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Heparan sulfate regulates amyloid precursor protein processing by BACE1, the Alzheimer's β-secretase

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Cleavage of amyloid precursor protein (APP) by the Alzheimer's β-secretase (BACE1) is a key step in generating amyloid β-peptide, the main component of amyloid plaques. Here we report evidence that heparan sulfate (HS) interacts with β-site APP-cleaving enzyme (BACE) 1 and regulates its cleavage of APP. We show that HS and heparin interact directly with BACE1 and inhibit in vitro processing of peptide and APP substrates. Inhibitory activity is dependent on saccharide size and specific structural characteristics, and the mechanism of action involves blocking access of substrate to the active site. In cellular assays, HS specifically inhibits BACE1 cleavage of APP but not alternative cleavage by α-secretase. Endogenous HS immunoprecipitates with BACE1 and colocalizes with BACE1 in the Golgi complex and at the cell surface, two of its putative sites of action. Furthermore, inhibition of cellular HS synthesis results in enhanced BACE1 activity. Our findings identify HS as a natural regulator of BACE1 and suggest a novel mechanism for control of APP processing.

Introduction

A characteristic feature of Alzheimer's disease (AD) is brain deposits of aggregated amyloid β-peptide (Aβ), which is considered by many as central to the pathogenesis of AD. Aβ is a predominantly 40–42–amino acid peptide produced from amyloid precursor protein (APP) by sequential proteolytic processing at the NH_2 and COOH termini of the Aβ domain (Howlett et al., 2000). After synthesis and post-translational modifications, a proportion of APP molecules are proteolytically cleaved by secretases in the Golgi complex, cell surface, and endosomal–lysosomal system to yield Aβ or alternative cleavage products (Hendriksen et al., 2002). Cleavage of APP by the recently identified aspartic protease β-secretase, also known as β-site APP-cleaving enzyme (BACE) 1, Asp-2, and memapsin-2 (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999; Lin et al., 2000), generates a soluble NH_2-terminal fragment (sAPPβ) and a membrane-bound COOH-terminal fragment (C99; see Fig. 1 A). Cleavage of the latter by an additional protease (γ-secretase) produces Aβ (Sisodia et al., 2001). Inhibition of BACE1 has therefore emerged as a key target for therapeutic intervention in AD (Howlett et al., 2000; Hendriksen et al., 2002).

A number of Aβ-associated molecules have been identified in tangles and senile plaques, including inflammatory proteins, apolipoproteins, and heparan sulfate proteoglycans (HSPGs; Verbeek et al., 1998). HSPGs play important roles in amyloidogenesis through interactions with various proteins. They are associated with the earliest stages of the formation of amyloid plaques (Snow et al., 1994); they colocalize with Aβ in neuritic plaques, cerebrovascular amyloid, and neurofibrillary tangles (Snow et al., 1990; Su et al., 1992); and they promote Aβ aggregation (McLaurin et al., 1999). Both heparan sulfate (HS) and heparin (a highly sulfated structural analogue of HS) bind fibrillar amyloid protein (Watson et al., 1997; Lindahl et al., 1999) and enhance fibril formation and stability (Castillo et al., 1997). Previous studies indicated that low molecular weight heparin saccharides inhibited APP secretion (Leveugle et al., 1998), prompting us to inves-

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Abbreviations used in this paper: Aβ, amyloid β-peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; BACE, β-site APP-cleaving enzyme; BLH, bovine lung heparin; C99, membrane-bound COOH-terminal fragment; DS, dermatan sulfate; HEK-BACE1, HEK293T cells stably transfected with myc-tagged BACE1; HS, heparan sulfate; HSPG, HS proteoglycan; PMHS, porcine mucosal HS; sAPPβ, soluble NH_2-terminal fragment; SHswAPP, SHSY5Y neuroblastoma cells expressing APP with the Swedish mutation; SWDC1, HEK293T cells expressing APP with the Swedish mutation.

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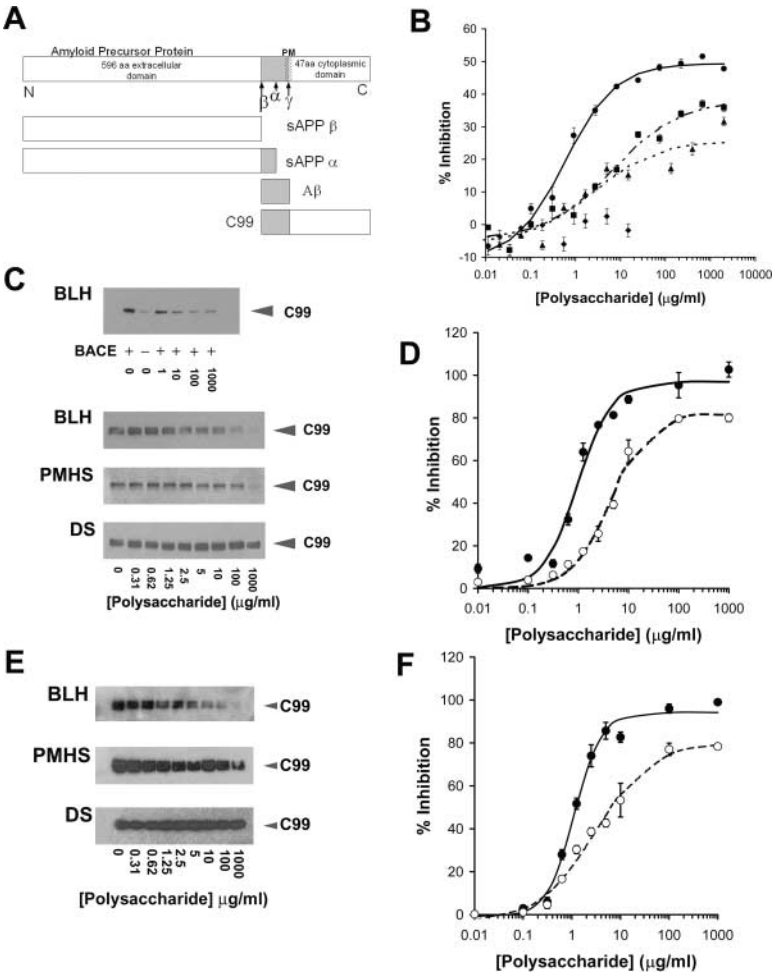

Figure 1. **Heparan sulfates inhibit BACE1 cleavage of APP.** (A) Schematic showing the structure of APP and the principal products generated by the action of α-, β-, and γ-secretases. (B) BACE1 activity at pH 4.5 was measured using an in vitro assay with a FRET peptide corresponding to the Swedish mutant β-cleavage site. Dose–response curves for BLH (●), PMHS (■), N1e neuroblastoma cell HS (▲), and DS (◆). Data are mean ± SD (n = 3). (C) BACE1 activity was also measured using an in vitro assay with authentic full-length APP substrate, pH 7. Dose responses to BLH, PMHS, and DS were assessed by Western blot detection of C99. (D) Graphical analysis of data derived by densitometry of C99 bands from C for BLH (●) and PMHS (○); data are mean ± SD (n = 2). (E and F) The same assay as in C and D but at pH 4.5.

tigate in more detail a potential role for cellular HS in APP metabolism. We report for the first time that HS interacts directly with BACE1 and regulates its activity. Our findings identify HS as a natural regulator of BACE1 and point to a novel mechanism for cellular modulation of APP processing.

Results

HS and heparin inhibit BACE1

Using a peptide cleavage FRET assay, we found that both HS and heparin inhibited BACE1 activity at its pH optimum of 4.5 (Fig. 1 B). A maximal level of inhibition of ~50–60% was observed in the presence of bovine lung heparin (BLH) with an IC_{50} of ~1–2 μg/ml (~100 nM). Porcine mucosal and bovine mucosal heparins exhibited similar activities (unpublished data). Porcine mucosal HS (PMHS) and HS purified from a neuroblastoma cell line (N1e) both displayed inhibitory activity (Fig. 1 B; $IC_{50} \approx 10$ μg/ml; ~400 nM), but required higher concentrations to achieve maximal inhibition (not depicted). The inhibitory activity was specific for HS-type polysaccharides because the sulfated galactosaminoglycan, dermatan sulfate (DS), was essentially inactive (Fig. 1 B), and chondroitin sulfate inhibited only weakly (~20% at 10–30 μg/ml) and did not inhibit at higher doses (not depicted). We observed similar inhibitory activities of BLH and PMHS in the presence of physiological concentrations of NaCl (unpublished data), indicating that these effects are not due to nonspecific electrostatic interactions at the low ionic strength of the acetate buffer. We also observed that BACE1 was active at neutral pH (7.0) in the FRET peptide assay, although its activity was ~60-fold lower than at its optima of pH 4.5 (unpublished data). In addition, we examined the possibility that HS might enhance BACE1 activity under appropriate conditions, in particular at lower concentrations. However, apart from a slight increase in activity at low concentrations in some assays (~5–10% at 10–100 ng/ml; Fig. 1 B), we did not observe any significant enhancement of BACE1 activity.

We confirmed the ability of heparin/HS to inhibit BACE1 activity with an assay using full-length APP substrate. Western blot analyses of cleavage of recombinant APP to produce the COOH-terminal fragment designated C99 (Fig. 1 A) demonstrated that both BLH and PMHS strongly inhibit BACE1 activity at both pH 7.0 (Fig. 2, C and D) and pH 4.5 (Fig. 2, E and F). In clear contrast to the peptide assay, maximal inhibition of ~90–100% was achieved. IC_{50} values of 1–2 μg/ml (~100 nM) for BLH and ~5 μg/ml (~200 nM) for PMHS were comparable to those observed in the peptide assay. Again, we obtained no evidence for enhancement of BACE1 activity by HS or heparin in these assays.

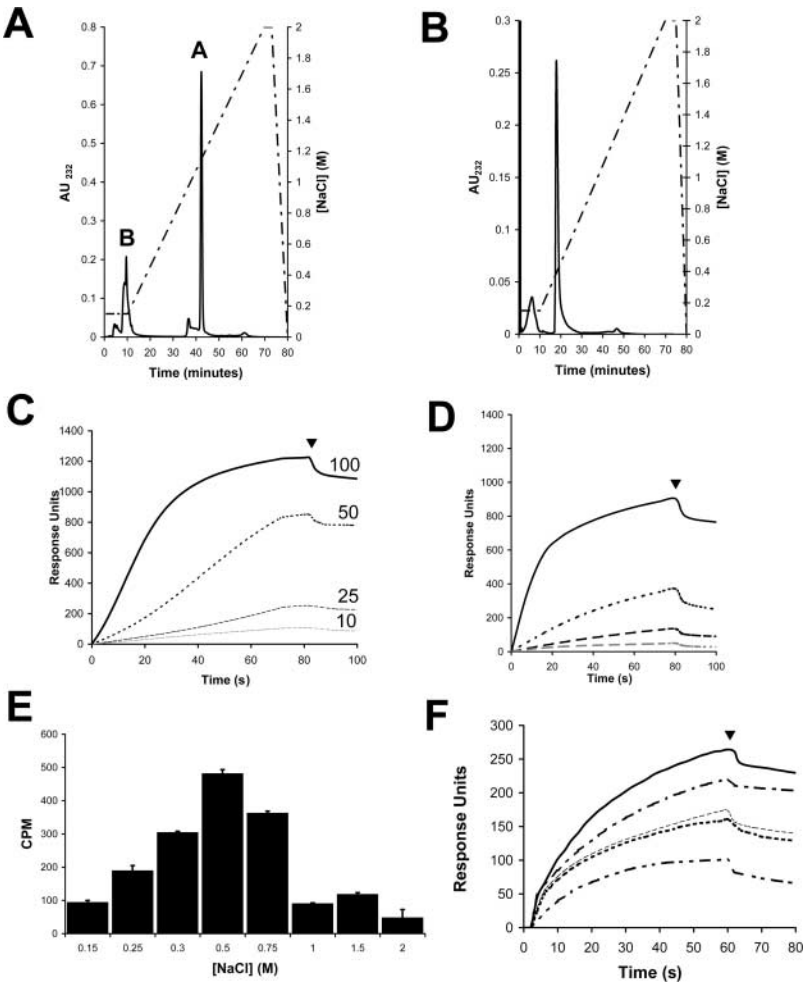

Figure 2. **BACE1 interacts directly with heparin and HS.** (A) Elution profile of BACE1-Fc from a heparin affinity column with an NaCl gradient (dashed-dotted line) at pH 7. (B) The same as in A but at pH 4.5. (C) Surface plasmon resonance biosensor binding profiles of the interaction of BACE1 at different concentrations with immobilized heparin at pH 7.0. Concentrations were 100 (solid line), 50 (dotted line), 25 (black dashed line) and 10 μg/ml (gray dashed line). (D) The same as in C but at pH 4.5. (E) Affinity profile of ^{3}H-labeled neuroblastoma HS (from N1e cells) from BACE1 protein immobilized in a filter binding assay (elution with a stepwise NaCl gradient). Data are mean ± SD ($n = 3$). (F) Inhibition of biosensor binding of BACE1 (25 μg/ml) to immobilized heparin, pH 7.0, by chemically modified heparins (25 μg/ml). Binding curves (from top): no inhibitor (solid line), 6-O-desulfated heparin (dash, single dot), unmodified BLH (dashed), oversulfated heparin (dotted), and N-acetylated heparin (dash, double dot). (arrowheads) Return to buffer alone; dissociation phase.

BACE1 is a heparin-binding protein

Using heparin affinity chromatography, we determined that the 10–amino acid FRET peptide does not bind to heparin at physiological ionic strength at pH 4.5 or 7.0 (unpublished data), suggesting that inhibition requires a direct interaction of HS with BACE1. To address this question, we subjected BACE1-Fc fusion protein to heparin affinity chromatography. The majority of the protein (~60%) bound tightly to the column at pH 7.0, eluting as a peak requiring ~1.2 M NaCl for elution (Fig. 2 A, peak A). Peak A contained 87% of the total BACE1 activity and displayed a high specific activity (4.7 U/μg) compared with the remainder of the protein that eluted at ~0.15 M NaCl (Fig. 2 A, peak B) with low specific activity (0.8 U/μg). Peak B's material contained some copurifying HS, as determined by dot blotting with an anti-HS antibody 10E4 (unpublished data), and this may explain its reduced activity. Binding was specific to BACE1 because Fc does not bind heparin (Powell et al., 2002). BACE1 also bound to heparin at pH 4.5 (the pH optima of BACE1) although with reduced affinity (elution at ~0.4 M NaCl; Fig. 2 B). We also determined the ability of BACE1 to interact with immobilized heparin using a surface plasmon resonance biosensor. BACE1 bound to heparin in a dose-dependent manner at both pH 7.0 (Fig. 2 C) and pH 4.5 (Fig. 2 D). The extent of binding at pH 4.5 was ~25% lower than at pH 7.0, supporting the above data that the interaction is weaker but still significant at the acidic pH.

To confirm that BACE1 also binds HS, we used an affinity filter binding assay. Metabolically radiolabeled HS from N1e neuroblastoma cells was eluted from nitrocellulose-bound BACE1 using stepwise salt washes (0.15–2.0 M NaCl). Essentially, all the HS chains bound to BACE1 and eluted predominantly at 0.3–0.75 M NaCl (Fig. 2 E). The spread of binding fractions is typical for HS in these assays, reflecting the structural heterogeneity of HS polysaccharides in comparison with heparin. These results demonstrate that biologically active BACE1 interacts directly with HS and heparin, and displays a high relative affinity for these polysaccharides.

BACE1 inhibition depends on specific HS structures

To establish whether inhibition of BACE1 by heparin/HS is dependent on saccharide size, we examined the activity of various size-defined heparins in the peptide cleavage assay (Fig. 3, A and B). Inhibition was clearly dependent on saccharide fragment length, with no inhibitory activity in fragments of <10 monosaccharide units (10mer; Fig. 3 A). The level of inhibitory activity increased with increasing fragment length, and ~24mers or larger displayed activity equivalent to full-length heparin (Fig. 3 B). Sized fragments from PMHS were also examined; ~14mers or larger were required for significant inhibition of BACE1 (unpublished data). This difference is probably due to the fact that these

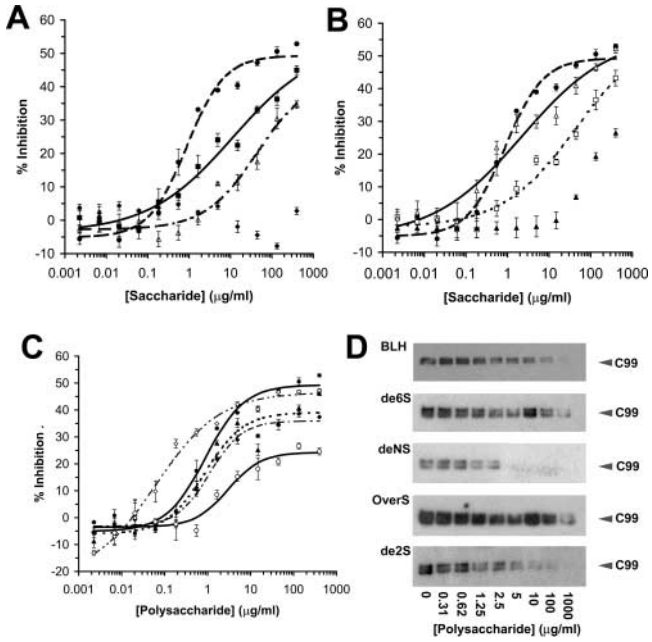

Figure 3. **Specific saccharide structures are required for inhibition of BACE1.** Structural specificity of the inhibition of BACE1 by HS/heparin was investigated using size-defined, and chemically modified, heparins in the FRET peptide cleavage assay, pH 4.5. (A and B) Dose–response curves of the inhibitory activity of size-defined fragments from BLH. (A) 8mer (◆), 12mer (△), 20mer (■), and full-length BLH (●); (B) 10mer (▲), 16mer (□), 24mer (△), and full-length BLH (●). (C) Dose–response curves of the inhibitory activity of chemically modified BLH: 80% 6-O-desulfated (de6S; ○), 2-O-desulfated (de2S; ◆), de-N-sulfated/reN-acetylated (deNS; ◇), oversulfated (OverS; ▲), and parental BLH (●). Data for A–C are mean ± SD ($n = 3$). (D) Inhibitory activity of the chemically modified heparins described in C measured using the APP substrate assay, pH 7, with Western blot detection of C99 product.

saccharides often contain lower sulfated flanking disaccharides (Guimond and Turnbull, 1999).

Next, we evaluated whether inhibition of BACE1 is dependent on specific structural features of heparin/HS by testing selectively chemically modified heparins in the peptide cleavage assay (Fig. 3 C). Removal of ~80% of 6-O-sulfates dramatically reduced inhibitory potency, whereas complete removal of 2-O-sulfates resulted in only a moderate reduction in activity compared with heparin. Over-sulfation of heparin also reduced its inhibitory activity, providing further evidence that potency is not related simply to sulfation level, and supporting the data indicating that other sulfated glycosaminoglycans have low potency (Fig. 1). Remarkably, removal of N-sulfate groups and their replacement by N-acetyl groups resulted in a substantial increase in potency (approximately fivefold) compared with heparin ($IC_{50} \approx 0.2$ μg/ml; ~16 nM). In contrast, complete desulfation (removal of all N- and O-sulfates) abolishes all activity (unpublished data). Thus, activity is dependent on 6-O-sulfate groups, is only partially dependent on 2-O-sulfate groups, and is enhanced by the presence of additional N-acetylated glucosamine residues. We obtained no evidence for any significant enhancement (>5–10%) of BACE1 activity with any of these modified polysaccharides, even at low concentrations (~1–100 ng/ml; Fig. 3 C).

The differential activities of chemically modified heparins were confirmed with APP as substrate and were in complete agreement with the peptide substrate data (Fig. 3 D). We also observed that the relative inhibitory activities of these compounds correlated directly with their differing abilities to act as soluble inhibitors of the BACE1–heparin interaction using surface plasmon resonance. N-acetylated heparin was more potent and 6-O-desulfated heparin less potent than parental BLH (Fig. 2 F). Together, these experiments clearly show that saccharide size and sulfation patterns are critical for the inhibitory activity of these molecules, and we obtained no evidence that any variant structures could enhance BACE1 activity. Furthermore, the data indicate that inhibitory potency correlates directly with ability to bind BACE1.

Cellular BACE1 is inhibited by heparin/HS

Next, we addressed the question of whether heparin/HS also inhibits BACE1 activity in cells by undertaking Western blot analyses of media from SHSY5Y neuroblastoma cells expressing APP with the Swedish mutation (SHswAPP). Treatment with either BLH or PMHS inhibited generation of the BACE1 cleavage product sAPPβ, whereas DS had no effect (Fig. 4 A, left panels; and Fig. 4 B, dose–response curves). In addition, chemically modified heparins produced levels of BACE1 inhibition, relative to heparin, identical to those observed in the peptide and APP in vitro cleavage assays. Importantly, the enhanced potency of N-acetylated heparin was confirmed. In contrast, there was no effect on generation of the α-secretase cleavage product sAPPα (Fig. 4 A, right panels), showing that inhibition was specific for BACE1. Similar results were obtained with HEK293T cells expressing APP with the Swedish mutatation (SWDC1cells; unpublished data). We confirmed these effects on cellular BACE1 activity by measurement of generation of $A\beta_{1\text{-}42}$ peptide by SWDC1 cells using an ELISA assay. Treatment with BLH or PMHS resulted in a dose-dependent inhibition of the levels of $A\beta_{1\text{-}42}$ secreted into the medium, and the N-acetylated heparin was again more potent than BLH (Fig. 4 C). Similar data on the inhibition of $A\beta_{1\text{-}42}$ secretion were obtained using SHswAPP cells (unpublished data). Furthermore, $A\beta_{1\text{-}40}$ secretion was also inhibited to an equivalent degree in both SWDC1 and SHswAPP cells (unpublished data). Although the apparent maximal level of BACE inhibition is higher, measured by sAPPβ levels (Fig. 4, A and B) compared with $A\beta_{1\text{-}42}$ levels (Fig. 4 C), the IC_{50} values were in good agreement in all cases and were closely similar to those obtained in the in vitro experiments (Figs. 1 and 3). Together, these results demonstrate that HS added exogenously to cells selectively inhibits BACE1 processing of APP, with no apparent effects on the α-secretase or γ-secretase processing events.

Endogenous HS interacts with and regulates BACE1

Next, we sought to determine whether BACE1 interacts with endogenous HS in cells. HEK293T cells express HSPGs that can be detected by Western blotting of cell lysates using an anti-HS antibody (10E4), and their identity was confirmed by susceptibility to heparitinase digestion (Fig. 5 A). We found that HSPGs were coimmunoprecipitated with an anti-BACE1 antibody from cell lysates of both parental HEK cells and HEK293T cells stably transfected with myc-tagged BACE1

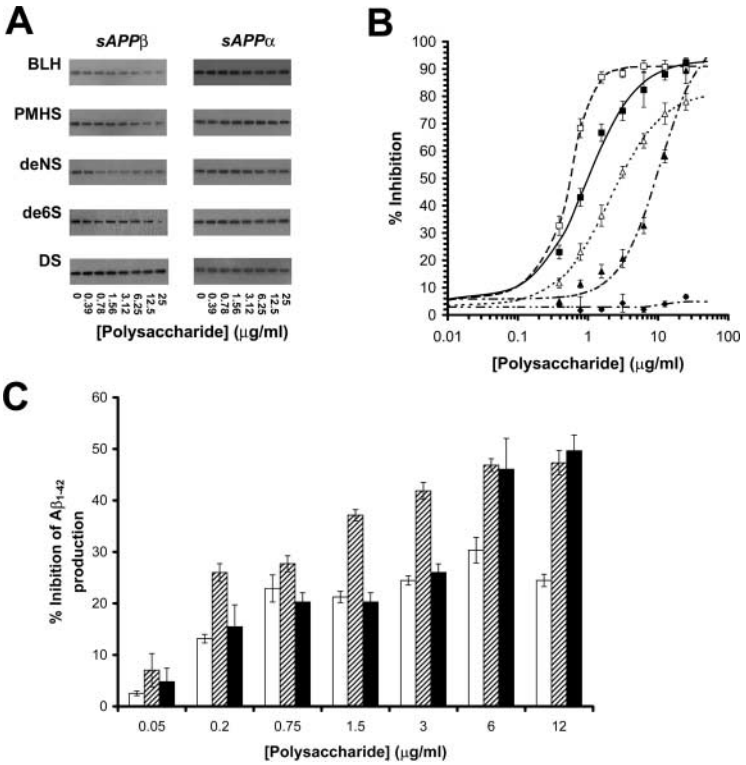

Figure 4. **Cleavage of APP by BACE1 in cells is inhibited by HS.** The effects of addition of exogenous HS to cells on BACE1 activity was tested in cells expressing swAPP. (A) The levels of sAPPβ and sAPPα cleavage products in the medium of SHswAPP exposed to different doses of HS/heparins (Fig. 3) were measured by Western blotting. (B) Graphical analysis of the of data derived by densitometry of bands from A. BLH (■), PMHS (△), de6S (▲), deNS (□) and DS control (◆). (C) The levels of $A\beta_{1-42}$ peptide in the medium of SWDC1 exposed to PMHS (white bar), BLH (black bar), and N-acetylated heparin (hatched bar) was measured by ELISA. Data are mean ± SD (n = 3).

(HEK-BACE1; Fig. 5 A), indicating that BACE1 interacts with endogenous HS and forms complexes. We also found that BACE1 coimmunoprecipitated with HS when an anti-HS antibody 10E4 was used (unpublished data). Quantitative measurements using a CCD camera (see Materials and methods) indicated that only a proportion of HSPGs (~10–20%) were coimmunoprecipitated from cells expressing endogenous BACE1 levels (parental HEK), whereas the amount coimmunoprecipitated increased in HEK-BACE1 cells, which overexpress BACE1 (unpublished data). It was noteworthy that BACE1 selectively immunoprecipitated only a fraction of the total HSPGs expressed in the HEK cells. In particular, the lower molecular mass HSPGs in the range 70–100 kD were preferentially immunoprecipitated due to their interaction with BACE1 protein. Specificity of the interaction was confirmed in three ways. First, we confirmed that the anti-BACE1 antibody was not binding nonspecifically to the HSPGs by demonstrating that an anion exchange purified HSPG extract devoid of BACE1 protein was not susceptible to immunoprecipitation (Fig. 5 B). Second, we showed that inclusion of heparin (a selective inhibitor of HS–protein interactions) abolished BACE1-mediated immunoprecipitation of HSPGs (Fig. 5 C). Third, using a sensitive protein stain, we also confirmed that BACE1 was the only protein immunoprecipitated in significant quantity (>95% of total protein) from the lysate, discounting the possibility that a secondary protein was mediating the HS–BACE1 interaction (unpublished data).

To identify the localization of these cellular BACE1–HS complexes, we performed immunofluorescence microscopy on HEK-BACE1 cells using antibodies specific for BACE1 and HS. myc-BACE1 was detected with an anti-myc antibody and was predominantly expressed intracellularly in the Golgi complex, and on the cell surface (Fig. 6 A, top panels). HS was expressed on the cell surface and also in the Golgi complex, and merged images demonstrated its colocalization with BACE1 (Fig. 6 A, top panels). To rule out the possibility that colocalization of BACE1 and HS was due to overexpression of BACE1, we also examined their localization in non-BACE–transfected SHswAPP cells. As observed for the HEK-BACE1 cells, HS was strongly expressed on the cell surface but also in the Golgi complex, and colocalized with BACE1 (detected with a rabbit anti-BACE1 antibody) (Fig. 6 A, bottom panels). Similar data for BACE1 localization in SHswAPP cells were also obtained with a mouse anti-BACE1 monoclonal antibody (unpublished data). We also performed confocal immunofluorescence analysis; the data clearly confirmed the colocalization of HS and BACE1 at the cell surface and in the Golgi complex in HEK-BACE1 cells (Fig. 6 B).

Golgi localization of both BACE1 and HS was confirmed by colocalization with a known Golgi marker, β-COP protein (Fig. 6 C). Similar results for BACE1 in HEK-BACE1 cells were obtained using rabbit anti-BACE1 and chicken anti-BACE1 polyclonal antibodies (unpublished data). Markers of late endosomal (Fig. 6 C, CD63) and recycling endosomal (CD71; not depicted) compartments were used to confirm that BACE1 is also expressed at these sites. Similar data were obtained with SHswAPP cells (unpublished data). HS was not detected in endosomes with 10E4 antibody, probably because this epitope is sensitive to partial degradation by heparanase in this compartment.

Overall, these results demonstrate that endogenous HS colocalizes with BACE1 in at least two of the cellular sites (cell surface and Golgi complex) identified as potential sites for cleavage of APP substrate by BACE1. If endogenous HS plays a role in restricting BACE1 activity, it might be expected that

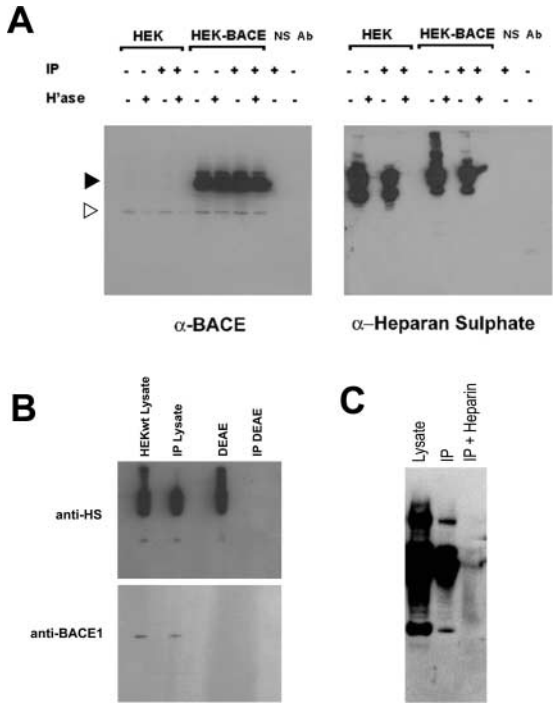

Figure 5. **BACE1 interacts with endogenous HS in cells.** The interaction of BACE1 with endogenous HS was examined using coimmunoprecipitation assays. (A) Western blot analyses were performed on cell lysates (20 μl) from myc-BACE1–transfected (HEK-BACE) and parental HEK293T (HEK) cells, either with or without immunoprecipitation of BACE1 with a chicken anti-BACE1 antibody (IP). Detection was achieved with a rabbit anti-BACE1 antibody (for BACE1) and 10E4 antibody (for HS). myc-tagged BACE1 was identified by reprobing with an anti-myc antibody (not depicted). (black arrowhead) myc-tagged BACE1; (white arrowhead) native BACE1; H'ase, heparitinase treated; NS, no sample (PBS); Ab, IP antibody only. (B) HSPGs from wild-type HEK 293T lysates were purified by DEAE anion exchange and subjected to Western blotting with or without immunoprecipitation as in A. The HEK lysate contained both HSPGs (top) and BACE1 (bottom), and both were immunoprecipitated by anti-BACE1. In contrast, the purified HSPG extract (DEAE), which did not contain BACE1 (bottom) was not immunoprecipitated by an anti-BACE1 antibody. (C) Lysates from HEK293T cells were subjected to immunoprecipitation of BACE1 as in (A), in the presence or absence of 50 μg/ml heparin (an inhibitor of HS–protein interactions).

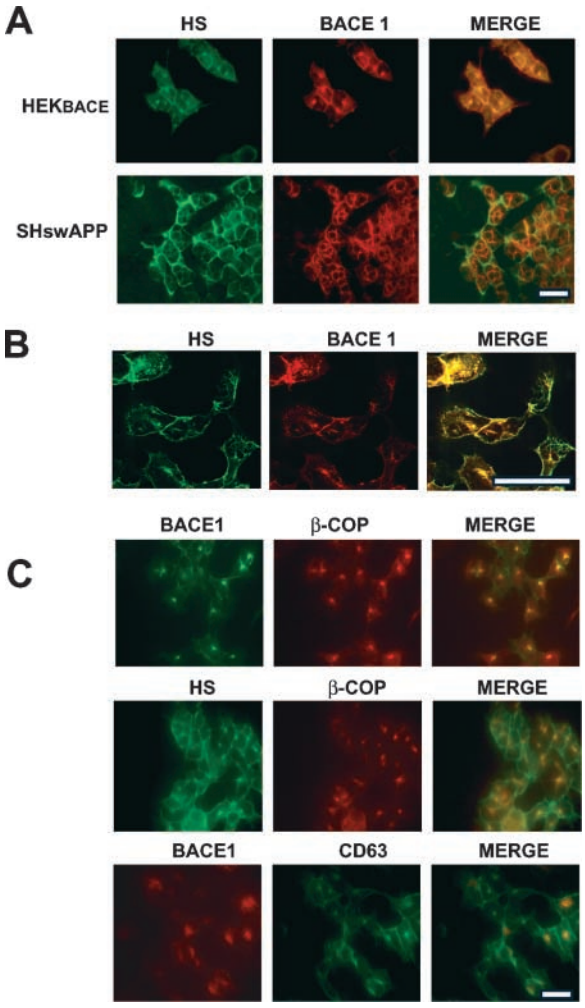

Figure 6. **BACE1 colocalizes with endogenous HS in cells.** The colocalization of BACE1 with endogenous HS was examined using immunofluorescence microscopy. (A) Immunofluorescence double staining of HEK-BACE and SHSY5YswAPP cells was performed to detect HS (green; 10E4 antibody) and BACE1 (red; anti-myc tag for HEK-BACE1 or rabbit anti-BACE1 for SHSY5Y cells). Merged images show colocalization of BACE1 and HS predominantly at the cell surface and in the Golgi complex. (B) Confocal immunofluorescence microscopy of HEK-BACE cells staining for HS (green; 10E4) and BACE1 (red; rabbit anti-BACE1). (C) Immunofluorescence of HEK-BACE cells staining for HS (green; 10E4) or BACE1 (green, top panels; red bottom panels; goat anti-BACE1), and comparison with known markers of the Golgi complex (red; β-COP) or late endosomes (green; CD63). Bars, 10 μm.

reducing cellular HS levels could enhance BACE1 activity. To explore this possibility, we examined the effect of chlorate treatment on cellular BACE1 activity. Chlorate is a competitive inhibitor of the formation of the sulfate donor (PAPS) required for sulfation in HS biosynthesis; chlorate treatment of cells resulted in abolition of binding of 10E4 antibody to cells, confirming the absence of the sulfated endogenous HS epitope (unpublished data). Under these conditions, the production of both $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}40}$ peptides was significantly elevated (by ~20 and 40%, respectively) in comparison with control cells (Fig. 7, A and B), indicating that endogenous HS is acting to restrict BACE1 activity in untreated cells. Elevated BACE1 activity in the chlorate-treated cells (as measured by Aβ levels) was susceptible to inhibition by exogenous heparin with equivalent IC_{50} values to those seen in normal cells (Fig. 7, C and D). Moreover, the level of sAPPα is completely unaffected by these treatments; however, sAPPβ levels were elevated by chlorate treatment, and could be inhibited in both control and chlorate-treated cells by addition of exogenous heparin (Fig. 7 E). These results indicate that elevated BACE1 activity was not due to secondary chlorate-induced alterations in trafficking or rates of production of APP. In addition, treatment of cells with a mixture of heparitinases (which remove cell surface HS) results in a marked elevation of BACE1 activity (Fig. 7 F). Together, these results strongly support the view that endogenous HS acts to restrict BACE activity.

Mechanism of HS inhibition of BACE1

To explore how exogenous heparin/HS can inhibit BACE1, we examined the time course of binding and uptake of flu-

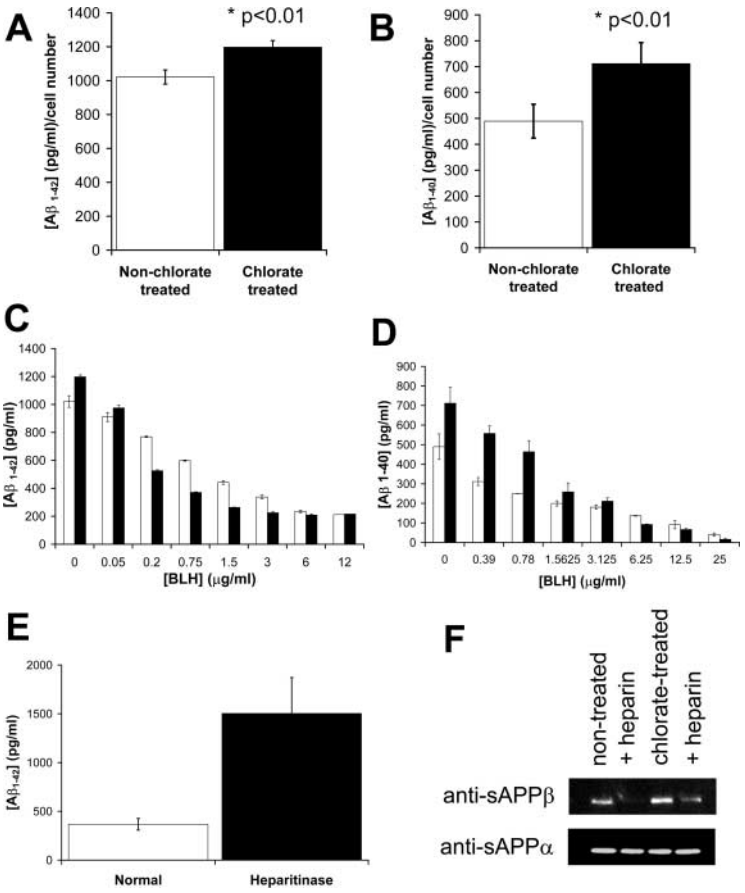

Figure 7. **Inhibition of endogenous HS synthesis or sulfation induce elevated levels of BACE1 activity.** The effect of chlorate on the generation of BACE1 cleavage products of APP in SHswAPP cells was assessed by immunoassay of $A\beta_{1\text{-}42}$ (A) and $A\beta_{1\text{-}40}$ (B) peptides in the medium of cells with or without chlorate treatment (see Materials and methods). *, $P < 0.01$ (*t* test). All data are mean ± SD ($n = 3$). The heparin dose response of cells treated with (black bars) or without (white bars) chlorate was assessed, measuring $A\beta_{1\text{-}42}$ (C) and $A\beta_{1\text{-}40}$ (D) peptides in medium by immunoassay (see Materials and methods). (E) SHswAPP cells were treated with 30 mM chlorate in the presence or absence of heparin (50 μg/ml) for 12 h, and measurement of medium sAPPα and sAPPβ was performed as described in Fig. 4. (F) SHswAPP cells were treated with a mixture of heparitinases (see Materials and methods) to remove cell surface HS, and the levels of medium $A\beta_{1\text{-}42}$ were measured by immunoassay. Chlorate or heparitinase treatments abolished binding of 10E4 anti-HS antibody to cells (not depicted).

orescent-labeled heparin by SWDC1 cells. Using Alexa 488–heparin, we detected specific binding at the cell surface within 30 min, strong uptake, and intracellular clustering within 2 h. Internalized heparin clearly colocalized with the endosomal marker CD-71 and, there was no evidence of localization in the Golgi complex (unpublished data). These data suggest that the inhibitory effects of exogenous heparin/HS are primarily exerted at the cell surface and in endosomal compartments. Studies to determine the mechanism of action of heparin/HS on BACE1 were undertaken using fluorescence polarization to examine the binding of a rhodamine-labeled noncleavable peptide substrate (R110) to BACE1. The R110 ligand binds to free BACE1 at the substrate binding site, increasing the polarization of light relative to unbound ligand. Binding of a known competitive ligand (GW 642976A) at the active site is detected as a decrease in polarization (due to dose-dependent displacement of the R110 ligand) whether added after or before preequilibration of R110 with BACE1 (Fig. 8, A and C). In contrast, we observed that if R110 is preequilibrated with BACE1, inhibition by BLH or N-acetylated heparin was abolished (Fig. 8 B); inhibition was achieved if BLH or N-acetylated heparin were preequilibrated with BACE1 (Fig. 8 D). No effect was observed for a DS control under either conditions (Fig. 8, B and D). These data suggest that HS acts to prevent entry of the substrate to the active site, and, furthermore, that if the ligand is prebound to BACE1, HS can effectively lock the ligand into the active site.

Discussion

HS interacts with BACE1 and regulates APP cleavage

Our in vitro assay data demonstrate direct inhibition of BACE1 cleavage of both peptide and full- length APP substrates by heparin/HS polysaccharides (Fig. 1). Inhibition of APP cleavage was confirmed in cell assays measuring formation of the BACE1 cleavage products sAPPβ and Aβ (Fig. 4), and was specific for BACE1 because no effect on α-secretase was evident (Fig. 4). Interestingly, our data differ from a previous study in which heparin was shown to enhance the levels of sAPPβ secreted by cells (Leveugle et al., 1997). We did not obtain any evidence for significant enhancement of BACE1 activity by any heparin/HS structures. The most likely explanation for these differences is that the previous experiments involved a 24-h recovery period after heparin treatment; hence, their data may reflect a transient rise in BACE1 activity in response to withdrawal of inhibition.

Our fluorescence polarization data (Fig. 8) provided an insight into the mechanism of action of HS. The data suggested that the principal mechanism for inhibition involves binding of HS to BACE1, probably at or near the active site in a manner that blocks access of the substrate (Fig. 9). The cocrystal structure of BACE with the peptide isoster analogue OM99-2 reveals the existence of a hairpin loop covering the active site (Hong et al., 2000). This loop is common to aspartyl proteases and is referred to as the flap. Our data agree with the binding of HS occurring with the closed conformation of the active site. Confirmation of this possibility

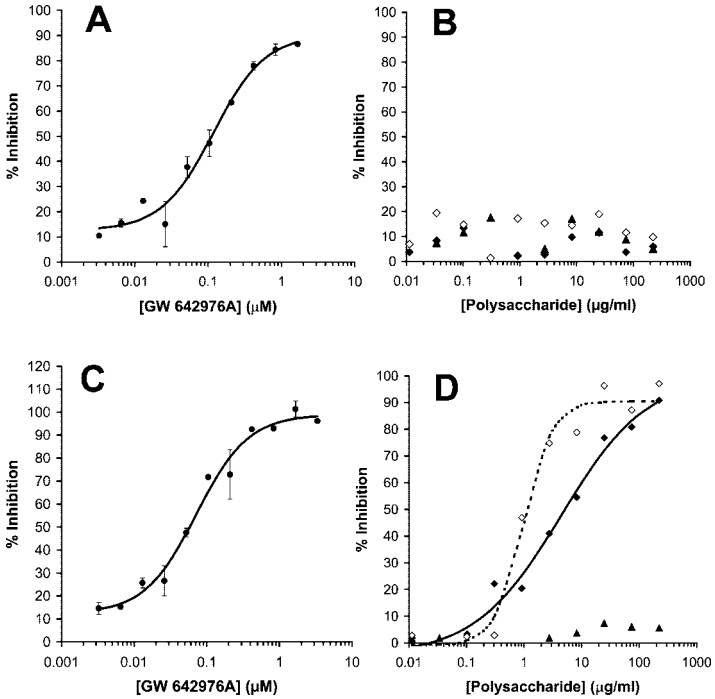

Figure 8. **Heparan sulfate blocks access of substrate to the BACE1 active site.** Fluorescence polarization measurements were made using a noncleavable fluorescent ligand R110 (see Materials and methods). Fluorescence polarization measures the binding or displacement of the fluorescent ligand by detecting changes in polarization. Dose responses are shown for a known competitive inhibitor (A and C; GW 642976A) or polysaccharides (B and D; BLH, ◆, solid line; NAc heparin, ◇, dashed line; and DS, ▲). BACE1 was equilibrated for 2 h with either R110 (A and B) or the inhibitor compounds (C and D), followed by a 6-h incubation to establish equilibrium. All data are mean ± SD ($n = 3$).

would require NMR and X-ray crystallography studies, which are in progress. Although natural inhibitors of aspartyl proteases are rare, another unusual inhibitor binding to proteinase A has recently been reported (Li et al., 2000).

To the best of our knowledge the data presented here are the first identification of a natural direct inhibitor of BACE1, and the first description of BACE1 as a heparin/HS–binding protein. It is well known that APP also interacts with heparin (Multhaup et al., 1995; Clarris et al., 1997), and we have recently determined that a subpopulation of neural HS chains bind APP with high affinity (unpublished data). Thus, our data demonstrate that HS can interact with both the protease and its substrate. This suggests an additional mechanism of inhibition of BACE1 in which HS could sequester APP away from the active site (Fig. 9). It is noteworthy that much higher levels of inhibition are apparent with authentic APP in comparison with peptide substrates. This suggests that binding of HS to BACE1 alone is insufficient for maximal inhibition and that binding of HS to APP may be an important component of the inhibition of cleavage of authentic protein substrate. Further studies are needed to identify the HS-binding site in BACE1 and to isolate and fully characterize the cellular HS structures responsible for interactions with BACE1. Surface charge modeling of the published crystal structure of a β-secretase dimer (Hong et al, 2000) reveals some strongly basic regions as potential binding sites (unpublished data). It is plausible that HS interacts with such sites to regulate substrate access, although we cannot exclude the possibility of conformational effects on the BACE1 protein that allosterically regulate the active site.

Structural determinants in HS for BACE1 inhibition

With respect to the HS structural specificity for BACE1 inhibition, inhibitory activity was present in heparin saccharides of at least 10 monosaccharide units (Fig. 3). Over-sulfated heparin (which serves as a control for high charge) and de–2O- or de–6-O-sulfated heparin have reduced potency compared with heparin, whereas substitution of N-sulfate with N-acetyl groups results in increased inhibitory activity (Fig. 3 C). This argues strongly that inhibitory activity is not simply a function of charge density but results from a significant degree of specificity in the glycan structures. This view was supported by experiments on the ability of the modified heparins to inhibit binding of BACE1 to heparin (Fig. 1 F). The removal of iduronate 2-O-sulfate or glucosamine 6-O-sulfate groups may impair molecular interactions of HS with BACE1. In contrast, replacement of N-sulfates with N-acetyl groups may increase inhibitory activity due to enhanced interactions of the uncharged N-acetyl function in the BACE1 binding site. However, there is also evidence that conformations of both sugar rings (in iduronate) and glycosidic linkages are altered upon various structural modifications (Yates et al., 2000). Hence, subtle conformational effects of altered substitution patterns may also underlie differences in activity. Overall, it is clear that there is a considerable degree of specificity in the size and structural characteristics of HS saccharides competent to inhibit BACE1 activity. This is important because there is emerging evidence that cells dynamically regulate HS structures, e.g., during neurogenesis (Ford-Perriss et al., 2002). Regulated synthesis of appropriate inhibitory HS structures, and modulation of their concentration levels at appropriate subcellular sites, may provide neural cells with a mechanism for modulating BACE1 processing of APP.

BACE1 interacts with endogenous cellular HS

We also addressed the crucial question of the ability of BACE1 to bind endogenous HS in cells. HS was immunoprecipitated by BACE1 from cell extracts (Fig. 5), which is consistent with the direct in vitro interaction we have de-

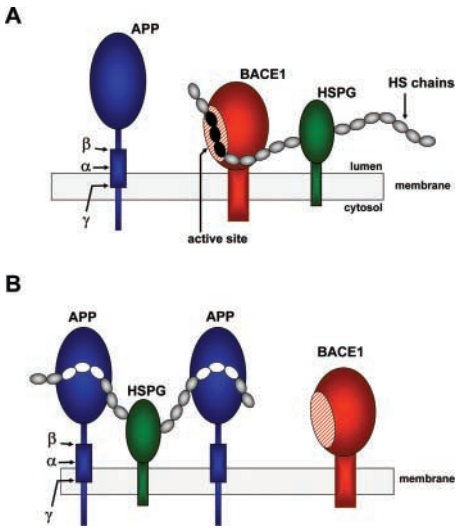

Figure 9. **Model for regulation of BACE1 processing of APP by HS.** (A) Direct inhibition of BACE1: HS chains (linked gray oval shapes) interact directly with the protease BACE1 (red), preventing access of the substrate APP (blue) to the active site (red diagonal hatching), or alternatively through conformational effects that allosterically regulate the active site. Specific HS structural motifs (black ovals) probably mediate this inhibitory interaction. The level and concentration of active HS structural motifs expressed on HSPGs in relevant cell compartments where BACE1 acts on APP could modulate the level of Aβ generated. (B) Sequestration of APP: HS also contains binding sites for APP (white ovals), providing an additional mechanism of action in which HS sequesters APP away from BACE1. This mechanism is supported by the enhanced BACE1 inhibitory action of HS with full-length versus peptide APP substrates. In addition, some neural HS chains have high affinity APP-binding sites distinct from those for BACE1 (unpublished data). Subpopulations of HSPGs carrying HS chains with APP-binding sites could segregate APP from BACE1. (arrows) α-, β- (BACE1), and γ-secretase cleavage sites.

scribed here (Fig. 2). The data indicated that selective cell-associated HSPG species bind to BACE1; likely candidates are syndecans or glypicans (see next paragraph), and the precise identity of these remain to be elucidated in future studies. BACE1 is thought to act on APP at several subcellular sites including the Golgi complex, cell surface, and endosomes (Haass et al., 1995; Thinakaran et al., 1996; Perez et al., 1999). Importantly, we have demonstrated here that BACE1 colocalizes with HS in both the Golgi complex and at the cell surface (Fig. 6), and that removal of endogenous HS or inhibition of HS sulfation results in increased BACE1 activity (Fig. 7). These data provide strong evidence that HS–BACE1 interactions are relevant in a cellular context, and also that cellular HS acts to restrict the activity of BACE1. We have not yet established colocalization of HS with BACE1 in endosomes, but it is well known that HS is present in endosomes during its trafficking to the lysosomes for degradation (Yanagishita, 1992; Egeberg et al., 2001). However, we have observed that exogenous heparin is taken up and concentrated in the endosomes (unpublished data), making this a likely site for the inhibitory action of exogenous HS/heparin, in addition to the cell surface. Furthermore, we have shown that HS and heparin bind and inhibit BACE1 under both neutral and acidic conditions (Figs. 1 and 2), indicating the potential for HS to modulate BACE1 activity at both the cell surface and in the acidic environment of endosomal compartments. Thus, HS is found at known cellular sites of BACE1 activity, supporting the notion that it has a potential physiological role in modulating its function.

We suggest that transmembrane or GPI-linked HSPGs (such as syndecan or glypican family members, respectively) are likely candidates for regulation of BACE1 activity. Because both BACE1 and APP are also membrane-bound proteins, HSPGs are ideally positioned to interact with, and control, the activities and functions of these molecules (Fig. 9). HSPGs may also play an important role in modulating the endocytic trafficking of APP and BACE from the cell surface to endosomal compartments. Interestingly, a recent study has indicated that access of β-secretase to APP, and thus Aβ generation, may be determined by dynamic interactions within lipid rafts (Ehehalt et al., 2003). Syndecan HSPGs have been shown to associate with lipid rafts (Fuki et al., 2000), and GPI-linked glypicans are also candidates for such an association. It is tempting to speculate that these HSPGs could participate in controlling the segregation of APP and BACE in lipid rafts, which serves to regulate the level of β-secretase cleavage (Ehehalt et al., 2003).

Potential role of HS in AD pathology

Our findings may also have significance for AD pathology because they suggest that HS could play an important role in regulating generation of the Aβ peptide. It is notable that HS from the cerebral cortex of AD patients exhibits structural differences in comparison with normal tissue (Lindahl et al., 1995). Subtle, but functionally significant, HS structural differences or the expression of region-specific HS species may occur in AD brain tissue. In addition, there is evidence for age-dependent changes in HS structure in human tissue (Feyzi et al., 1998). Such age- and disease-related changes in brain HS could alter its ability to modulate BACE1 activity, and thus contribute to the production of elevated levels of Aβ peptide. Interestingly, the activities of the size-defined and chemically modified heparins show that variant structures can differentially inhibit BACE1. Heparin oligosaccharides have been shown to pass the blood–brain barrier (Leveugle et al., 1998) and could also act by interfering with HS-stimulated Aβ aggregation, as described for small molecule anionic sulfonates and sulfates (Kisilevsky et al., 1995). Thus, compounds based on HS saccharides have potential as novel drugs for reducing both the generation of Aβ and its aggregation into amyloid plaques. It is striking that the most potent inhibition of BACE1 was observed with N-acetylated heparin, which has ~1,000-fold lower anticoagulant activity than parental heparin (Guimond, S., personal communication). Because anticoagulant activity is a major side effect of heparin in novel clinical applications (Lever and Page, 2002), our data indicate the potential for creation of "designer" heparin-based drugs as BACE1 inhibitors.

In summary, we have identified a novel role for HS as a regulator of BACE1 processing of APP, in which it acts by direct binding to the aspartyl protease and interfering with access of APP substrate to the active site. Our findings suggest that endogenous cellular HS could play an in vivo role

in controlling BACE1 cleavage of APP, and thereby regulate the levels of Aβ peptide generated. Targeting this interaction may form the basis of a novel therapeutic strategy for reducing Aβ production in the treatment of AD.

Materials and methods

Polysaccharides

Heparins, DS, and CS were purchased from Sigma-Aldrich; PMHS was a gift from Organon. BLH and PMHS were purified by treatment with chondroitinase ABC, DEAE anion exchange, and ethanol precipitation. Fluorescent-labeled heparin was prepared by reacting AlexaFluor 488 carboxylic acid succinimidyl ester (Molecular Probes) with BLH in saturated $NaHCO_3$ buffer.

In vitro peptide cleavage assay

Reaction reagents were dispensed in triplicate in 384-well black assay plates (Nunc) to give final assay concentrations of 10 nM BACE1, 5 μM FRET peptide substrate (GW 684854X; GlaxoSmithKline), 0.06% Triton X-100, and 100 mM sodium acetate, pH 4.5. Recombinant BACE1 (residues 1–460) was expressed in HEK293T cells as an Fc fusion protein, and purified by protein A affinity (provided by C. Plumpton, GlaxoSmithKline, Stevenage, UK). The FRET peptide was based on a previously described substrate with the Swedish mutation (Yan et al., 1999). The reaction was performed in the dark (25°C, 60 min) and the plate was read using a Tecan Ultra Fluorimeter (485/535 nm). A known statin BACE1 inhibitor (GW 642976A; GlaxoSmithKline) was used in control assays.

In vitro APP cleavage assay

APP was purified from HEK293T cells transfected with human wild-type APP. Supernatant from lysed cells was purified using Q-Sepharose, Heparin Hi-Trap, Mono Q, and reactive yellow dye ligand binding as described previously (Moir et al., 1992; de La Fourniere-Bessueille et al., 1997; Ohshita et al., 1998). Reactions were prepared with 50 μM APP, 100 nM BACE1, 0.06% Triton X-100, and 100 mM sodium acetate, pH 4.5 or 7.0, and incubated (25°C, 1 h) before denaturation in sample buffer and SDS-PAGE on 4–20% Tris-glycine gels (Invitrogen). Cleavage products were detected by Western blotting using rabbit anti–APP-β (Zymed Laboratories); this antibody recognizes a 22–amino acid COOH-terminal region sequence of human APP and detects C99/C89 BACE1 cleavage products (resolved as doublets in some experiments) as well as full-length APP. Only a small proportion of the APP (<5%) was cleaved under the conditions used. Supersignal West Pico chemiluminescence reagents (Pierce Chemical Co.) and BioMax film (Kodak) were used for Western blot detection. Quantitation was achieved by densitometry and validated by additionally exposing the blots on a CCD chemiluminescence camera system (Syngene) with a wide dynamic range.

Binding assays

For heparin affinity chromatography, BACE1 in PBS was loaded onto a 5-ml HPLC HiTrap Heparin column (Amersham Biosciences; flow rate 1 ml/min). Unbound material was eluted (10 ml PBS) and bound material was eluted with a 60-ml linear gradient of 0–2.0 M NaCl. Surface plasmon resonance binding experiments were performed on a Biacore 2000 in HBS buffer (flow rate 10 μl/min; Amersham Biosciences). BLH was biotinylated and immobilized on streptavidin-coated chips as described (Warner et al., 2002). For filter binding assays, ^{3}H-labeled HS was purified from the N1e neural cell as described by Brickman et al. (1998). The HS was agitated (4°C, 12 h) with 25 μg BACE1 and bound material was step eluted with NaCl in a filter binding assay as described previously (Brickman et al., 1998).

Chemically modified heparins

Chemically modified heparins were prepared as described previously (Yates et al., 1996). Size-defined heparin saccharides were purified by partial cleavage of BLH with heparitinase III followed by gel filtration as described previously (Turnbull et al., 1999).

Cellular assays for BACE1 cleavage of APP

SHswAPP and SWDC1 were seeded in 96-well plates at 10^5 cells/ml and left to attach (37°C, 12 h) before incubation with compounds (24 h). Media were tested using $Aβ_{1-42}$ (Innogenetics) or $Aβ_{1-40}$ (Biosource International) immunoassay kits; control experiments confirmed that the presence of heparin/HS does not interfere with peptide detection in this assay. To assess sAPPβ and sAPPα levels, cells were exposed to test compounds as described for the Aβ immunoassays. Media were run on 10% Tris-glycine gels. The products were detected by Western blotting using antibodies against sAPPβ (G27; GlaxoSmithKline) or sAPPα (6E10; Signet Labs). Heparin or HS did not affect cell viability in these assays, as measured by MTT assay (Guimond and Turnbull, 1999). Cells were treated with 30 mM chlorate as described previously (Guimond et al., 1993; 12-h pretreatment plus one passage, with continuous treatment throughout the experiment). Both cell lines exhibited slightly reduced growth but no evidence of apoptosis under these conditions. Heparitinase treatment was also used to remove endogenous cell surface HS. Cells were treated with 2.5 mU each of heparitinases I, II, and III (Ibex) in 15 ml of media (12-h pretreatment plus one passage, with continuous treatment throughout the experiment).

Cellular assays for BACE1–HS interactions

In colocalization studies HEK-BACE1, as described previously (Hussain et al., 1999), or SHswAPP cells were grown on poly-L-lysine–coated coverslips, washed with PBS, fixed and permeabilized with ice-cold methanol, rinsed in PBS, and blocked with 0.1% BSA in PBS (30 min). The slides were incubated with primary antibodies (either singly or in tandem) for 1 h, washed in PBS, incubated with relevant secondary antibodies (20°C, 1 h), and mounted using Fluorsave (Calbiochem) on Superfrost slides (BDH-Merck). HS was detected by an anti-HS antibody 10E4 (Seikagaku) and Cy2-conjugated or rhodamine red-X–conjugated goat anti–mouse secondary antibodies (Jackson ImmunoResearch Laboratories). Native BACE1 was detected with rabbit anti-BACE1 polyclonal (Biosource International), mouse anti-BACE1 monoclonal (Chemicon International), or chicken anti-BACE1 polyclonal (GlaxoSmithKline) and AlexaFluor 594, 488, or 568 secondary antibodies. myc-BACE1 was detected with FITC-conjugated 9E10 anti-myc secondary antibody (Santa Cruz Biotechnology, Inc.). Markers used were antibodies against CD71, for recycling endosomes, and CD63, for late endosomes (Neomarkers), and β-COP for Golgi complex (Affinity BioReagents, Inc.). For standard immunofluorescence, the cells were visualized using a microscope (DMBR; Leica), camera (ORCA; Hamamatsu), and Open Lab software (Improvision). Confocal images were taken with an Eclipse E600 (Nikon) and a Radiance 2000MP system (Bio-Rad Laboratories) using LaserSharp 2000 software (Bio-Rad Laboratories). For endogenous HS pull downs, lysates were prepared from HEK293T and HEK-BACE1 cells in 50 mM Tris, pH 7.5 (with 150 mM NaCl, 100 μM benzamide, 10 μM E64, 1 μM pepstatin A, 100 μM chymostatin, 1 μM 1,10-phenanthrolin, 2 mM EDTA, and 2% [wt/vol] octyl-β-glucoside), and 20 μl was incubated with a chicken anti-BACE1 antibody for 1 h at 4°C. In some cases, the lysates were treated with heparitinase I to specifically digest HS chains before immunoprecipitation. The complexes were incubated with protein A–Sepharose (Amersham Biosciences; 4°C, 30 min), centrifuged (1,000 *g*, 1 min), and washed. Bound proteins were eluted with sample buffer, heated (100°C, 5 min), and run on 4–20% Tris-glycine gels. The products were detected by Western blotting using anti-HS (10E4), anti-myc (9E10), and rabbit anti-BACE1 polyclonal antibodies. Protein staining (detection sensitivity ~ 5 ng) was performed using the SimplyBlue Safestain (Invitrogen) microwave procedure.

Fluorescence polarization

Changes in fluorescence polarization upon binding of a rhodamine-labeled peptide ligand (R110; GlaxoSmithKline) to BACE 1 were measured using an LJL Analyst instrument. R110 corresponded to the FRET peptide described in In vitro peptide cleavage assay but contained a noncleavable statin group at the cleavage site.

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