NP-40 (0.1%) and protease inhibitors. The cell lysates and conditioned media were incubated with either MAB9311 antibody or 1D4 antibody and protein G–agarose overnight, and immunoprecipitates were subjected to 10% SDS–PAGE. The gel was dried and analyzed using a BAS5000 bio-image analyzer (Fuji Film, Tokyo, Japan).

Cell surface biotinylation. Cell surface biotinylation was performed using a Sulfo-NHS-LC-Biotinylation Kit (Pierce, Rockford, IL, USA) essentially as described [30]. Briefly, cells cultured on 6-well plates were rinsed with ice-cold PBS and incubated with PBS containing 0.5 mg/ml Sulfo-NHS-LC-Biotin for 30 min at 4 °C. Cells were rinsed three times with PBS containing 20 mM glycine and twice with PBS alone, and were then returned to growth media (serum-free DMEM/F12 containing N2 supplements) and incubated at 37 °C for 1–3 h. Conditioned media were harvested and cells were lysed in RIPA buffer as above. The media and cell lysates were incubated with avidin–agarose, and bound proteins were eluted by incubation in 2× Laemmli sample buffer at 95 °C for 20 min, and analyzed by immunoblotting with anti-BACE1 antibodies.

Results

Identification of the shedding-associated BACE1 cleavage site and detection of full-length BACE1 in conditioned media

To investigate the mechanisms of BACE1 protein processing, we transfected human neuroblastoma SH-SY5Y cells with a plasmid encoding BACE1 with a short C-terminal rhodopsin tag [24] (Fig. 1A). We successfully established a stable transformant with high expression of BACE1 (designated SH-BA cells). Cell lysates were immunoblotted with a 1D4 antibody raised against the rhodopsin tag, which detected not only full-length BACE1 (FL-BACE1) but also a ~10 kDa C-terminal fragment (CTF) of BACE1 (Fig. 1B). To determine the shedding-associated cleavage site within BACE1, we purified the tagged BACE1 CTF with glycerol gradient fractionation followed by immunoprecipitation with the 1D4 antibody and subjected the purified protein to amino acid sequencing. The N-terminal sequence of the BACE1 CTF was VEGPxxTL, indicating that cleavage occurred between Ala429 and Val430 (Fig. 1A).

Immunoprecipitation Western blot analyses using antibodies against the BACE1 ectodomain (MAB9311 and NBA) revealed that soluble BACE1 (~63 kDa) was present in conditioned media from SH-BA cells (Fig. 1C). Interestingly, we also detected a minor band of ~70 kDa that was immunoreactive with the anti-BACE1 N-terminal antibody (NBA). This band was also recognized by the anti-BACE1 C-terminal antibody (M-83), and had the same molecular size as mature BACE1 in cell lysates (Fig. 1C), indicating the presence of FL-BACE1 in the conditioned media.