Characterization of the Ectodomain Shedding of the β-Site Amyloid Precursor Protein-cleaving Enzyme 1 (BACE1)*

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Ishrut Hussain‡, Julie Hawkins, Aarti Shikotra, David R. Riddell, Andrew Faller, and Colin Dingwall.

From the Neurology and Gastrointestinal Centre of Excellence for Drug Discovery, GlaxoSmithKline Research & Development Limited, New Frontiers Science Park, Third Avenue, Harlow, Essex, CM19 5AW, United Kingdom

Generation of the amyloid peptide through proteolytic processing of the amyloid precursor protein by β- and γ-secretases is central to the etiology of Alzheimer’s disease. β-secretase, known more widely as the β-site amyloid precursor protein cleaving enzyme 1 (BACE1), has been identified as a transmembrane aspartic proteinase, and its ectodomain has been reported to be cleaved and secreted from cells in a soluble form. The extracellular domains of many diverse proteins are known to be cleaved and secreted from cells by a process known as ectodomain shedding. Here we confirm that the ectodomain of BACE1 is secreted from cells and that this process is up-regulated by agents that activate protein kinase C. A metalloproteinase is involved in the cleavage of BACE1 as hydroxamic acid-based metalloproteinase inhibitors abolish the release of shed BACE1. Using potent and selective inhibitors, we demonstrate that ADAM10 is a strong candidate for the BACE1 sheddase. In addition, we show that the BACE1 sheddase is distinct from α-secretase and, importantly, that inhibition of BACE1 shedding does not influence amyloid precursor protein processing at the β-site.

The extracellular domains of a diverse range of cell proteins are cleaved and released from cells in a soluble form by a process known as ectodomain shedding. Membrane-anchored proteins that undergo such cleavage include cytokines, cytokine receptors, growth factors, growth factor receptors, cell adhesion molecules (1), and proteins of unknown function, including the amyloid precursor protein (APP) (2). The ectodomain shedding of these proteins exhibits three main characteristics. First, shedding is enhanced by agents, such as phorbol esters, that activate protein kinase C (3, 4). Second, shedding is sensitive to hydroxamic acid-based metalloproteinase inhibitors (1, 5, 6). Third, shedding appears to occur at or near the cell surface (7–9).

A number of enzymes are known to be involved in the ectodomain shedding of cell surface proteins. The first sheddase to be identified was the tumor necrosis factor α (TNFα)−converting enzyme (TACE) (10, 11), which cleaves the ectodomain of pro-TNFα. TACE, also known as ADAM17, is a member of the α-disintegrin and metalloproteinase (ADAM) family of metalloproteinases (12, 13) and has been reported to mediate ectodomain shedding of other proteins such as tumor necrosis factor (14), interleukin-1 receptor (15), and APP by cleavage at the α-site (16, 17). In addition to TACE, two other members of the ADAM family of metalloproteinases, ADAM9 and ADAM10, have also been proposed as α-secretases (18, 19). ADAM9 and ADAM10, in common with other sheddase activities, exhibit regulation by protein kinase C and inhibition by hydroxamic acid-based metalloproteinase inhibitors.

Proteolytic processing of the APP appears to be central to the etiology of Alzheimer’s disease (AD). Cleavage of APP by β- and γ-secretases generates the amyloidogenic peptide Aβ, which aggregates to form senile plaque (20). In an alternative non-amyloidogenic pathway, cleavage of APP by α-secretase within the β-amyloid region of APP precludes the release of intact Aβ. β-Secretase has been identified as a novel transmembrane aspartic proteinase, BACE1, and this proteinase fulfills all the key criteria for β-secretase (21–26). BACE1 is highly expressed in neurons in the brain, and overexpression of BACE1 in cultured cells and transgenic mice using a neuronal specific promoter results in an increased production of β-secretase-derived APP cleavage products (21, 22, 27). In addition, BACE1 knockout mice appear phenotypically normal (28, 29), making BACE1 a very attractive therapeutic target for the treatment of AD.

Recently it was reported that the extracellular domain of BACE1 is cleaved and released from cells in a soluble form (30). The extent to which BACE1 shedding is regulated or the possible impact this may have on the amyloidogenic processing of APP is not known. However, it has been suggested that inhibition of BACE1 shedding could have value as an alternative strategy in the treatment of AD (30). Here we show that BACE1 shedding is indeed increased by agents that stimulate protein kinase C and that a metalloproteinase, possibly ADAM10, is involved in this process. As BACE1 is known to be re-internalized and targeted to the endosomes (31), which are a major site of APP processing, it is possible that an alteration in the level of BACE1 at the cell surface would alter the levels of BACE1 available for re-internalization. This would clearly have implications for the processing of APP at the β-site and, hence, Aβ production. To address this issue, we identified BACE1 sheddase-selective hydroxamate inhibitors and investigated the effect of inhibiting BACE1 shedding on the processing of APP. Interestingly, abolishing the ectodomain shedding of BACE1 appeared to have no effect on the processing of APP at the β-site.
EXPERIMENTAL PROCEDURES

Expression of BACE1 Constructs—BACE1-MycHis6/pC DNA3.1 has been described previously (21). Mutant constructs were generated by QuikChange site-directed mutagenesis (Stratagene) as described by the manufacturer and sequenced. Human embryonic kidney (HEK) 293 cells transiently expressing wild type (WT) or mutant (D93N and D289N) BACE1-MycHis6 were cultured in serum-free media for 20 h. Whole cell lysates and medium samples were incubated in the absence (−) or presence (+) of endoglycosidase H (Endo-H) or N-glycosidase F (N-glycosid. F) at 37 °C for 16 h prior to Western blot analysis with a 9B21 antibody. Shed BACE1 is endoglycosidase H-resistant but is sensitive to N-glycosidase F, suggesting that shed BACE1 is generated from mature BACE1 in cells. d, HEK 293 cells transiently expressing wild type (WT) or mutant (D93N and D289N) BACE1-MycHis6 were cultured in serum-free media for 20 h. Whole cell lysates and medium samples were generated and resolved on polyacrylamide gels for Western blot analysis with anti-Myc (top panel) or 9B21 antibodies (bottom panel). Similar levels of shed BACE1 are secreted from BACE1 WT and mutant cells.

RESULTS

Ectodomain Shedding of BACE1 from HEK 293 Cells—To determine whether the extracellular domain of BACE1 is cleaved and released from cells, we generated HEK 293 cells stably expressing BACE1 with a C-terminal MycHis6 tag (BACE1-MycHis6). Western blot analysis of whole cell lysates with an anti-Myc antibody confirmed BACE1 expression in these cells (Fig. 1a). Subsequent analysis of the medium with antibody 9B21, which recognizes the catalytic domain of BACE1, allowed detection of soluble shed BACE1. Shed BACE1 in the medium was not immunoreactive with anti-Myc antibody (data not shown) and migrated as a band of lower Mr than full-length BACE1 in cells, confirming that the extracellular domain of BACE1 is cleaved and released from cells. The appearance of shed BACE1 in the medium should be concomitant with the generation of an intracellular C-terminal fragment (CTF). Western blot analysis of whole cell lysates with anti-Myc antibody failed to detect a BACE1 CTF even upon prolonged exposure, suggesting that it may be rapidly turned over in the cell (data not shown). To eliminate any effect of the MycHis6 tag at the C terminus of BACE1 on its ability to be cleaved and released from cells, medium from HEK 293 cells expressing untagged BACE1 (Fig. 1b) was also subjected to Western blot analysis with antibody 9B21. Shed BACE1 was detected in the medium from these cells, indicating that the C-terminal tags do not influence BACE1 cleavage and release from cells. In addition, both the tagged and non-tagged forms of the protein expressed in cells gave the same overall pattern of mature and immature forms (Fig. 1), indicating that the tag had no effect on the trafficking and processing of the proteins in the endomembrane system.

To determine which form of BACE1 is shed from cells, deglycosylation experiments were carried out. Shed BACE1 in the medium is resistant to endoglycosidase H treatment (Fig. 1c), whereas treatment with N-glycosidase F causes a shift in Mr, indicating that it is the mature form of BACE1 in cells that is subject to cleavage. As BACE1 is a protease, we went on to investigate whether BACE1 could mediate its own ectodomain shedding. HEK 293 cells were transfected with either wild type BACE1-MycHis6 or mutants in which the aspartic acid residues in the active site had been mutated to asparagine (D93N and D289N), which, as we have shown previously, abolish the catalytic activity of the protease (21). Both wild type and mutant BACE1 were expressed in cells to similar levels (Fig. 1d, top panel). Analysis of the medium from these cells revealed a similar level of soluble shed BACE1 from both wild type and mutant protein (Fig. 1d, bottom panel), indicating that BACE1 does not mediate its own cleavage and release from cells.

Characteristics of BACE1 Shedding—The ectodomain shedding of membrane proteins can be up-regulated by agents that activate protein kinase C (32). To investigate whether ectodomain shedding of BACE1 is controlled by a similar mechanism, HEK 293 cells expressing BACE1-MycHis6 were grown in the absence or presence of the phorbol ester PMA, which is known to activate protein kinase C. Treatment of cells with PMA led to an increase of −2.5-fold in the level of shed BACE1 in the medium (Fig. 2a, bottom panel) indicating that activation of protein kinase C increases BACE1 shedding.
Hydroxamic acid-based metalloproteinase inhibitors have been reported to inhibit the shedding of other cell surface proteins (1, 5, 6). We went on to determine whether the ectodomain shedding of BACE1 is sensitive to inhibitors of this class by exposing HEK 293 cells expressing BACE1-MycHis6 to two hydroxamate inhibitors, SB-202968 or SB-242113 (Fig. 2b). The presence of either SB-202968 or SB-242113 reduced the release of soluble BACE1 in the medium to <20% of the level seen in untreated cells, clearly indicating the involvement of a metalloproteinase in the ectodomain shedding of BACE1. Taken together, this set of results indicates that BACE1 shedding shows all the characteristics of ectodomain shedding reported previously for other proteins, namely up-regulation by phorbol esters and cleavage by a metalloproteinase.

Inhibition of BACE1 Shedding Does Not Regulate APP Processing at the β-Site, and BACE1 Sheddase Is Distinct from α-Secretase—The phorbol ester and hydroxamate inhibitors described so far are non-selective and are known to affect the ectodomain shedding of other cell surface proteins, including α-secretase cleavage of APP (5). To determine whether inhibition of BACE1 shedding could influence APP processing at the β-site (for example, by reducing the amount of BACE1 re-internalized to the endosomes, which is a major site of APP processing), we needed to identify fully selective BACE1 sheddase inhibitors, which had no effect on α-secretase processing of APP. Therefore, a range of hydroxamate-based inhibitors were screened for their effects on BACE1 shedding and APP processing by α-secretase.

A number of hydroxamate inhibitors were found to inhibit ectodomain shedding of BACE1 from HEK 293 cells (Fig. 3a). To determine whether these compounds affected APP processing, HEK 293 cells expressing BACE1-MycHis6 were transiently transfected with APP695 followed by treatment with the inhibitors. None of the hydroxamate inhibitors had any effect on the processing of APP by α-secretase, as similar levels of sAPPα and intracellular CTFα were detected in the control and treated cells (Fig. 3b). This different inhibition profile for the BACE1 sheddase and α-secretase suggests that the BACE1 sheddase is distinct from α-secretase. This was further confirmed by the identification of a hydroxamate inhibitor, SB-451916, which had no effect on BACE1 shedding (Fig. 3c) but significantly reduced the level of sAPPα (Fig. 3d).

The selective BACE1 sheddase inhibitors were subsequently used to determine whether inhibition of BACE1 shedding affects β-secretase cleavage of APP in these cells. None of the hydroxamate inhibitors had any effect on secreted sAPPβ levels or intracellular CTFβ levels, indicating that the inhibition of BACE1 shedding has no effect on APP processing at the β-site (Fig. 3b). In addition, the inhibitors had no effect on the level of Aβ40 and Aβ42 peptides secreted into the medium from these cells (data not shown).

ADAM10-selective Hydroxamate Inhibitor Blocks Ectodomain Shedding of BACE1—Members of the ADAM family of metalloproteases are known to mediate the ectodomain shedding of a variety of cell surface proteins (34). To establish the identity of the BACE1 sheddase, the potency and selectivity of two hydroxamate inhibitors to ADAM10 and TACE in vitro were exploited (33). GW4023 is a highly potent and selective ADAM10 inhibitor (IC50, 5 nM) being 100-fold less potent against TACE (IC50, 541 nM). In contrast, GW0264 shows similar potency for ADAM10 (IC50, 11 nM) and TACE (IC50, 8 nM) (Fig. 4a). Treatment of HEK-BACE1 cells with the indicated concentrations of either inhibitor caused a dose-dependent inhibition of the release of shed BACE1 (Fig. 4b). Densitometric analysis of these blots allowed concentration response curves and EC50 values for inhibition of BACE1 shedding to be generated (Fig. 4c). Both compounds inhibited the generation of shed BACE1 with potencies similar to those described for these.
inhibitors against ADAM10 in vitro. As GW4023 abolished BACE1 shedding at concentrations far below the reported IC50 for this compound against TACE, it is unlikely that TACE mediates the ectodomain shedding of BACE1. Rather, our data show ADAM10 to be a potential candidate for the BACE1 sheddase.

**DISCUSSION**

The extracellular domains of many diverse cell surface proteins are cleaved and released from cells by a process known as ectodomain shedding. Here we have shown that the ectodomain of BACE1 undergoes a similar cleavage, resulting in the release of soluble shed protein from cells. As reported for other shed proteins, ectodomain shedding of BACE1 is stimulated by agents that activate protein kinase C and is inhibited by hydroxamic acid-based metalloproteinase inhibitors, suggesting a common processing mechanism. Hence, BACE1 belongs to that group of transmembrane proteins that is subjected to regulated ectodomain shedding and includes APP, TNFα, and L-selectin.

Using potent and selective ADAM10 inhibitors, we demonstrate that ADAM10, but not TACE, is a strong candidate for the BACE1 sheddase in HEK cells. However, we can not rule out the possibility that other metalloproteinases that may be
Our findings suggest that ADAM10 is not the major ADAMs contributing to 

tase activity (16). All of these findings suggest that multiple 

cations for APP processing, and, therefore, inhibition of BACE1 

crease in the Aβ40 levels. However, it is possible that the 

on BACE1 shedding would have the opposite effect and reduce the level of full-length BACE1 that is in cells and available for re-internalization and, therefore, decrease APP processing at the β-site. The possibility that the ectodomain shedding of BACE1 may enhance the amyloidogenic potential of this protease was proposed in a previous study that employed a secreted form of the protein (30). Expression of a soluble form of BACE1 in cells that resembled the shed protein led to a marked increase in Aβ40 levels but, interestingly, a reduction in CTFβ levels. However, it is possible that the increase in the Aβ40 level reported in this study was merely a reflection of the faster rate of transit in cells of soluble BACE1 as compared with that of the full-length protein rather than an indication that shed BACE1 is more amyloidogenic. To address this point in a more physiological manner, we exploited the availability of selective and potent inhibitors of the BACE1 sheddase and clearly established that none of these compounds have any significant effect on β-secretase cleavage of APP. However, subtle changes in β-secretase activity due to inhibition of BACE1 shedding cannot be ruled out.

In conclusion, we have shown that BACE1, a key therapeutic target for treatment of AD, is subject to ectodomain shedding. Although the physiological significance of shed BACE1 is unclear at present, it appears to have no major functional implications for APP processing, and, therefore, inhibition of BACE1 shedding is unlikely to have therapeutic potential in Alzheimer's Disease.

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