Heparin Activates β-Secretase (BACE1) of Alzheimer's Disease and Increases Autocatalysis of the Enzyme†

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ABSTRACT: BACE1 is an aspartic protease that generates the N-terminus of the β-amyloid protein (Aβ) from the β-amyloid precursor protein (APP). BACE1 is a key target for Alzheimer drug development. However, little is known about the physiological regulation of the enzyme. Heparin can promote β-secretase cleavage of APP in neuroblastoma cells. However, heparin has also been reported to directly inhibit BACE1 activity in vitro. To clarify the role of heparin in regulating BACE1, we examined the effect of heparin on the activity of recombinant human BACE1 (rBACE1) in vitro. Low concentrations (1 μg/mL) of heparin were found to stimulate rBACE1, increasing enzyme V_{max} and decreasing the K_M. In contrast, higher concentrations of heparin (10 or 100 μg/mL) were inhibitory. Heparin affinity chromatography demonstrated that heparin interacted strongly with the zymogen form of rBACE1 and bound to a peptide homologous to the N-terminal pro sequence of BACE1. Mature (pro sequence cleaved) enzyme lacked the capacity to be stimulated by heparin, indicating that the pro domain was necessary for the stimulation by heparin. Furthermore, in the presence of stimulatory concentrations of heparin, there was an increase in autocatalytic cleavage of the protease domain and a subsequent loss of enzyme activity in vitro. Our results strongly suggest that heparin stimulates the partially active BACE1 zymogen, and we propose that the activation is mediated by high-affinity binding of heparin to the pro domain. Our study provides evidence that heparan sulfate proteoglycans could regulate the rate of Aβ production in vivo.

Alzheimer's disease (AD)[1] is a progressive neurodegenerative disorder that is characterized by synaptic and neuronal loss (*1*) and the deposition of protein aggregates in the intracellular and extracellular compartments of the brain. The extracellular deposits or amyloid plaques consist primarily of the β-amyloid protein (Aβ) (*2*), whereas the intracellular deposits or neurofibrillary tangles contain the microtubule-associated protein tau (*3*).

Aβ accumulation in the brain is associated with the progression of AD, and anti-amyloid therapeutic strategies are therefore regarded as promising for treatment of the disease (*4*). Aβ is produced from the β-amyloid precursor protein (APP) by sequential cleavage at the N- and C-termini of the Aβ domain by β- and γ-secretase, respectively (*5*). β-Secretase cleavage also generates a larger N-terminal fragment of APP (sAPPβ). The protease responsible for β-secretase activity in neurons has been identified as the β-site APP cleaving enzyme 1 (BACE1), also known as Asp2 or memapsin 2 (*6*−*10*). Although other substrates for BACE1 have been identified (*11*−*16*), the range of substrates may be limited in vivo. BACE1 knockout mice can survive to adulthood without any major phenotypic abnormality (*17*−*20*). Therefore, decreasing Aβ load in the brain via inhibition of BACE1 activity has become an attractive strategy for Alzheimer drug development. Recently, BACE1 suppression using small interfering RNAs was shown to ameliorate the neuropathology in an AD transgenic model (*21*).

BACE1 belongs to the family of aspartic proteases, but it differs, along with its homologue BACE2, from other family members by being membrane anchored (*8*). Full-length BACE1 contains 501 amino acid residues including a signal peptide sequence and a 24 amino acid residue pro domain. The protease domain is located between residues 46−460 and is followed by a transmembrane domain and a short cytoplasmic tail. Because the catalytic ectodomain of BACE1 can be shed from the membrane, the enzyme also exists in a soluble form (*22*, *23*).

During its maturation in the secretory pathway, BACE1 undergoes complex N-linked glycosylation (*24*, *25*). The pro domain, which is important for exit of proBACE1 from the endoplasmic reticulum (*22*) and for proper folding of the protease domain (*26*), is removed, possibly by furin or other members of the proprotein convertase (PC) family (*27*, *28*). Removal of the pro sequence increases enzyme activity (*26*). However, unlike many other aspartic proteases, the pro domain does not completely suppress the activity of the zymogen.

Heparan sulfate (HS) chains, attached to core proteins forming HS proteoglycans (HSPGs), and heparin, a highly

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[1] Abbreviations: Aβ, β-amyloid protein; AD, Alzheimer's disease; APP, β-amyloid precursor protein; BACE1, β-site APP cleaving enzyme 1; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; PC, proprotein convertase.

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sulfated analogue of HS, are known to regulate the activity of a number of proteases (*29*−*34*). Interestingly, heparin has been reported to promote β-site cleavage of APP in cell culture (*35*). However, HS or heparin reportedly inhibits BACE1 activity in vitro (*36*).

Therefore, to clarify the role of HS in regulating BACE1 activity, we have reexamined the effect of heparin on BACE1 using recombinant human BACE1 (rBACE1) in vitro. In this study, we show that a low concentration of heparin (1 μg/mL) can stimulate rBACE1 activity. In addition, we show that the stimulation by heparin leads to increased autocatalytic cleavage of the protease domain and a subsequent loss of enzyme activity in vitro. We also present evidence for the identification of novel autocatalytic cleavage sites in BACE1.

MATERIALS AND METHODS

Materials. rBACE1, amino acid residues 1−460 with or without a C-terminal His tag, was obtained from R&D Systems (931-AS; BioScientific, Gymea, Australia) and Invitrogen (P2947; Mount Waverley, Victoria, Australia), respectively. The substrate peptide MCA-SEVNLDAEFRK-(DNP)RR-NH_2 was purchased from MP Biomedicals (Seven Hills, NSW, Australia), and microplates (OptiPlate-96 F) were from PerkinElmer Life Sciences (Boston, MA). Coomassie blue R-250, heparin from porcine intestinal mucosa (H3393), human recombinant apolipoprotein E4 (apoE4), polyclonal N-terminal anti-BACE1 antibody (immunogen peptide comprising of amino acids 46−62 of human BACE1; EE-17), and protein A−Sepharose 4B Fast Flow were all obtained from Sigma-Aldrich (Sydney, Australia). HiTrap heparin HP columns, anti-rabbit IgG HRP, and ECL reagent were from Amersham Biosciences (Castle Hill, Australia), and Centricon YM-10 and Microcon YM-30 concentrators, Immobilon-P membrane (0.45 μm), and C18 ZipTips were from Millipore (North Ryde, Australia). A peptide homologous to the pro domain of BACE1 (TQHGIRLPLRSGLG-GAPLGLRLPR-NH_2) was synthesized by Auspep (Parkville, Australia). Polyclonal anti-proBACE1 antibody (PRB622C, Covance) was purchased from Chemicon (Boronia, Australia), and the Penta-His HRP conjugate kit was from Qiagen (Doncaster, Australia). Bio-Rad silver stain and Sequi-Blot PVDF membrane were obtained from Bio-Rad Laboratories (Regents Park, Australia), and syringe filters were from Pall Corp. (Cheltenham, Australia). The Brownlee Spheri-5 RP-18 column was purchased from Applied Biosystems (Scoresby, Australia).

BACE1 Activity Assay. BACE1 activity was measured using a quenched fluorescence assay with a peptide substrate homologous to the Swedish APP mutant β-cleavage site (*8*, *37*). Assays were performed in a buffer of 0.1 M sodium acetate pH 4, and 5% dimethyl sulfoxide (DMSO) using 0.8 μg of rBACE1 (unless otherwise specified) and 1−30 μM substrate peptide in a total volume of 100 μL in black 96-well plates. Heparin was dissolved in water and sterile filtered with a 0.45 μm syringe filter. The increase in fluorescence intensity (*F*) produced by substrate hydrolysis was monitored at 37 °C on a fluorescence microplate reader (FLUOstar; BMG Lab Technologies, Offenburg, Germany) with excitation and emission wavelengths of 320 and 405 nm, respectively. Enzyme activity was calculated from the change in *F* per minute (ΔF/min) during the initial linear phase of the reaction and plotted against increasing substrate concentrations. The data were fitted to the Michaelis−Menten equation by the method of least squares using GraphPad Prism software (version 4.00) to calculate V_{max} and K_M.

Gel Electrophoresis and Western Blotting. Samples (10−25 μL) were mixed with 2× SDS sample buffer and heated to 95 °C for 5 min prior to gel loading. The proteins were separated by 10% SDS−PAGE (*38*) and either silver stained using Bio-Rad silver stain reagent or electroblotted onto an Immobilon-P membrane for 1 h at 350 mA. For immunoblot analysis with the polyclonal antibodies EE-17 and anti-proBACE1, the membrane was blocked in 5% skim milk powder in PBS with 0.05% Tween 20, pH 7.4, and incubated with the antibodies diluted to 1:2000 and 1:1000, respectively, in blocking solution. HRP-conjugated secondary antibody was used for chemiluminescent detection by ECL. For detection of the His tag, a Penta-His HRP conjugate kit was used according to the manufacturer's instructions (Qiagen, Doncaster, Australia), and immunoblots were developed using ECL. An image of the signals obtained on X-ray film was acquired, and band intensities were measured using AlphaImager with AlphaEase Software (Quantum Scientific, Victoria, Australia).

N-Terminal Amino Acid Sequencing. Samples of rBACE1 (6−7.5 μg) were separated by 10% SDS−PAGE and electroblotted onto a Sequi-Blot PVDF membrane using 10 mM CAPS buffer with 10% methanol (*39*). The membrane was stained with Coomassie blue R-250 (0.1% in 40% methanol and 1% acetic acid) and then destained in 50% methanol until the background was light blue. The membrane was then washed in water and air-dried, and the band of interest was excised. Proteins on the excised membrane were subjected to N-terminal amino acid sequencing by an automated Edman degradation procedure using the Procise protein sequencing system (Applied Biosystems, Scoresby, Australia).

Heparin Affinity Chromatography. For heparin affinity chromatography, 15 μg of rBACE1 (100 μg/mL in distilled water) or 250 μg of peptide (1 mM in DMSO) was diluted to 1 mL with 10 mM sodium acetate buffer, pH 5 (acetate buffer), and then applied to a 1 mL HiTrap heparin HP column preequilibrated with acetate buffer. Unbound material was eluted with 2 × 3 mL of acetate buffer, and then bound material was eluted with 1 × 3 mL washes of 0.15, 0.5, and 1.2 M NaCl in acetate buffer at a flow rate of 1 mL/min. For rBACE1, fractions of 3 mL were collected and then concentrated (11-fold), and the buffer was exchanged to 0.1 M sodium acetate, pH 4, using Centricon YM-10.

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC). Elution of a synthetic peptide homologous to the pro domain of BACE1 from the heparin column was monitored by RP-HPLC. A portion (0.9 mL) of the heparin column wash fractions or a standard amount of the peptide (90 μg) was applied to a 4.6 × 220 mm Brownlee Spheri-5 RP18 column (5 μm bead size, 80 Å pore size) using an Agilent Technologies 1100 series solvent delivery system (Forest Hill, Australia). Samples were filtered through a 0.45 μm syringe filter before injection onto the column. After an initial wash for 5 min with solvent A [0.1% trifluoroacetic acid (TFA) in water], the peptide was eluted with a linear gradient of 0−70% solvent B (0.1% TFA in 90% acetonitrile/

water) over 35 min at a flow rate of 1 mL/min. The elution of the peptide from the column was monitored by measuring the absorbance at a wavelength of 218 nm.

Immunoprecipitation. Immunoprecipitation was performed with the anti-proBACE1 antibody (1 μL) and protein A−Sepharose (30 μL). Briefly, protein A beads were preloaded with the antibody in PBS, and the mixture was rotated for 1 h at room temperature. The beads were centrifuged and washed in PBS, after which 2 μg of rBACE1 (R&D Systems) in PBS (500 μL) was added. The mixture was rotated overnight at 4 °C. The beads were centrifuged, and the supernatant fraction was collected and concentrated (7.5-fold) using a Microcon YM-30. A sample (20%) of the concentrated supernatant was subjected to western blot analysis, and enzyme activity was measured in the absence or presence of heparin (1 μg/mL) in the remaining part of the fraction.

Analysis by Mass Spectrometry (MS). MALDI-TOF MS analysis was performed with a 4700 Proteomics analyzer (Applied Biosystems, Scoresby, Australia). Prior to loading on the sample plate, the samples were desalted using C18 ZipTips according to the manufacturer's instructions. The instrument was operated in positive polarity in a linear mode or reflector mode using an α-cyano-4-hydroxycinnamic acid matrix (Agilent Technologies, Forest Hill, Australia) and calibrated against six peptides of the Applied Biosystems supplied 4700 mix. The matrix (0.5 μL) was spotted on the sample plate and allowed to air-dry. Samples (0.5 μL) were subsequently spotted on the dried matrix and allowed to air-dry. Data were collected and processed with the instrument manufacturer's 4000 series Explorer software (version 3). Tandem MS analysis (MSMS) was performed by precursor selection of the mass of the peptide of interest, and the masses of the resulting MSMS spectrum were compared with the theoretical fragment masses of the expected sequence generated by the ion fragmentation calculator contained as a part of the 4000 series Explorer software.

RESULTS

Effect of Heparin on rBACE1 Activity. Initially, the effect of various concentrations of heparin on BACE1 activity was examined. Similar to a previous report (*36*), heparin was found to inhibit the activity of rBACE1. However, this inhibition occurred only at relatively high concentrations of heparin (10 or 100 μg/mL) (Figure 1A). At a lower concentration of heparin (1 μg/mL), the enzyme activity was increased (Figure 1A). To examine whether this increase in activity was due to an effect on the V_{max} or K_M of the enzyme reaction, the ability of 1 μg/mL heparin to stimulate activity over a range of substrate concentrations (1−30 μM) was examined. The results showed that the V_{max} was increased to almost 200% of the control value in the presence of 1 μg/mL heparin and the K_M was decreased from approximately 11.4 μM in the absence of heparin to 6.5 μM in the presence of 1 μg/mL heparin (Figure 1B).

To investigate the specificity of the heparin-induced stimulation of BACE1, we tested whether a heparin-binding protein, apolipoprotein E (apoE) (*40*), could compete with rBACE1 for binding to heparin and thereby inhibit the stimulation. Consistent with this idea, the stimulatory effect of heparin on enzyme activity was inhibited by apoE (Figure

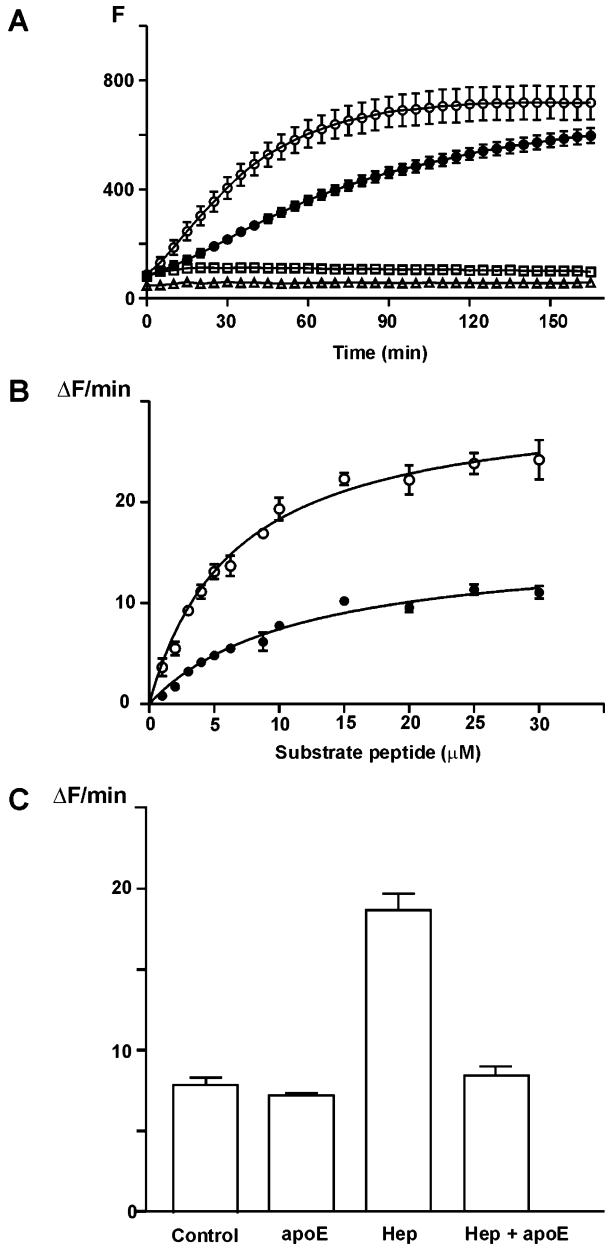


FIGURE 1: Effect of heparin on rBACE1 activity. (A) Increase in relative fluorescence (F) during rBACE1 hydrolysis of 5 μM substrate peptide in the presence of 0 μg/mL (closed circles), 1 μg/mL (open circles), 10 μg/mL (open squares), or 100 μg/mL (open triangles) heparin. Data from incubations with 0 and 1 μg/mL heparin are expressed as means ± SEM, $n = 4$. Enzyme activity in the presence of 1 μg/mL heparin was significantly different from controls lacking heparin ($P < 0.0001$, Student's t-test). (B) Velocity (ΔF/min) of product formation by rBACE1 as a function of substrate concentration in the absence (closed circles) or presence (open circles) of 1 μg/mL heparin. Data for 1−30 μM substrate peptide are expressed as means ± SEM, $n = 2-9$. The velocity of the reaction in the presence of 1 μg/mL heparin was significantly different from controls ($P < 0.0001$, Student's t-test). Curves shown are calculated from a best fit of the data to the Michaelis−Menten equation. In the absence or presence of heparin, respectively, the V_{max} was calculated as 15.9 ± 0.9 ΔF/min and 30.3 ± 1.6 ΔF/min SEM, and the K_M was 11.4 ± 1.4 μM and 6.5 ± 0.9 μM SEM. (C) Velocity (ΔF/min) of product formation by rBACE1 using 10 μM substrate peptide in the absence (control) or presence of 4 μg/mL apoE (apoE), 1 μg/mL heparin (Hep), and 1 μg/mL heparin with 4 μg/mL apoE (Hep + apoE). Similar results were obtained in three separate experiments. The figure shows data expressed as means ± range, $n = 2$, for one representative experiment.

1C). ApoE had no effect on basal enzyme activity in the absence of heparin, indicating that the ability of apoE to block the heparin-induced stimulation was due to an interaction between apoE and heparin and not between apoE and BACE1.

The rBACE1 used in the experiments described above was obtained from R&D Systems and contained a His tag at the C-terminus. To exclude the possibility that the stimulatory effect of heparin might be due to an interaction between heparin and the His tag, we examined the ability of heparin to stimulate rBACE1 activity using an enzyme preparation from Invitrogen, which lacked a His tag. The activity of the Invitrogen rBACE1 was increased by 54% in the presence of 1 μg/mL heparin when using 5 μM substrate peptide ($n = 3$, $P < 0.005$, Student's *t*-test). Thus, heparin was able to stimulate rBACE1 activity independently of the His tag. In all further experiments, we used the His-tagged enzyme.

Analysis of the time course of activation showed that 1 μg/mL heparin had a dual effect on rBACE1 (Figure 2). In an initial phase, heparin increased enzyme activity. However, the increased activity was followed by a second phase in which enzyme activity was lost. The effect of heparin was dependent on the concentration of the substrate peptide. At a very low substrate concentration (0.5 μM), the inhibition of substrate peptide cleavage occurred very fast (Figure 2A). When the concentration of substrate was increased to 1 μM, significant stimulation of enzyme activity was seen during the first 15 min of incubation (Figure 2B). After the phase of activation in the presence of 1 μM substrate, there was a lower rate of substrate hydrolysis as compared to the control incubations lacking heparin. The observed loss of enzyme activity was not due to consumption of the substrate. In the incubations with heparin, the rate of substrate cleavage at later time points (between 90 and 165 min) was lower than in control incubations both at 0.5 μM and at 1 μM substrate ($n = 2-6$, $P < 0.0001$, Student's *t*-test). This showed that the inhibitory phase was due to a decline in enzyme activity. At higher substrate concentrations (5 or 20 μM), the heparin-induced stimulation phase was more prolonged than at the lower concentrations, and the subsequent phase of inhibition was delayed (Figures 1A and 2). Thus, the length of the stimulatory phase induced by heparin was dependent on the concentration of substrate peptide.

Effect of Heparin on Autocatalytic Cleavage of rBACE1. BACE1 is known to undergo autocatalytic cleavage in the protease domain (*10*). Because the rate of autocatalysis would be lower at higher substrate concentrations, we hypothesized that the delayed appearance of the inhibitory phase at higher substrate concentrations may have been due to a lower rate of autocatalysis at these higher concentrations. Therefore, we investigated whether heparin could promote autocatalytic cleavage of rBACE1. rBACE1 was incubated in the absence or presence of heparin, and aliquots taken at different time points were analyzed on silver-stained SDS−polyacrylamide gels and by western blotting. The results showed that when using a low molar ratio of heparin to rBACE1 (1 μg/mL heparin:8 μg/mL rBACE1), which stimulates enzyme activity, autocatalytic cleavage was increased. In contrast, little autocatalysis was observed at a concentration of heparin (100 μg/mL) that inhibited enzyme activity.

Two major bands (M_r = 70−73 and 65−68 kDa) of rBACE1 were observed on silver-stained gels (control, Figure 3A). Western blotting using the antibody EE-17 directed against amino acids 46−62 of BACE1 or an antibody directed against the C-terminal His tag also revealed two immunoreactive bands of rBACE1, a major band at 70−73 kDa and a minor band at 65−68 kDa (control, Figure 3C,E). The difference in molecular mass between the two bands (5 kDa) was greater than would have been expected from cleavage of the 2.5 kDa pro domain sequence alone. The protein migrating at 70−73 kDa was recognized by an antibody directed against the pro sequence of BACE1 (control, Figure 3C,E). A small amount of proenzyme immunoreactivity was also detected in association with the 65−68 kDa band. N-Terminal amino acid sequencing showed that the upper 70−73 kDa band predominantly contained the zymogen (pro-rBACE1) beginning at threonine-22, but it also contained a small amount of mature

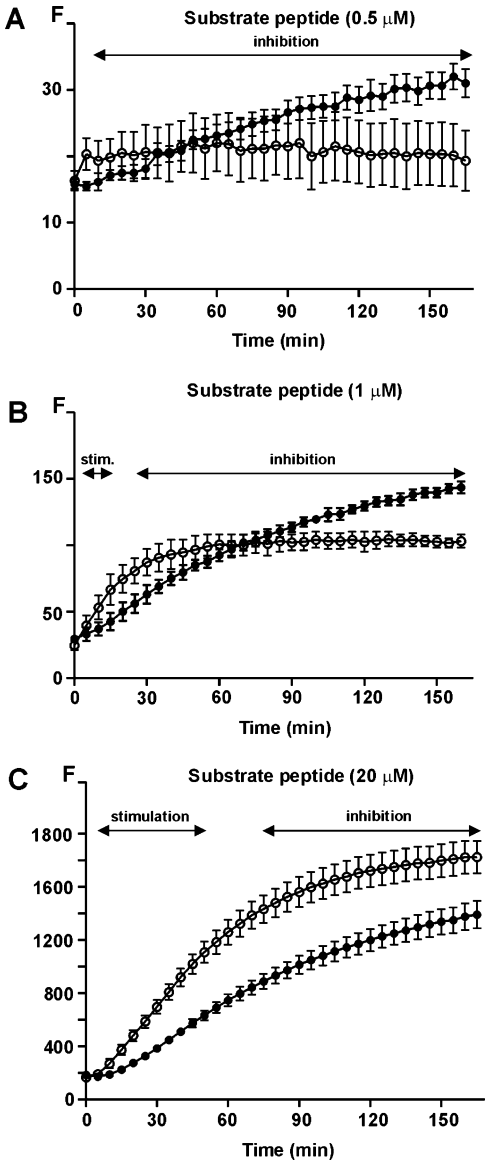


FIGURE 2: Time course of activation of rBACE1 by heparin. (A−C) Increase in *F* during rBACE1 hydrolysis at different concentrations of substrate peptide as indicated in the absence (closed circles) or presence (open circles) of 1 μg/mL heparin. Data are expressed as means ± SEM, $n = 2-6$. Enzyme activity in the presence of heparin was significantly different from controls lacking heparin at all of the substrate concentrations tested ($P < 0.0001$ for 0.5 and 20 μM substrate and $P < 0.01$ for 1 μM substrate, Student's *t*-test). The phase of stimulation (stim.) and inhibition is shown.

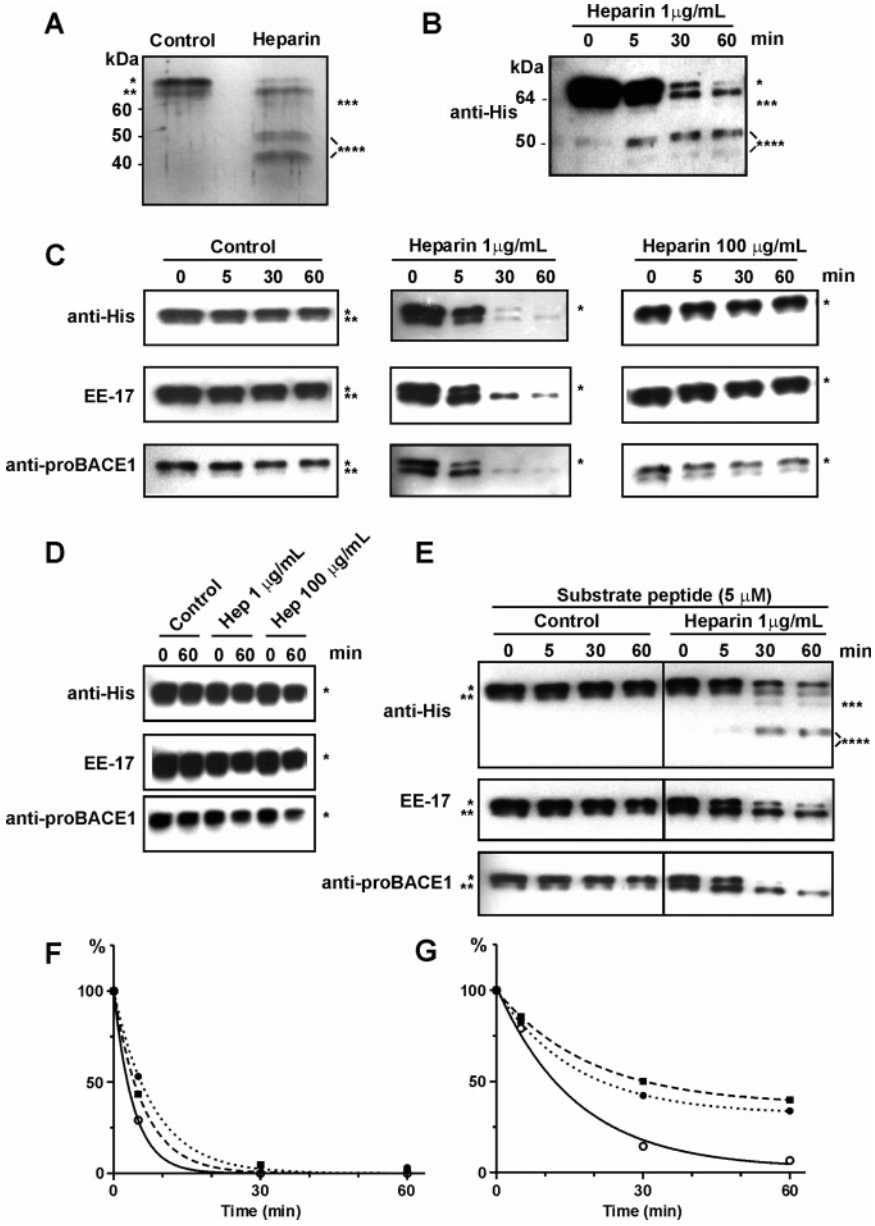


FIGURE 3: Effect of heparin on autocatalytic cleavage of rBACE1. (A) Silver-stained gel after SDS−PAGE. rBACE1 (1.6 μg in 100 μL, pH 4, 5% DMSO) was incubated in the absence (control) or presence of heparin (2 μg/mL) for 15 min at 37 °C, and an aliquot (25 μL) from each incubation was analyzed. (B−E) Western blot analysis of rBACE1 using the indicated antibodies. For each incubation, one representative blot of two to three separate experiments is shown. (B) rBACE1 (0.8 μg) was incubated in a volume of 100 μL of 0.1 M sodium acetate buffer (pH 4, 5% DMSO) at 37 °C, and aliquots of 10 μL were analyzed after 0−60 min incubation in the presence of 1 μg/mL heparin. (C) Incubation of rBACE1 as in (B) in the presence of 0 μg/mL (control), 1 μg/mL, or 100 μg/mL heparin. The immunoblots were probed, stripped, and reprobed using the antibodies in the following order: anti-His, EE-17, and anti-proBACE1. (D) Incubation of rBACE1 as in (B) except that 0.1 M sodium phosphate buffer (pH 7) was used. Incubations contained 0 μg/mL (control), 1 μg/mL, or 100 μg/mL heparin. (E) rBACE1 (0.8 μg in 100 μL, pH 4, 5% DMSO) was incubated with 5 μM substrate peptide in the absence (control) or presence of 1 μg/mL heparin at 37 °C. Aliquots of 10 μL were analyzed after 0−60 min. The immunoblots were probed, stripped, and reprobed using the antibodies in the following order: anti-His, anti-proBACE1, and EE-17. (F, G) Quantification of the immunoreactivity of the 70−73 kDa protein to the anti-proBACE1 (open circles), the EE-17 (closed circles), and the anti-His (closed squares) antibodies. rBACE1 was incubated for 0−60 min with heparin (1 μg/mL) in the absence (F) or presence (G) of substrate peptide (5 μM). For each antibody, the intensity of the signals obtained on the film was measured, and the data are expressed relative to the intensity at time 0. (A−G) Time 0 represents 10 s after the start of the reaction. Key: *, 70−73 kDa form; **, 65−68 kDa form; ***, 62 kDa form; ****, 40−50 kDa forms.

enzyme beginning at glutamate-46. However, because the 65−68 kDa band also contained a small amount of pro-rBACE1, this indicated that proteolytic cleavage of the pro domain was not solely responsible for the difference in molecular mass. Instead, it is likely that differential glycosylation accounted for some of this difference. Therefore, the preparation of rBACE1 consisted mainly of the zymogen, which preferentially migrated at an apparent molecular mass of 70−73 kDa.

After incubation with heparin (1 μg/mL) for 15 min, the amount of the 70−73 kDa form decreased, and several fragments of lower relative molecular mass (40−62 kDa) were generated (Figure 3A). These fragments were recognized by an antibody directed against the C-terminal His tag, identifying them as C-terminal fragments (Figure 3B). Western blot analysis using the anti-proBACE1 antibody

showed that the 70−73 kDa proenzyme was decreased after 5 min of incubation with heparin (1 μg/mL) (Figure 3C). The rate of loss of the zymogen after incubation with 1 μg/mL heparin was much higher at pH 4 (Figure 3C) than at pH 7 (Figure 3D), confirming that the breakdown was due to autocatalytic cleavage, as BACE1 activity is much higher at acidic pH. In the presence of a high concentration of heparin (100 μg/mL), there was little autocatalytic cleavage both at acidic and at neutral pH (Figure 3C,D).

Effect of Substrate Peptide on Heparin-Stimulated Autocatalytic Cleavage of rBACE1. As previous experiments showed that high concentrations of substrate peptide delayed the secondary inhibitory phase of enzyme activity, we examined whether the substrate peptide also affected the rate of autocatalysis. Heparin-induced autocatalytic cleavage was found to proceed at a slower rate in the presence of substrate peptide (5 μM) than in its absence (Figure 3E). This was also demonstrated by quantification of the amount of pro domain and total (i.e., C-terminal His and EE-17) BACE1 immunoreactivity associated with the 70−73 kDa band after western blotting (Figure 3F,G). Because higher concentrations of substrate peptide both decreased the rate of autocatalysis and prolonged the heparin-induced stimulation, this suggested that the later inhibitory phase that followed after the activation was due to autocatalytic cleavage of the enzyme.

Analysis of the pro-rBACE1 immunoreactivity indicated that it was more rapidly lost in the presence of heparin (1 μg/mL) than total rBACE1 immunoreactivity (Figure 3F,G). This suggested that N-terminal pro sequence cleavage might occur at a faster rate than other autocatalytic cleavages, thus providing a mechanism for the increased activity of the enzyme. Alternatively, the zymogen may be more sensitive to autocatalytic degradation than the mature enzyme. To distinguish between these possibilities, the 70−73 kDa form of rBACE1 was subjected to N-terminal amino acid sequencing after activation of rBACE1 for 5 min with heparin. The result showed that, after incubation with heparin, the enzyme was predominantly in the zymogen form starting at threonine-22, indicating that heparin did not increase production of the mature form. Therefore, the observed loss of pro-rBACE1 immunoreactivity most likely indicated that the zymogen was more susceptible to autodegradation than the mature form. In addition, proteolytic removal of the pro domain appeared not to be necessary for the activation by heparin.

Heparin Affinity Chromatography of rBACE1. Heparin is known to bind to clusters of positively charged residues on the surface of proteins (*41*). Analysis of the tertiary folding of mature BACE1 did not reveal any obvious domain to us that could form a high-affinity binding site for heparin. However, as the main preparation of rBACE1 used in this study contained a mixture of both the pro and the mature (pro sequence cleaved) enzyme, and as the pro sequence (residues 22−45) contains five positively charged residues, we hypothesized that the heparin-induced stimulation of BACE1 was caused by binding of heparin to the pro sequence.

To analyze the binding of BACE1 to heparin further, rBACE1 was applied to a heparin column, which was then eluted with buffers of increasing salt concentration. Western blot analysis of the salt washes showed that pro-rBACE1

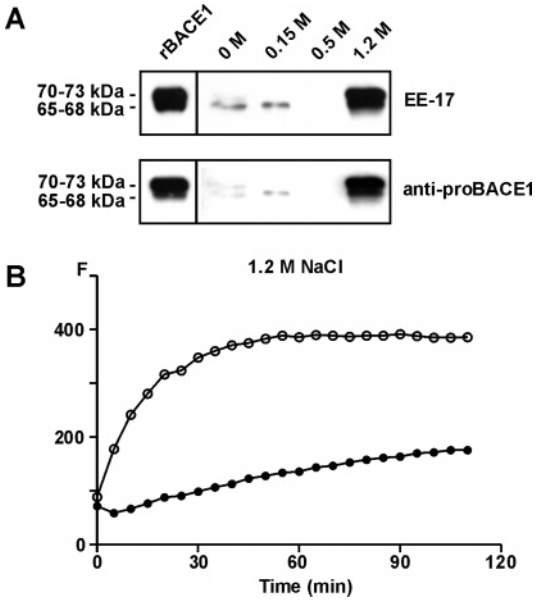


FIGURE 4: The proenzyme form of rBACE1 interacts strongly with heparin. (A) Western blot analysis of an aliquot (20 μL) from the fractions eluted from a heparin affinity column with increasing concentrations of NaCl as indicated. rBACE1 (15 μg) was applied to the column. Lane 1: 100 ng of rBACE1. The immunoblot was first incubated with anti-proBACE1 antibody and then stripped and reprobed with the EE-17 antibody. (B) Activity of rBACE1 during hydrolysis of 10 μM substrate peptide in the enzyme fraction recovered in the 1.2 M NaCl wash. Incubations were performed in the absence (closed circles) or presence (open circles) of 1 μg/mL heparin in 0.1 M sodium acetate buffer (pH 4). Similar results were obtained in two separate experiments.

bound strongly to heparin as it eluted primarily in the 1.2 M NaCl fraction (Figure 4A). Next, we analyzed the enzyme activity in the fractions collected from the heparin column. The enzyme eluting in the 1.2 M NaCl fraction was strongly activated by heparin (1 μg/mL) (Figure 4B). As the pro domain immunoreactivity was recovered predominantly in this fraction, this result supported the view that the zymogen was the target for the heparin-induced stimulation.

Heparin Affinity Chromatography of a Synthetic BACE1 Propeptide. To assess the heparin-binding capacity of the pro domain, we examined whether a synthetic peptide homologous to the pro domain could bind to the heparin column. A pro domain peptide was applied to the column and eluted with a stepwise salt gradient, and the collected fractions were assayed for the peptide by RP-HPLC. The peptide bound to the column and was recovered exclusively in the 0.5 M NaCl fraction (Figure 5), supporting the view that the BACE1 propeptide is able to interact with heparin.

Effect of Heparin on Mature rBACE1. Our data indicated that heparin-induced activation of enzyme activity may be mediated by an interaction of heparin with the pro domain of the enzyme. This would mean that the mature (pro sequence cleaved) enzyme should be insensitive to activation by heparin. To examine this possibility, the zymogen was removed from the rBACE1 preparation by immunoprecipitation with the anti-proBACE1 antibody. The supernatant fraction after immunoprecipitation was enriched in a 65−68 kDa band that lacked immunoreactivity to the anti-proBACE1 antibody (Figure 6). Removal of the proenzyme was found to abolish the stimulatory effect of heparin (1 μg/mL). In contrast, the activity in incubations of the original

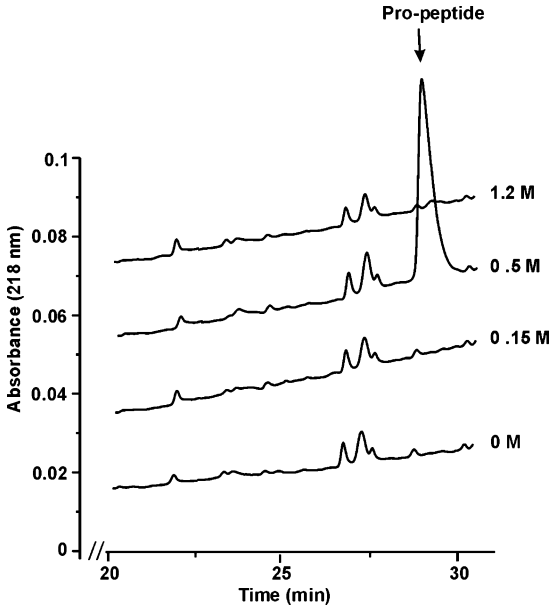


FIGURE 5: A synthetic peptide homologous to the pro sequence of BACE1 binds heparin. RP-HPLC analysis of fractions eluted with increasing concentrations of NaCl as indicated during heparin affinity chromatography of the synthetic propeptide. The propeptide (retention time = 28.4 min, arrow) was recovered almost exclusively in the 0.5 M fraction.

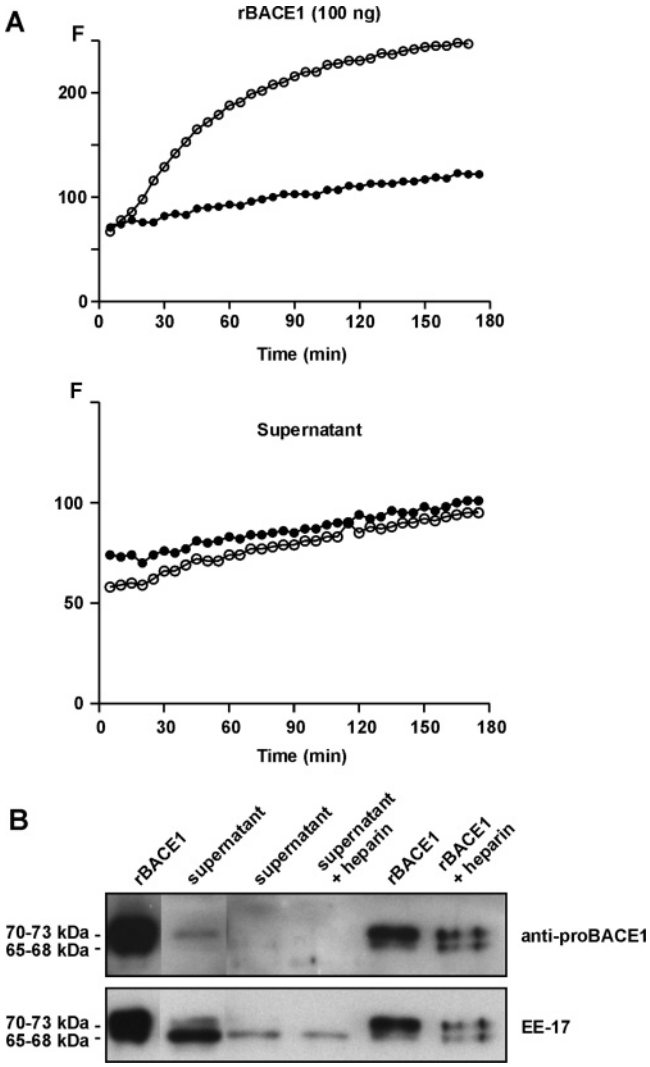


FIGURE 6: Effect of heparin on mature rBACE1. (A) BACE1 activity (*F*) during hydrolysis of 10 μM substrate peptide by 100 ng of rBACE1 (upper panel) or by a proenzyme-depleted supernatant fraction obtained after immunoprecipitation of rBACE1 with the anti-proBACE1 antibody (lower panel). Incubations were performed in the absence (closed circles) or presence (open circles) of 1 μg/mL heparin in a volume of 100 μL at pH 4. (B) Western blot analysis of rBACE1: lane 1, 100 ng of rBACE1; lane 2, supernatant fraction; lanes 3−6, analysis of aliquots (15 μL) taken after the enzyme activity assay in (A); lane 3, supernatant fraction; lane 4, supernatant fraction incubated with heparin (1 μg/mL); lane 5, rBACE1; lane 6, rBACE1 incubated with heparin (1 μg/mL). The immunoblot was first incubated with anti-proBACE1 antibody and then stripped and reprobed with the EE-17 antibody. Similar results were obtained in two separate experiments.

rBACE1 preparation, which primarily consisted of the zymogen, was strongly stimulated by heparin. As noted previously (Figure 3), the amount of 70−73 kDa enzyme, which was present in the original preparation and recognized by both the anti-proBACE1 and the EE-17 antibodies, was strongly decreased after incubation with heparin (1 μg/mL) (Figure 6B). In contrast, there was no loss of the 65−68 kDa form, which lacked the pro domain, after incubation with heparin (1 μg/mL). These results indicated that mature rBACE1 lacked the capacity to be activated by heparin and that stimulation of enzyme activity depended on the presence of the pro domain.

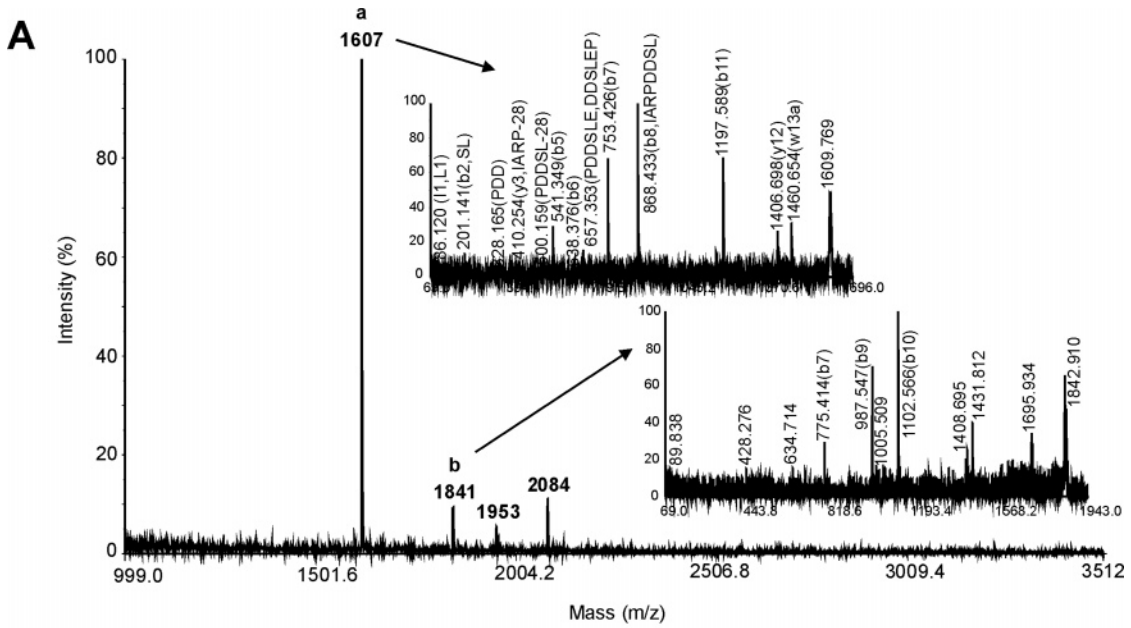
Analysis of rBACE1 Autocatalytic Cleavage Products by Mass Spectrometry. To identify products of heparin-stimulated autocatalytic cleavage of BACE1, MALDI-TOF MS analysis was performed. rBACE1 was activated by heparin for 10 min in sodium acetate buffer (pH 4) at 37 °C. A sample from this incubation and samples from control incubations containing rBACE1 or heparin alone were then subjected to MALDI-TOF MS analysis.

A number of autocatalytic cleavage products were identified by this method (Figure 7). However, we were unable to identify a peak corresponding to the mass (2536 Da) of the intact propeptide which would have been produced by cleavage of the zymogen on the C-terminal side of arginine-45 (Figure 7A).

We observed two major peaks at a *m*/*z* ratio of 1607 and 1841 in the rBACE1 sample incubated with heparin, which were not found in the control incubations (Figure 7A). To identify the peptides corresponding to the 1607 and 1841 peaks, the peptides in each fraction were subjected to MSMS analysis. The product ion spectrum of peak 1607 strongly suggested that this peptide was BACE1(185−198), which would have been generated by cleavage at a known autocatalytic cleavage site on the C-terminal side of tyrosine-184 (*10*) and at a novel cleavage site on the C-terminal side of phenylalanine-198 (Figure 7A, inset, and Figure 7C,D). Although fewer fragments were generated, the product ion spectrum of peak 1841 was consistent with a peptide that was produced by cleavage on the C-terminal side of leucine-182 and phenylalanine-198, i.e., BACE1(183−198) (Figure 7A, inset, and Figure 7C,D).

A peak with the *m*/*z* ratio of 2084 was also present in samples containing rBACE1 alone; i.e., it was not specifically generated by heparin treatment. One peak in the lower molecular mass range that was found in the incubation of rBACE1 with heparin (*m*/*z* ratio of 1953) remained to be identified.

In the higher molecular mass range, we observed two peaks at a *m*/*z* ratio of 5922 and 8441 that were found only



C

Peak	Observed mass	Peptide	Theoretical mass
a	1607	BACE1(185-198)	1607
b	1841	BACE1(183-198)	1841
c	5922	BACE1(46-99)	5922
d	8441	BACE1(22-99)	8440

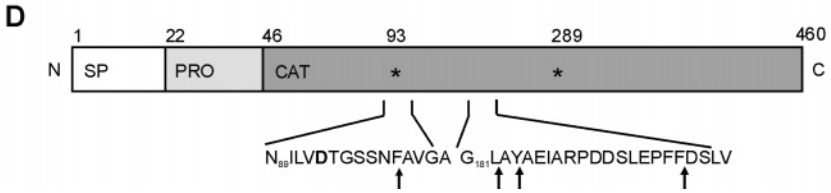


FIGURE 7: MALDI-TOF MS analysis of autocatalytic cleavage products of rBACE1. (A) MALDI-TOF MS spectra showing the percentage intensity against mass/charge (*m*/*z*) ratio in the range of 1000−3500 (A) and 3500−15000 (B) obtained from a sample of rBACE1 which had been activated by heparin. The inset in (A) shows the product ion spectra (MSMS) of the peptides corresponding to peaks a and b. (C) The observed masses of peaks a−d were consistent with the theoretical monoisotopic masses of the peptides shown. (D) Schematic illustration of the amino acid sequence and reported functional domains of BACE1(1−460) (*6*). Abbreviations: SP, signal peptide; PRO, pro domain; CAT, catalytic domain. Asterisks denote catalytically active aspartates, and numbers indicate amino acid position. Arrows show the putative heparin-induced cleavage sites as identified from the MS and tandem MS analysis. The figure also shows the catalytically active aspartate-93 (D) in bold. The representation of BACE1 is not drawn to scale.

in incubations of rBACE1 with heparin (Figure 7B). The observed masses were consistent with the masses of the N-terminal product of mature BACE1 and proBACE1, respectively, when cleaved at another known autocatalytic cleavage site on the C-terminal side of phenylalanine-99 (*10*) (Figure 7B−D).

For the 70−73 kDa zymogen, the cleavage at phenylalanine-99 would result in removal of an N-terminal fragment

of 8.4 kDa, producing a C-terminal fragment of rBACE1 with an apparent molecular mass of approximately 62−65 kDa. In support of this cleavage, a 62 kDa C-terminal fragment recognized by the anti-His antibody was observed after incubation with heparin (1 μg/mL) (Figure 3E). This 62 kDa fragment was not recognized by the N-terminal antibody directed against amino acids 46−62 of BACE1.

DISCUSSION

BACE1 is considered to be an important target for AD drug development because of its direct involvement in Aβ production. Since the first reports of its identification in 1999, BACE1 has been intensely studied (*6*−*9*, *42*). Several studies suggest that the activity of BACE1 is altered in AD and may be involved in the pathogenesis of the disease. For example, BACE1 protein expression and enzymatic activity were found to be increased in the brains of patients with AD (*43*−*45*). BACE1 activity, but not protein expression, was also reported to increase in human brains with aging (*46*). Recently, increased activity of human BACE1 in neurons of transgenic mice was shown to cause neurological deficits (*47*). Despite these studies, little is known about the mechanisms by which BACE1 activity may be regulated.

In this study, we show that a low concentration of heparin (1 μg/mL) stimulates rBACE1 and increases autocatalysis of the enzyme. The heparin-induced stimulation was due to both an increase in enzyme V_{max} and an increase in the enzyme's affinity for the substrate, as judged by the reduced K_M in the presence of heparin. In addition, our results strongly suggest that the pro domain is required for the stimulation of rBACE1 by heparin. The high-affinity heparin-binding fraction of rBACE1 was enriched in pro-rBACE1 immunoreactivity, and this fraction was strongly activated by heparin, indicating that the partially active zymogen was the target for stimulation by heparin. A preparation of mature rBACE1 lacked the capacity to be stimulated by heparin, indicating the importance of the presence of the pro domain for the activation by heparin. Furthermore, stimulatory concentrations of heparin induced autocatalytic cleavage of the enzyme involving a preferential loss of the zymogen form. Finally, we showed that a synthetic peptide homologous to the pro domain of BACE1 bound to heparin. Taken together, our results strongly suggest that the BACE1 zymogen is stimulated by low concentrations of heparin, and we propose that the stimulation is mediated by high-affinity binding of heparin to the pro domain of the enzyme.

There is increasing evidence that interactions between specific structural motifs in HS and HS-binding proteins are involved in the regulation of protein activities (*29*), including the stimulation of a number of proteases (*30*−*34*). The 24 amino acid long propeptide sequence of BACE1 contains five positively charged residues, of which four are arginines. In general, basic amino acids and especially arginines are known to interact strongly with the sulfate groups of heparin (*41*). It is also noteworthy that the propeptide is rich in leucine residues that apparently occur more commonly in known heparin-binding sites than statistically expected (*41*). We found that the binding of pro-rBACE1 to a heparin column was stronger than that of a synthetic peptide homologous to the pro sequence. Although it is possible that this difference in affinity is due to the fact that there are additional binding sites in other regions of the protein, it is also possible that the propeptide bound more weakly because its secondary and tertiary conformations differ from the pro domain in native BACE1. Notwithstanding the possibility that heparin may also bind elsewhere in the enzyme to inhibit activity (*36*), we propose that a high-affinity binding site for heparin, which is involved in the stimulation of enzyme activity, is located in the pro domain of BACE1.

The N-terminal pro domain of proteases can control enzyme activity by preventing entry of the substrate to the active site, and for many proteases full activation of the enzyme requires removal of the pro sequence (*48*). Cleavage of the pro sequence can occur by limited proteolysis by other proteases or by an autocatalytic mechanism. For many aspartic proteases, activation occurs at acidic pH through autocatalysis (*48*, *49*). Mature BACE1 purified from human brain has been reported to contain a single N-terminal sequence starting at glutamate-46 (*7*). Immediately preceding glutamate-46 is a PC cleavage recognition motif (RXXR) (*50*), and the PC furin is able to cleave proBACE1 and generate the mature enzyme (*27*, *28*).

Prolonged incubation of BACE1 at acidic pH (*51*) has been reported to generate various N-terminal sequences, including sequences beginning at glutamate-46. In view of the known cleavage specificity of BACE1, an autocatalytic cleavage on the C-terminal side of arginine-45 would be atypical, and using mass spectrometry we did not find any evidence for such cleavage when rBACE1 was incubated with heparin. Furthermore, N-terminal sequencing of rBACE1 after heparin-induced stimulation showed that the enzyme was predominantly in the zymogen form. Thus, our results indicate that the stimulatory effect of heparin does not depend on autocatalytic cleavage of the pro domain.

Autocatalytic removal of the pro sequence, or a major part of it, has been shown to increase BACE1 activity (*26*, *52*). In addition, pro sequence removal has been reported to induce local conformational changes in the N-terminal lobe of BACE1 (*53*). Our results are consistent with a model in which the binding of heparin to proBACE1 is sufficient to induce activation of the enzyme. It is possible that the binding of heparin to the proenzyme weakens the interaction between the pro domain and the catalytic cleft, similar to what has been suggested for the activation of lysosomal proteases by heparin (*54*). It may also be speculated that the binding of heparin to the proenzyme may, in part, induce conformational changes similar to those induced by proteolytic pro domain removal and, thereby, increase enzyme activity.

In our study, the initial phase of heparin-stimulated enzyme activity was followed by a second phase in which enzyme activity was lost. Consistent with an overall increased enzyme activity, prolonged incubations of rBACE1 with a low concentration of heparin resulted in extensive autocatalytic cleavage within the protease domain. In these incubations, cleavage most likely occurred on the C-terminal side of phenylalanine-99 and tyrosine-184. Previously, BACE1 expressed in *Escherichia coli* has been reported to be autocatalytically cleaved at these sites after prolonged incubation under acidic conditions (*10*). Phenylalanine-99 is located near the C-terminal side of the first catalytically active aspartate (aspartate-93), suggesting that cleavage at this site would inactivate the enzyme. In addition, a novel cleavage site on the C-terminal side of phenylalanine-198

was identified by tandem MS analysis. Combined cleavage on the C-terminal side of tyrosine-184 and phenylalanine-198 generated a 14 amino acid long peptide (1607 Da). Cleavage at phenylalanine-198 was also consistent with the production of another peptide (1841 Da), produced by combined cleavage on the C-terminal side of leucine-182 and phenylalanine-198. Analysis of the position of leucine-182, tyrosine-184, and phenylalanine-198 in the BACE1 crystal structure (PDB ID 1SGZ) (*55*) showed that cleavage at these sites would result in the removal of either a 14 or 16 amino acid long fragment from a surface-exposed loop in the N-terminal lobe of BACE1. This would separate the two catalytically active aspartates (aspartate-93 and aspartate-289), likely inactivating the enzyme. The present study is the first report of an autocatalytic cleavage on the C-terminal side of leucine-182 and phenylalanine-198. Importantly, both cleavage sites are consistent with the substrate specificity of the S_1 subsite in BACE1, which is the most stringent site preferring bulky hydrophobic residues such as leucine or phenylalanine (*56*, *57*). Taken together, our results strongly suggest that the loss in enzyme activity that follows heparin-induced stimulation is caused by autocatalytic cleavage at several sites in the protease domain. The physiological relevance, if any, of this mechanism remains to be elucidated.

In contrast to the inhibition that was produced with prolonged incubation at low concentrations of heparin, higher concentrations (100 μg/mL), which also inhibited enzyme activity, had very little effect on the autocatalysis of rBACE1. This demonstrates that inhibition of rBACE1 at the higher concentrations involved an entirely different mechanism than the inhibition observed with prolonged incubation at low heparin concentrations. In a previous study, Scholefield and colleagues showed that heparin inhibited BACE1, possibly by blocking access of the substrate to the active site (*36*). However, in that study no significant heparin-induced stimulation of BACE1 was reported. In our study, detection of the stimulatory effect depended on a low molar ratio of heparin to BACE1 and a relatively short incubation period because autocatalytic cleavage masked the stimulatory effect. Thus, it is possible that the ability of heparin to activate BACE1 was not detected by Scholefield et al. because of differences in their assay conditions. In addition, in the previous study (*36*), a BACE1-Fc fusion protein was used, and it is unclear to what extent this fusion protein carried the pro domain, as this was not reported in that study.

Our results are in agreement with the previous finding of Leveugle and colleagues that heparin promotes β-secretase cleavage in human neuroblastoma cells (*35*). The results presented here in this study support the view that HSPGs may play an important role in the regulation of BACE1 activity in vivo. Alterations in BACE1 activity or trafficking would be expected to change the rate of Aβ production in vivo. From a drug development perspective, targeting a BACE1 regulatory mechanism could be a strategy for lowering Aβ in AD. In view of this possibility, further studies on the nature of the interaction between HS and BACE1 are warranted.

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