Convertases other than furin cleave β-secretase to its mature form[1]

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SPECIFIC AIMS

A novel aspartyl protease, beta site APP cleaving enzyme (BACE) was identified as the β-secretase that cleaves at the NH_2 terminus of Aβ. The major goal of this study is to determine the role of furin and other proprotein convertases in the proteolytic processing of BACE to its mature form.

PRINCIPAL FINDINGS

1. ProBACE is cleaved to its mature activated form in the furin-deficient mutant RPE.40

The amyloid precursor protein (APP) is cleaved by β-secretase to a secreted derivative sAPPβ and membrane-associated carboxyl-terminal fragment CTFβ. The latter is cleaved by γ-secretase to the secreted amyloid β protein (Aβ) of 39–43 residues. All known mutations linked to familial Alzheimer's disease (FAD) increase levels of the longer 42 residue form of Aβ (Aβ42), highlighting its role in the pathogenesis of this disease. This makes reduction of Aβ an important therapeutic goal and the β- and γ-secretases important therapeutic targets. Like other aspartyl proteases, BACE is synthesized as a preproprotein that is sequentially cleaved by signal peptidase and another protease to remove the leader peptide and the prodomain. Mature BACE is 300% more active in vitro than the prodomain-containing form (proBACE). It was previously suggested that BACE maturation is not required for β-secretase activity as the levels of Aβ are not reduced in the RPE.40 cell line. However, an alternative explanation is that BACE maturation occurs in the absence of furin. To determine whether BACE is cleaved by other proprotein convertases in the furin-deficient RPE.40 cells, we compared the stability of proBACE with that of mature BACE by pulse labeling with Tran (35)S-label™ (ICN, Plain View, NY), followed by a chase in media supplemented with an excess of nonradioactive cysteine and methionine. Immunoprecipitates using an antibody against a peptide derived from the proBACE sequence ($BACE_{26-45}$) show that proBACE is synthesized as an ~65 kDa core-glycosylated protein that becomes a larger ~75 kDa protein upon elaboration of sugars after a 0.5 h chase (**Fig. 1*A***). Both 65 and 75 kDa bands become fainter at 1 h of chase and are very faint after 2 h, indicating that proBACE is rapidly turned over in these cells (Fig. 1*A*). In contrast, an antibody against the 15 carboxyl-terminal residues of BACE ($BACE_{CT15}$) showed that total (pro+mature) BACE was quite stable even after 2 h (Fig. 1*B*), indicating that the loss of proBACE signal was not due to turnover of BACE but to cleavage of its proregion. To independently determine whether BACE maturation occurs in the absence of furin, we treated cultures with decanoyl-RVKR-chloromethylketone (d-RVKR-CMK), an inhibitor of the prohormone convertase family, and found that this treatment increased the signal of proBACE in RPE.40 like wild-type CHO-K1 without similarly affecting total BACE (not shown here). This increase in proBACE signal in the presence of an inhibitor indicates that cleavage by a prohormone convertase leads to loss of the proBACE signal even in the furin-deficient RPE.40 cells.

2. Other propeptide convertases can cleave proBACE in transfected cells

To examine the possibility that other proprotein convertases can cleave the BACE propeptide, we determined whether proBACE levels are reduced in RPE.40 cells transfected with furin, PACE4, PC5/6, and PC7, the proprotein convertases located in the constitutive secretory pathway. The data show that proBACE is reduced to varying degrees in cultures transfected with each of these convertases (**Fig. 2*A, C***). The reduction in proBACE signal is not accompanied by a simultaneous reduction in total BACE detected with antibodies against the first 20 residues ($BACE_{46-65}$) or the last 15 residues ($BACE_{CT15}$) of the mature protein (Fig. 2*B–D*). The proBACE signal was significantly reduced

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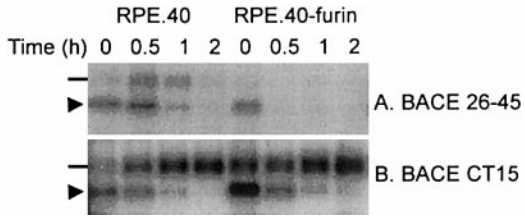

Figure 1. Proteolytic maturation of BACE in the furin-deficient mutant line RPE.40. Stable transfectants of RPE.40 expressing BACE (RPE.40) and BACE + furin (RPE.40 furin) were pulse labeled for 45 min with Trans (35)S-label™, chased with an excess of nonradioactive cysteine and methionine. Equal amounts of lysates were immunoprecipitated with either $BACE_{26-45}$, an antibody against residues 26–45 within the prodomain of the precursor protein (*A*), or $BACE_{CT15}$, an antibody against the carboxyl-terminal 15 residues of the cytoplasmic tail (*B*). The line and the arrowhead point to the 75 kDa and 65 kDa forms of BACE, respectively.

($P<0.05$) for furin, PC7, and PC5/6 but the reduction was more modest and not significant ($P=0.07$) for PACE4 (Fig. 2*D*). These results show that all the proprotein convertases tested augment cleavage of BACE to its mature form at least in transiently transfected cells expressing high levels of these enzymes.

Transfection with PC5/6 reduced levels of the 65 kDa core-glycosylated band of proBACE (Fig. 2*A*). This band is endoglycosidase H-sensitive and therefore represents the endoplasmic reticulum pool of BACE, which is expected to contain more of the intact prodomain. Consistent with this prediction, the relative signal of the 65 kDa band is stronger when the proBACE-specific antibody $BACE_{26-45}$ is used for detection by Western blotting (Fig. 2*A*) than with antibodies against total BACE (Fig. 2*B, C*). Thus, PC5/6 may cleave BACE at least partly before elaboration of the sugars, which is consistent with findings by Capell et al. that BACE maturation starts in early secretory compartments.

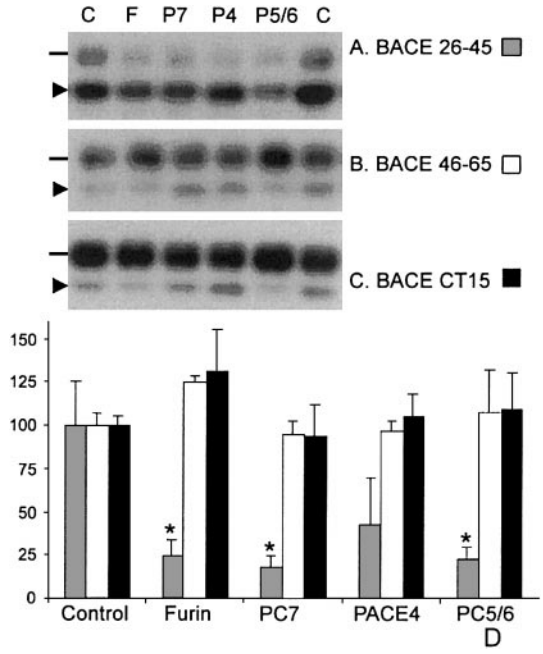

Figure 2. Several convertases augment proBACE processing in transfected cells. RPE.40 was transfected with vector control (C), furin (F), PC7 (P7), PACE4 (P4), and PC5/6 (P6) using Lipofectamine (Invitrogen, San Diego, CA). Western blots show proBACE signal detected with $BACE_{26-45}$ (*A*). Total BACE was detected with $BACE_{46-65}$ (*B*) and $BACE_{CT15}$ (*C*). The horizontal line and the arrowhead point to the 75 kDa and 65 kDa forms of BACE, respectively. Quantitative data obtained by densitometry (mean±SD) are shown as bar graphs (*D*) color-coded as indicated in panels *A–C*. $^*P < 0.05$ by a two-tailed *t* test.

SIGNIFICANCE AND CONCLUSIONS

Some of the proprotein convertases that cleave proBACE in transfected RPE.40 cells may play an important role in its processing in vivo (**Fig. 3**). Since many of these enzymes (except furin) are not essential for survival of knockout animals, it may be possible to significantly reduce their activity without deleterious consequences. The furin-deficient RPE.40 cells, which clearly are capable of processing proBACE, do not show detectable PACE4, PC1/2, or PC5/6 activity (J. Sucic, unpublished observations). Based on reports that the major proprotein convertases in CHO-K1 cells are furin and PC7, the latter is a likely candidate for BACE maturation in RPE.40 cells. However, given the com-

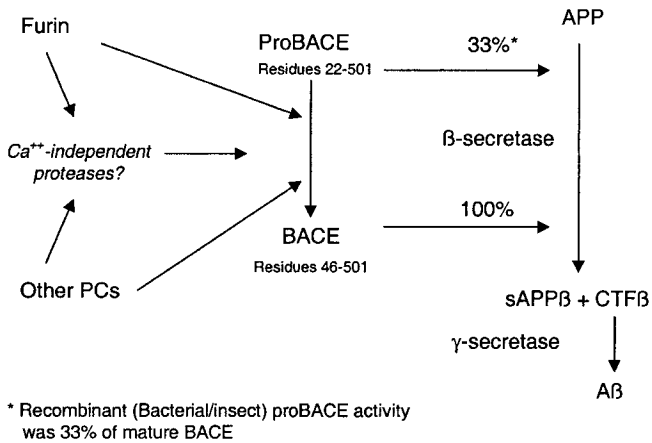

Figure 3. Schematic diagram of β-secretase regulation by proteolytic maturation. BACE is synthesized with a short prodomain that is post-translationally removed by proteolysis. This is an often-repeated paradigm in aspartyl proteases, with the proprotein being an inactive zymogen that is activated by proteolytic processing. It was shown that BACE processing by furin increases activity by 300% in vitro. Our study shows that proprotein convertases can cleave proBACE to its mature form in a cell line deficient in furin. Together with the inefficient in vitro processing of proBACE by furin, the study raises the possibility that the major proprotein convertase involved in BACE maturation in vivo is not furin. Unlike furin, some of these proprotein convertases are not essential for survival and may be inhibited with minimal toxicity. The proprotein convertase responsible for BACE maturation may be a useful therapeutic target for inhibition of β-secretase activity. This may be important considering that BACE is believed to be a difficult target enzyme for inhibition using small molecules.

plexity of the proteolytic pathways in the cell, we cannot rule out the possibility that the BACE prodomain is cleaved by a protease that is activated by furin and other proprotein convertases. For example, membranes from RPE.40 transfected with PACE4 also contain a Ca^{2+}-independent protease that cleaves HIV GP160. Despite similarities in the substrate specificity of this protease, its lack of Ca^{2+} requirement points away from the subtilisin like proprotein convertases. This alternative pathway is depicted in italics as another potential pathway for BACE maturation (Fig. 3).

While this manuscript was in preparation, two prepress reports provided evidence for proBACE cleavage by furin. One report indicated that the mutant RPE.40 cells are deficient in BACE maturation and that β-secretase activity was not reduced in BACE-transfected RPE.40 cells. Although the authors acknowledge the possibility that proBACE may be cleaved in RPE.40, they argue against it and suggest that this maturation event is unimportant for β-secretase activity. Our studies show that despite the furin deficiency, proBACE is converted to its mature form in RPE.40 cells. This leaves open the possibility that proteolytic cleavage stimulates β-secretase activity in these cells and, by extension, in the AD brain.

To determine relevance of proteolytic maturation in regulating β-secretase activity, we need a cell line that fails to generate mature BACE. Although peptide inhibitors (e.g., d-RVKR-CMK) of the proprotein convertase pathways are known, they are unstable. It is not feasible to examine the effect of pure proBACE or APP processing by eliminating its preexisting mature pool after treatment with the known unstable inhibitors, because mature BACE is very stable in cultured cells. In addition, these studies cannot be conducted in transfected cells as proBACE levels are increased by several orders of magnitude, which may prevent us from detecting the effect of increasing BACE activity by threefold upon proteolytic maturation. Since the known furin-deficient mutant cells are capable of cleaving HIV GP160 using alternative proprotein convertases, we are exploring alternative methods to eliminate these activities in order to study the physiological role of this maturation process and examine whether BACE maturation is involved in regulating Aβ levels in AD.

The reports that proBACE activity is 66% lower than mature BACE generated by either furin or clostripain cleavage suggest that this pathway is capable of regulating BACE activity in vivo. Since β-secretase activity in most cells is low, accounting for only ~10% of APP cleavage to secreted derivatives, BACE levels should be low and limiting. Thus, the predicted 66% inhibition of BACE upon failure of maturation should be physiologically relevant.

Given that it is not essential for the survival of knockout mice, BACE is expected to be a good therapeutic target. However, conventional drug screens have not identified a small molecule inhibitor for this activity. It has been suggested that it may be difficult to identify a nonpeptide inhibitor (with useful bioavailability in the brain) for this enzyme due to its large catalytic pocket. The enzymes responsible for cleavage of the BACE propeptide may be good drug targets for inhibiting BACE indirectly. In addition, proteolytic maturation of proBACE may play an important role in regulating BACE activity in the brain and therefore play a role in the pathogenesis of AD. FJ