Immunocytochemistry—Astrocytes grown on poly-L-lysine-coated coverslips were incubated with a mixture of Aβ (250 nM) and LPL (2 μg/ml) at 37 °C for 5 h. After treatment, the cells were fixed with 4% paraformaldehyde in PBS at room temperature for 10 min, blocked, and permeabilized with 10% normal goat serum and 0.05% saponin in PBS at room temperature for 20 min. In some experiments, cells were washed twice with DMEM followed by incubation at 37 °C for 3 h in DMEM and fixed. The cells were then incubated with primary antibodies followed by Cy3- and FITC-conjugated secondary antibodies. The stained specimens were mounted with Fluor-Save reagents (Calbiochem) and examined under an LSM 510 confocal laser microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

Statistical Analysis—The collected data were analyzed by one-way analysis of variance (ANOVA) including appropriate variables followed by the Dunnett's test or unpaired Student's *t* test. Results were considered significant when $p < 0.05$.

RESULTS

LPL Binds to Aβ in Vitro—LPL was incubated with freshly prepared Aβ42 *in vitro*, and the complexes formed were immunoprecipitated with an anti-LPL antibody coupled with magnetic beads, followed by probing Western blots of protein complexes using an anti-Aβ antibody (Fig. 1*A*). Aβ42 was immunoprecipitated with an anti-LPL antibody, but not with control IgG. The levels of Aβ42 recovered in the immunoprecipitates from samples in the presence of 2–5 μg/ml LPL were significantly higher than those from samples in the presence of 0, 0.5, or 1 μg/ml of LPL (Fig. 1, *B* and *C*), suggesting that LPL directly interacts with Aβ42, and these two molecules form a complex in an LPL dose-dependent manner. Furthermore, endogenous mouse Aβ was immunoprecipitated with the anti-LPL antibody from brain homogenates prepared from C57BL/6 mice (Fig. 1*D*), indicating that endogenous mouse LPL directly interacts with endogenous mouse Aβ. We also determined the assembly state of Aβ that forms complex with LPL. Solutions containing Aβ oligomers were subjected to immunoprecipitation/immunoblot analysis, and Aβ42 monomers were immunoprecipitated by an anti-LPL antibody (supplemental Fig. 1).

LPL Promotes Cell Surface Binding and Cellular Uptake of Aβ in Astrocytes—We then determined whether LPL affects the cellular binding of Aβ to astrocytes. Soluble Aβ42 and various concentrations of LPL were added to primarily cultured astrocytes prepared from WT mice and then incubated at 4 °C. LPL (2–5 μg/ml) of significantly augmented Aβ42 binding to astrocytes by 5.8- to 9-fold of that in the case without LPL (Fig. 2, *A* and *B*). To examine the effect of LPL on the cellular uptake of Aβ, we incubated primary astrocytes with soluble Aβ42 at 37 °C for 5 h. Apparently, the level of Aβ uptake by astrocytes increased in the presence of LPL at concentrations of 2 to 5 μg/ml (Fig. 2*C*, lysate). Consistent with the increase in the level of cellular uptake of Aβ, the level of Aβ remaining in culture medium was decreased (Fig. 2*C*, medium). The Aβ levels in the cell lysate quantified are shown in Fig. 2*D*, indicating that Aβ levels were significantly increased by 5–8-fold that in astrocytes incubated without LPL. Next,

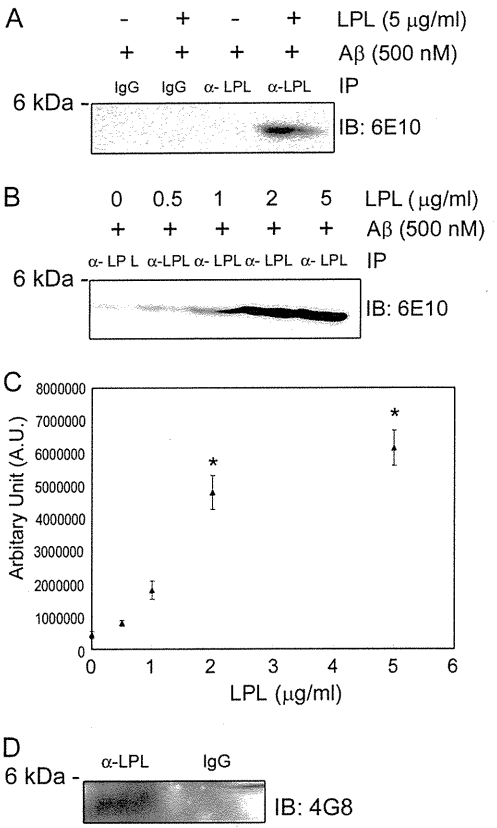

FIGURE 1. **LPL binds to Aβ *in vitro*.** *A*, LPL (5 μg/ml) and Aβ (500 nM) were incubated in DMEM at 37 °C for 3 h. Protein complexes formed were immunoprecipitated with an anti-LPL antibody (α-LPL), and the immunoprecipitates (*IP*) were analyzed by Western blotting using 6E10, an anti-Aβ antibody. These data are representative of three independent experiments. *B*, LPL at various concentrations of 0, 0.5, 1, 2, and 5 μg/ml and Aβ at 500 nM were incubated in DMEM at 37 °C for 3 h. Protein complexes formed were immunoprecipitated with an α-LPL, and the immunoprecipitates were subjected to Western blotting using 6E10. *C*, quantification of Aβ immunoprecipitated with α-LPL. The data presented are the means ± S.D. of three independent experiments. *, $p < 0.001$ *versus* samples without LPL treatment. *D*, the mouse cerebrum was homogenized by sonication in 4 volumes of PBS containing a protease inhibitor mixture and centrifuged at 1000 × *g* for 10 min at 4 °C. The supernatants were harvested. LPL-Aβ complexes in the supernatant were immunoprecipitated with an α-LPL, and the Aβ in the immunoprecipitates was detected by Western blotting using 4G8, an anti-Aβ antibody. *IB*, immunoblot.

we determined the time-dependent effect of LPL-mediated Aβ uptake into astrocytes. Astrocyte cultures were incubated with Aβ (500 nM) and LPL (2 μg/ml) at 37 °C for various hours, and the Aβ level in the cell lysate was determined. The level of Aβ in the cell lysate increased in a time-dependent manner (Fig. 2*E*). The Aβ levels in the astrocytes incubated for 3 and 5 h were significantly higher by 9–14-fold of that in astrocytes incubated without LPL (Fig. 2*F*). These concentrations of LPL are comparable with the concentrations with which LPL could act as "bridging molecules" (2, 20). There were no significant differences among the values for cultures without LPL (one-way ANOVA, $p = 0.1386$). No change in cellular morphology or cell number in astrocyte cultures was observed during the incubation (data not shown). To examine the involvement of LPL expressed by astrocytes, we carried out experiments using the gene silencing technique for LPL. The transient knockdown of LPL expression was achieved by the transfection of siRNA specific for LPL. After transfection,

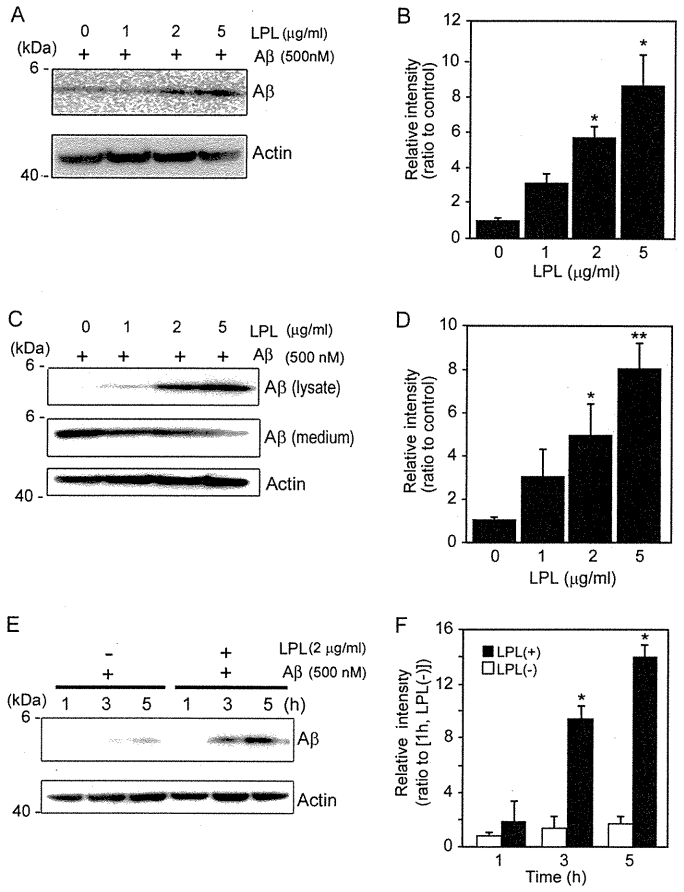

FIGURE 2. **LPL augments cell-surface association and cellular uptake of Aβ in astrocytes.** *A*, mouse primary astrocytes were incubated with LPL (0–5 μg/ml) and Aβ (500 nM) at 4 °C for 3 h. The astrocytes were washed in cold PBS three times, and the cells were harvested using a scraper. The level of Aβ on the cell surface was determined by Western blotting in a detergent extract of whole cells. *B*, quantification of cell-surface-associated Aβ. The data are the means ± S.D. of three independent experiments. *, $p < 0.001$ *versus* LPL at 0 μg/ml. *C* and *D*, astrocytes were incubated with Aβ (500 nM) and LPL (0, 1, 2, and 5 μg/ml) at 37 °C for 3 h. The cultured cells were then washed thoroughly in PBS for three times, and the cells were collected. The level of Aβ in the whole cell lysate (*lysate*), and the conditioned medium of cultured cells (*medium*) were determined by Western blotting using 6E10 antibody. The level of actin demonstrated by Western blotting using an anti-β-actin antibody was used as the loading control. These data are representative of at least three independent experiments. *D*, quantification of cellular Aβ is shown. The data presented are the means ± S.D. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$ *versus* LPL at 0 μg/ml. *E* and *F*, astrocytes were incubated with Aβ (500 nM) and LPL (2 μg/ml) at 37 °C for 0, 3, and 5 h. The cultured cells were then washed thoroughly in PBS three times, and the cells were collected. The amount of Aβ in the whole cell lysate was determined by Western blotting using 6E10 antibody. The level of actin demonstrated by Western blotting using the anti-β-actin antibody was used as the loading control. These data are representative of at least three independent experiments. *F*, quantification of cellular Aβ is shown. The data are the means ± S.D. of three independent experiments. *, $p < 0.001$ *versus* LPL (+) at 1 h.

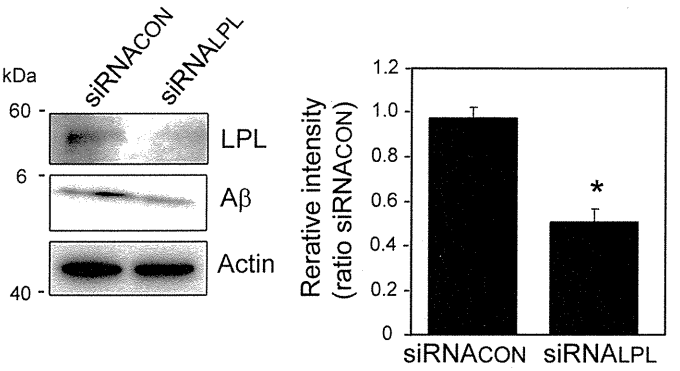

FIGURE 3. **Effect of LPL knockdown on cell-surface association of Aβ in cultured astrocytes.** Astrocytes were transfected with 10 nM siRNA specific for LPL (*siRNALPL*) and control siRNA (*siRNACON*). Forty-eight hours after transfection, cells were treated with Aβ42 (1 μM) at 4 °C for 3 h. The cells were washed in cold PBS three times, and the cells were harvested using a scraper. The level of Aβ42 on the cell surface was determined by Western blotting in a detergent extract of whole cells. The graph shows the levels of cell-surface-associated Aβ. The data are the means ± S.D. of three independent experiments. *, $p < 0.001$ *versus* control siRNA by unpaired Student's *t* test.

cells were treated with Aβ42 (1 μM) and then incubated at 4 °C for 3 h. As shown in Fig. 3, the cellular binding of Aβ42 to astrocytes was significantly decreased by LPL protein knockdown.

Degradation of Internalized Aβ in a Lysosomal Pathway in Astrocytes—Next, we examined the degradation of internalized Aβ. Mouse primary astrocytes were incubated with soluble Aβ42 and LPL at 37 °C for 5 h, washed in DMEM three times, and cultured at 37 °C for additional time (0, 3, 5, 12, and 24 h). Cells were then harvested, and the Aβ level in the cell lysate was analyzed by Western blotting. The strong signal representing internalized Aβ during the initial incubation for 5 h was detected in the cell lysate at the point of 0 min after washing (Fig. 4*A*). Three to five hours after washing, the level of Aβ remaining in the cell lysate partially disappeared (Fig. 4*A*). Twelve and twenty-four hours after washing, the internalized Aβ completely disappeared, indicating that the internalized Aβ was degraded in astrocytes in a time-dependent manner (Fig. 4*A*). To gain insight into the degradation pathway of the internalized Aβ, we investigated the localization of Aβ by immunocytochemical analysis. Mouse primary astrocytes were plated on poly-L-lysine-coated coverglasses and incubated with Aβ42 (500 nM) and LPL (2 μg/ml) at 37 °C for 5 h. In some experiments, cells were washed in DMEM three times and further incubated in serum-free DMEM for 3 h. Cells were then permeabilized and stained with an anti-Aβ antibody, 6E10, and an anti-LAMP2 antibody, whose staining signal is considered as a marker of late endosomes/lysosomes (21). We found that some portions of anti-Aβ antibody-positive signals were co-localized with staining signals reactive to the anti-LAMP2 antibody, showing that the internalized Aβ was trafficked into late endosomal/lysosomal compartments (Fig. 4*B*). To confirm the involvement of a lysosomal pathway in the degradation of LPL-mediated internalized Aβ, we determined the effect of chloroquine on the localization of Aβ internalized in an LPL-mediated manner. Chloroquine is a weak base and is taken up by cells, which results in the neutralization of acidic organelles such as lysosomes and impairment of their functions (22, 23). Chloroquine treatment at concentrations of 25 and 50 μg/ml prevented the degradation of internalized Aβ 12 h after washing out (Fig. 4*C*). We also tested inhibitors of neprilysin, an insulin-degrading enzyme, and cathepsin B, all of which are known to degrade Aβ. These inhibitors failed to suppress the degradation of internalized Aβ in astrocytes (data not shown). Thus, Aβ internalized in an LPL-mediated manner was degraded in a lysosomal pathway in astrocytes.

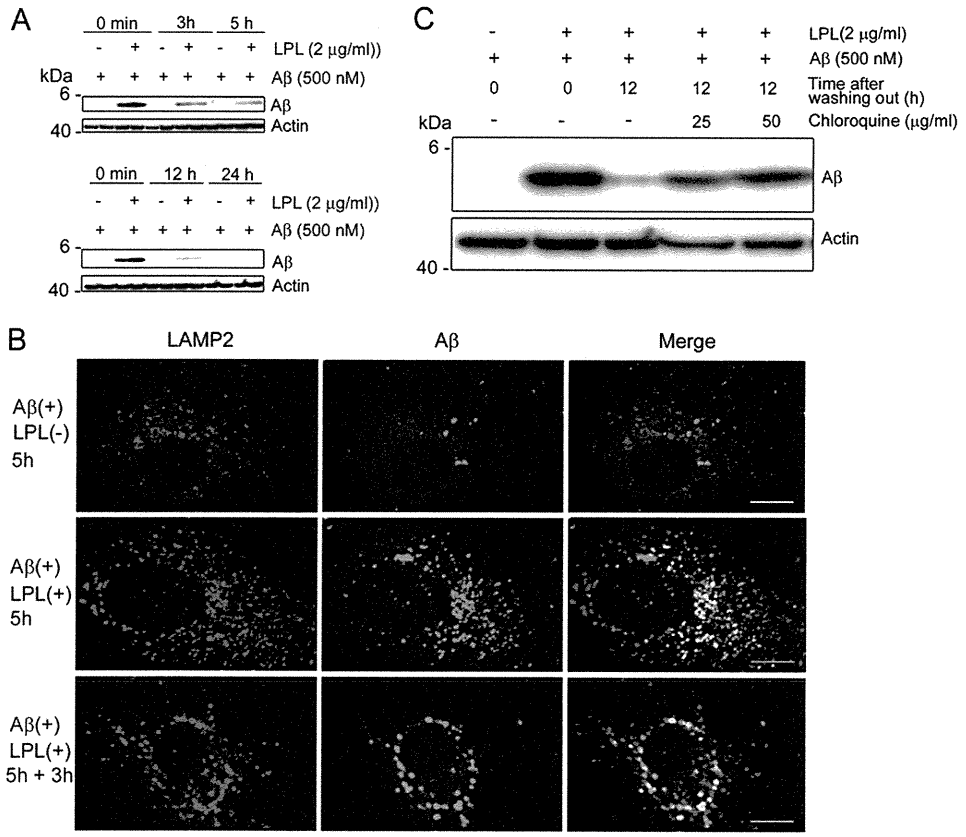

FIGURE 4. **Aβ is trafficked to late endosomal/lysosomal compartments and degraded after the LPL-mediated uptake.** *A*, mouse primary astrocytes were incubated with LPL (2 μg/ml) and Aβ (500 nM) at 37 °C for 5 h. Cells were washed in DMEM three times and then incubated in DMEM at 37 °C for 0, 3, 5, 12, and 24 h. The amount of Aβ remaining in the cells was determined by Western blotting using the anti-Aβ antibody, 6E10, in a detergent extract of whole cells. *B*, astrocytes were plated on poly-L-lysine-coated coverglasses and incubated with LPL (2 μg/ml) and Aβ (250 nM) at 37 °C for 5 h. Then, cells were permeabilized and double stained with an anti-LAMP2 antibody and 2C8. Bound antibodies were visualized with Cy3-conjugated (*red*) and FITC-conjugated (*green*) secondary antibodies for the anti-LAMP2 antibody and 6E10, respectively. Astrocytes incubated without Aβ did not show any anti-Aβ antibody-positive signals (not shown). *Scale bar*, 10 μm. *C*, astrocytes were incubated with LPL (2 μg/ml) and Aβ (500 nM) at 37 °C for 5 h. Cells were then washed in DMEM and cultured with or without chloroquine in DMEM at 37 °C for an additional 12 h. The level of Aβ in the detergent extract of whole cells was determined by Western blotting with 6E10. These are representative data of at least three independent experiments.

LPL Promotes Cellular Uptake of Aβ in a Heparan Sulfate- and Chondroitin Sulfate-dependent Manner—LPL has a high affinity with heparan sulfate (HS) and chondroitin sulfate (CS) (5, 24, 25). Therefore, we next investigated whether HS and CS are involved in the LPL-mediated cellular binding and cellular uptake of Aβ in astrocytes. Mouse primary astrocytes were pretreated with a mixture of heparinase II and heparinase III and/or chondroitinase ABC for 24 h at 37 °C, followed by incubation with Aβ42 and LPL at 4 °C for 3 h. There were no significant differences among the values in the absence of LPL (one-way ANOVA; $p = 0.0929$ for cell-surface-associated Aβ, $p = 0.4350$ for cellular Aβ). Pretreatment with heparinases or chondroitinase ABC partially decreased the level of LPL-mediated cellular binding of Aβ in astrocytes to 40 or 50% of that observed in the nontreated control, respectively (Fig. 5*A*). Interestingly, pretreatment with both heparinases and chondroitinase ABC decreased the level of LPL-mediated binding of Aβ to astrocytes to 20% of that observed in nontreated control (Fig. 5*A*). Next, we determined the effect of HS and/or CS on the LPL-mediated cellular uptake of Aβ. In conjunction with the effect of LPL on Aβ binding, heparinases and chondroitinase ABC decreased the level of LPL-mediated cellular uptake of Aβ in astrocytes to 30 and 50% of that observed in the nontreated control incubated with LPL, respectively (Fig. 5*B*). Pretreatment with both heparinases and chondroitinase ABC did not show an additive effect on the attenuation of LPL-promoted Aβ uptake (Fig. 5*B*). These findings indicate that HS and CS expressed in astrocytes are involved in the LPL-mediated association of Aβ with astrocytes and Aβ cellular uptake.

To further confirm the involvement of HS and CS in LPL-mediated Aβ uptake, we incubated astrocytes with various glycosaminoglycans. Heparin, which is a structural analog of HS, substantially suppressed the effect of LPL on Aβ uptake at a concentration of 3 μg/ml (Fig. 5*C*). The suppressive effect of heparin on LPL-mediated Aβ uptake was also observed in the presence of de-*N*-sulfated heparin, whereas either de-2-*O*-sulfated heparin or de-6-*O*-sulfated heparin had no effect on LPL-mediated Aβ uptake (Fig. 5*C*). None of these heparins interfered with the interaction between LPL and Aβ (Fig. 5*D*). In addition, 4-*O*-, 6-*O*-disulfated chondroitin sulfate (3 μg/ml) completely suppressed the promotive effect of LPL on Aβ uptake (Fig. 5*E*). 4-*O*-Sulfated chondroitin sulfate and 6-*O*-sulfated chondroitin sulfate moderately attenuated the function of LPL, whereas chondroitin (a nonsulfated form of chondroitin sulfate) and 2-*O*-, 6-*O*-disulfated chondroitin

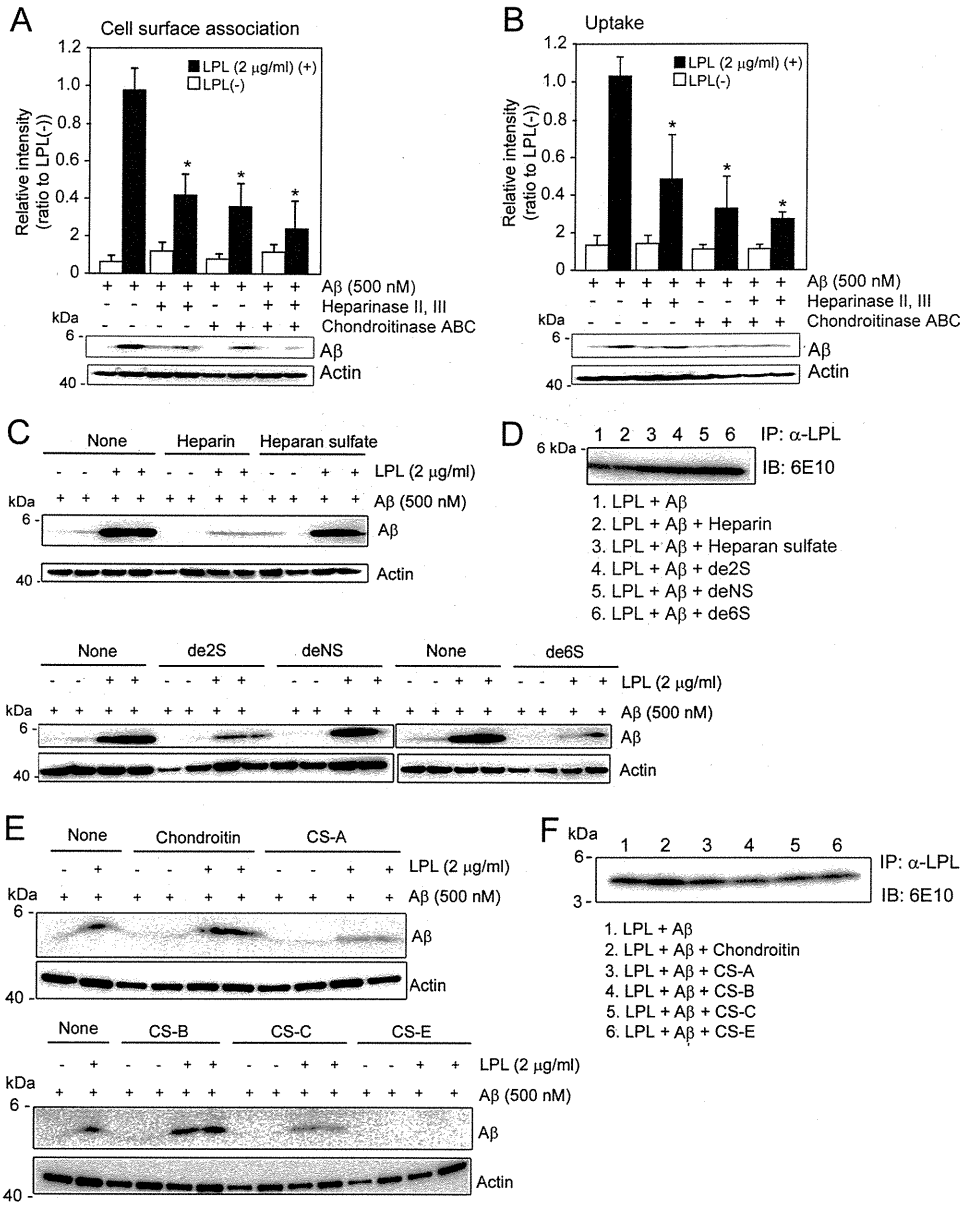

FIGURE 5. **LPL-mediated cellular binding and uptake of Aβ depends on heparan sulfate and chondroitin sulfate in astrocytes.** *A* and *B*, astrocytes from wild-type mice were pretreated with a mixture of heparinase II (0.03 μg/ml) and heparinase III (0.03 μg/ml), and/or chondroitinase ABC (0.03 μg/ml) at 37 °C for 24 h. After washing in DMEM three times, cells were incubated with LPL (2 μg/ml) and Aβ (500 nM) at 4 °C for 3 h (for cell surface association) (*A*) or 37 °C for 3 h (for uptake) (*B*). The level of Aβ in the detergent extract of whole cells was determined by Western blotting using 6E10. The quantitative assessment of cell-surface-associated Aβ (*A*) and cellular Aβ (*B*) in the present (closed bars) or absence (open bars) of LPL are shown. The data presented are the means ± S.D. of three independent experiments. * $p < 0.001$ *versus* levels of LPL (-). (*C*) Mouse primary astrocytes were incubated with Aβ (500 nM) or LPL (2 μg/ml) and Aβ (500 nM) in the presence or absence of heparin or chemically modified heparins at a concentration of 3 μg/ml at 37 °C for 5 h. The level of Aβ in the detergent extract of whole cells was determined using 6E10. (*D*) LPL (2 μg/ml) and Aβ (500 nM) were incubated in DMEM at 37 °C for 3 h in the presence or absence of heparin, heparan sulfate, or chemically modified heparins at a concentration of 3 μg/ml. Protein complexes in DMEM were immunoprecipitated (*IP*) with an anti-LPL antibody (α-LPL) and the Aβ recovered in the immunoprecipitates was analyzed by Western blotting using 6E10. These data are representative of at least three independent experiments. *de2S*, 2-*O*-desulfated heparin; *de6S*, 6-*O*-desulfated heparin; *deNS*, *N*-desulfated heparin. *E*, astrocytes were incubated with LPL (2 μg/ml) and Aβ (500 nM) in the presence or absence of chondroitin sulfates (chondroitin, chondroitin 4-sulfate (*CS-A*), 2-*O*-, 6-*O*-disulfated chondroitin sulfate (*CS-B*), 6-*O*-sulfated chondroitin sulfate (*CS-C*), and chondroitin 4,6-disulfate (*CS-E*)) at a concentration of 3 μg/ml at 37 °C for 5 h. The level of Aβ in a detergent extract of whole cells was determined by Western blotting using 6E10. *F*, LPL (2 μg/ml) and Aβ (500 nM) were incubated in DMEM at 37 °C for 3 h in the presence or absence of chondroitin sulfates at a concentration of 3 μg/ml. Protein complexes were immunoprecipitated with the anti-LPL antibody (α-LPL), and the Aβ recovered in the immunoprecipitates was analyzed by Western blotting using 6E10. The data are representative of at least three independent experiments. *IB*, immunoblot.

sulfate (also known as dermatan sulfate) did not (Fig. 5*E*). None of these CS interfered with the interaction between LPL and Aβ *in vitro* (Fig. 5*F*).

ApoE Is Dispensable for LPL-mediated Cellular Uptake of Aβ in Astrocytes—Because ApoE is reported to be involved in the metabolism of Aβ, including its aggregation and clearance (26), we analyzed the effects of ApoE on the LPL-mediated cellular uptake of Aβ in astrocytes. We collected culture media of primary astrocytes prepared from ApoE-KO mice and C57BL/6 (WT) mice. The astrocyte cultures prepared from wild-type mouse cortices were incubated in conditioned media in the presence of Aβ42 and LPL. As shown in Fig. 6A, Aβ

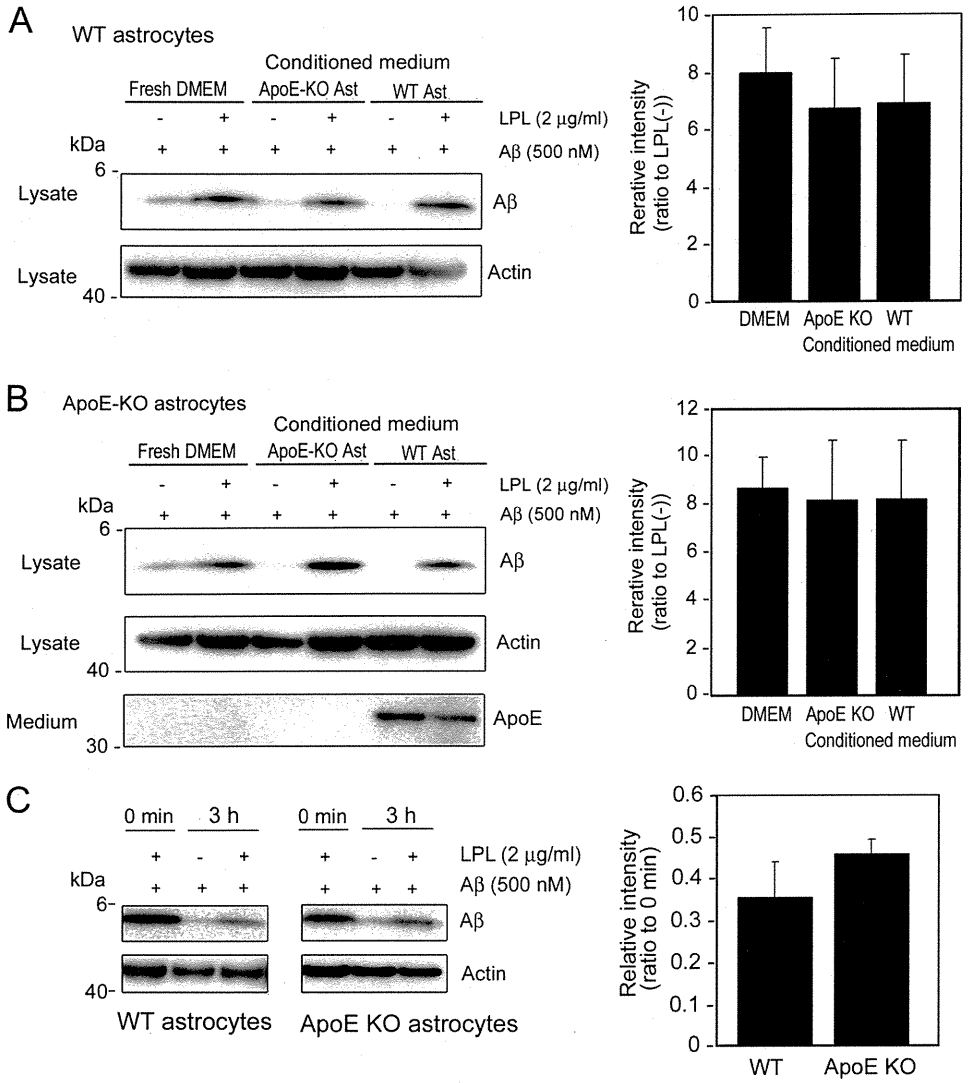

FIGURE 6. **ApoE is dispensable for the LPL-mediated cellular uptake of Aβ in astrocytes.** The astrocyte cultures prepared from WT or ApoE knock-out (KO) mice were incubated in fresh serum-free DMEM for 3 days at 37 °C. The conditioned media of these cultures were then collected. The astrocytes prepared from WT (*A*) or ApoE-KO (*B*) mouse brains were incubated in the conditioned medium of ApoE-KO astrocyte cultures or conditioned medium of WT astrocyte cultures, and LPL (2 μg/ml) and Aβ (500 nM) were added into each culture; the cultures were then maintained for another 5 h at 37 °C. After the incubation, the cultures were harvested, and the amount of cellular Aβ in a detergent extract of whole cells (*lysate*) was determined by Western blotting using 6E10. The amount of ApoE in the conditioned medium of cultured cells (*medium*) was determined by Western blotting using an anti-ApoE antibody, AB947. These data are representative of at least three independent experiments. The graphs show the cellular Aβ levels. The data are the means ± S.D. of three independent experiments. *CM*, conditioned medium; *Ast*, astrocytes. *C*, mouse primary astrocytes from WT and ApoE-KO mice were incubated with soluble Aβ42 in the presence or absence of LPL at 37 °C for 5 h, washed in DMEM three times, and further incubated at 37 °C for 3 h. Cells were then harvested, and the Aβ levels in the lysate was analyzed by Western blotting. The graph shows the cellular Aβ levels. The data are the means ± S.D. of three independent experiments.

uptake was promoted by LPL in astrocytes prepared from WT mice incubated in a fresh medium, the conditioned medium from ApoE-KO astrocytes, and the conditioned medium from WT astrocytes. There were no significant differences between these three groups (one-way ANOVA; $p = 0.6419$). This is also the case for ApoE-KO astrocytes (one-way ANOVA; $p = 0.9467$) (Fig. 6*B*). These findings indicate that ApoE is dispensable for the LPL-promoted cellular uptake of Aβ in astrocytes. We also examined the effects of ApoE on the degradation of internalized Aβ. Primary astrocytes from WT and ApoE-KO mice were incubated with soluble Aβ42 and LPL at 37 °C for 5 h, washed in DMEM three times, and further incubated at 37 °C for 3 h. Cells were then harvested, and the Aβ level in the cell lysate was analyzed by Western blotting. As shown in Fig. 6*C*, there were no significant differences between the levels of Aβ remaining in the lysate of WT astrocytes and ApoE-KO astrocytes ($p = 0.1031$).

DISCUSSION

Previous studies have shown that the mRNA expression of the LPL gene and the enzymatically active LPL are found in the brain in several mammalian species (6, 7, 27). However, considering that the main fraction of lipoproteins in the brain is HDL, which contains negligible or no triacylglycerols, and that the brain lacks an essential cofactor, apoCII, it is conceivable that LPL has a different function in the brain from that in the systemic circulation serving as an enzyme with the cofactor apoCII to catalyze the hydrolysis of triacylglycerols (28). In

the present study, we found a novel function of LPL serving as an Aβ binding molecule; that is, exogenous LPL binds to Aβ and promotes cellular binding and uptake of Aβ in astrocytes. The internalized Aβ was degraded within 12 h, mainly in a lysosomal pathway. Furthermore, we have demonstrated that HS and CS glycosaminoglycans are involved in the promotion of the LPL-mediated cellular uptake of Aβ in astrocytes.

Astrocytes are a major glial cell type in the CNS and play a crucial role in neuronal development, maintenance of synapse functions, and CNS repair after injury. Additionally, astrocytes have phagocytic and proteolytic activities (29, 30) and ingest Aβ (15, 31, 32). Our results indicate that LPL strongly enhances cellular uptake of Aβ, leading to increased degradation of Aβ in astrocytes. Previous studies have shown that SNPs in the coding region of the LPL gene are associated with AD development (33) and the severity of AD pathophysiological features (12), with the molecular mechanisms underlying this association remaining unknown. It may be possible that altered function of LPL shown in this study would result in impaired Aβ clearance and subsequent accumulation of Aβ, accelerating AD development. Because the accumulation of Aβ in the extracellular space is considered to trigger Aβ aggregation and deposition, the function of LPL to enhance Aβ binding, uptake, and degradation in astrocytes may decrease Aβ levels in the brain. However, because LPL is known to regulate the uptake and transport of vitamin E to the brain, of which deficiency results in increased Aβ accumulation and presynaptic defects accompanied by impaired learning and memory function *in vivo* (34, 35), there may be other possibilities as well, that the altered LPL function regulating vitamin E transport may enhance Aβ accumulation and impair synaptic function.

It has been suggested that lysosomal dysfunction plays a major role in Aβ accumulation, thereby causing neuronal cell death (36, 37) and that chloroquine, which disrupts lysosomal pH balance, enhances Aβ accumulation in a microglial cell line (38). Our results show that almost all of the internalized Aβ was localized in lysosomes and degraded in a time-dependent manner, and this degradation was markedly inhibited by the treatment with chloroquine, suggesting that Aβ was degraded mainly in a lysosomal pathway. These findings suggest that lysosomal pathways play a critical role in the degradation of Aβ that is internalized via a novel pathway as LPL-Aβ complexes by astrocytes.

It has been shown that LPL associates with lipoproteins and the formed LPL-bound lipoprotein complexes bind to cell-surface HS proteoglycans and CS proteoglycans (1, 5, 39), promoting the cellular uptake of lipoproteins by acting as a bridging molecule (2, 40). HS proteoglycans and CS proteoglycans are present in astrocytes (41–43). We found that pretreatment of astrocytes with a mixture of heparinases or chondroitinase ABC partially attenuated the LPL-mediated Aβ uptake, and cotreatment with heparinases and chondroitinase ABC completely suppressed the LPL-mediated cellular uptake of Aβ (Fig. 4), indicating that the LPL-mediated cellular uptake of Aβ is mediated via HS proteoglycans and CS proteoglycans. Interestingly, heparin, a highly sulfated form of HS, and 4-*O*-, 6-*O*-disulfated chondroitin sulfate, a highly sulfated CS, selectively suppressed the promotion of Aβ uptake in astrocytes. These findings suggest that LPL could act as a bridging molecule between not only cell-surface GAGs and lipoproteins but also cell-surface GAGs and Aβ and facilitate the cellular uptake of Aβ in astrocytes and that certain domains modified by multiple sulfate groups are necessary for LPL to function in astrocytes.

ApoE is one of the major apolipoproteins in the brain and plays a key role in lipid transport in the brain. ApoE affects the aggregation of Aβ *in vitro* (26). PDAPP and Tg2576 transgenic mice exhibit extensive cerebral Aβ deposition. When these transgenic mice lack the murine *apoE* gene, a significant decrease in amyloid plaque formation was observed (44, 45). Furthermore, two *in vitro* studies have demonstrated that ApoE can facilitate the cellular degradation of Aβ (16, 31). These lines of evidence suggest that ApoE affects Aβ metabolism. Thus, we examined whether ApoE could be involved in the LPL-mediated cellular uptake of Aβ. LPL promoted the cellular uptake of Aβ in wild-type and ApoE-deficient astrocytes in culture. The presence or absence of ApoE in the conditioned medium of astrocytes did not alter the levels of Aβ internalized in an LPL-mediated manner. These results suggest that ApoE is not required for the LPL-mediated cellular uptake of Aβ in astrocytes.

In this study, we demonstrated a novel LPL function; that is, LPL binds to Aβ and enhances the cellular uptake of Aβ in a sulfated glycosaminoglycan-dependent manner, and the internalized Aβ is degraded in a lysosomal pathway. Although further studies will be needed to confirm the role of LPL in the clearance of Aβ *in vivo*, our findings provide a new insight into the molecular pathogenesis of AD and a potential strategy for AD therapy.

REFERENCES

1. Williams, K. J., Fless, G. M., Petrie, K. A., Snyder, M. L., Brocia, R. W., and Swenson, T. L. (1992) *J. Biol. Chem.* **267,** 13284–13292
2. Mulder, M., Lombardi, P., Jansen, H., van Berkel, T. J., Frants, R. R., and Havekes, L. M. (1993) *J. Biol. Chem.* **268,** 9369–9375
3. Kreuger, J., Spillmann, D., Li, J. P., and Lindahl, U. (2006) *J. Cell Biol.* **174,** 323–327
4. Edwards, I. J., Goldberg, I. J., Parks, J. S., Xu, H., and Wagner, W. D. (1993) *J. Lipid Res.* **34,** 1155–1163
5. Edwards, I. J., Xu, H., Obunike, J. C., Goldberg, I. J., and Wagner, W. D. (1995) *Arterioscler. Thromb. Vasc. Biol.* **15,** 400–409
6. Goldberg, I. J., Soprano, D. R., Wyatt, M. L., Vanni, T. M., Kirchgessner, T. G., and Schotz, M. C. (1989) *J. Lipid Res.* **30,** 1569–1577
7. Yacoub, L. K., Vanni, T. M., and Goldberg, I. J. (1990) *J. Lipid Res.* **31,** 1845–1852
8. Eckel, R. H., and Robbins, R. J. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81,** 7604–7607
9. Havel, R. J., Fielding, C. J., Olivecrona, T., Shore, V. G., Fielding, P. E., and Egelrud, T. (1973) *Biochemistry* **12,** 1828–1833
10. Zannis, V. I., Cole, F. S., Jackson, C. L., Kurnit, D. M., and Karathanasis, S. K. (1985) *Biochemistry* **24,** 4450–4455
11. Rebeck, G. W., Harr, S. D., Strickland, D. K., and Hyman, B. T. (1995) *Ann. Neurol.* **37,** 211–217
12. Blain, J. F., Aumont, N., Théroux, L., Dea, D., and Poirier, J. (2006) *Eur. J. Neurosci.* **24,** 1245–1251
13. Iwatsubo, T., Odaka, A., Suzuki, N., Mizusawa, H., Nukina, N., and Ihara, Y. (1994) *Neuron* **13,** 45–53
14. Tanzi, R. E., Moir, R. D., and Wagner, S. L. (2004) *Neuron* **43,** 605–608
15. Wyss-Coray, T., Loike, J. D., Brionne, T. C., Lu, E., Anankov, R., Yan, F.,

Silverstein, S. C., and Husemann, J. (2003) *Nat. Med.* **9,** 453–457
16. Jiang, Q., Lee, C. Y., Mandrekar, S., Wilkinson, B., Cramer, P., Zelcer, N., Mann, K., Lamb, B., Willson, T. M., Collins, J. L., Richardson, J. C., Smith, J. D., Comery, T. A., Riddell, D., Holtzman, D. M., Tontonoz, P., and Landreth, G. E. (2008) *Neuron* **58,** 681–693
17. Majumdar, A., Cruz, D., Asamoah, N., Buxbaum, A., Sohar, I., Lobel, P., and Maxfield, F. R. (2007) *Mol. Biol. Cell* **18,** 1490–1496
18. Mandrekar, S., Jiang, Q., Lee, C. Y., Koenigsknecht-Talboo, J., Holtzman, D. M., and Landreth, G. E. (2009) *J. Neurosci.* **29,** 4252–4262
19. Michikawa, M., Gong, J. S., Fan, Q. W., Sawamura, N., and Yanagisawa, K. (2001) *J Neurosci.* **21,** 7226–7235
20. Fernández-Borja, M., Bellido, D., Vilella, E., Olivecrona, G., and Vilaró, S. (1996) *J. Lipid Res.* **37,** 464–481
21. Fukuda, M. (1991) *J. Biol. Chem.* **266,** 21327–21330
22. de Duve, C., de Barsy, T., Poole, B., Trouet, A., Tulkens, P., and Van Hoof, F. (1974) *Biochem. Pharmacol.* **23,** 2495–2531
23. Poole, B., and Ohkuma, S. (1981) *J. Cell Biol.* **90,** 665–669
24. Bengtsson, G., Olivecrona, T., Höök, M., Riesenfeld, J., and Lindahl, U. (1980) *Biochem. J.* **189,** 625–633
25. Pillarisetti, S., Paka, L., Sasaki, A., Vanni-Reyes, T., Yin, B., Parthasarathy, N., Wagner, W. D., and Goldberg, I. J. (1997) *J. Biol. Chem.* **272,** 15753–15759
26. Kim, J., Basak, J. M., and Holtzman, D. M. (2009) *Neuron* **63,** 287–303
27. Brecher, P., and Kuan, H. T. (1979) *J. Lipid Res.* **20,** 464–471
28. Koch, S., Donarski, N., Goetze, K., Kreckel, M., Stuerenburg, H. J., Buhmann, C., and Beisiegel, U. (2001) *J. Lipid Res.* **42,** 1143–1151
29. al-Ali, S. Y., and al-Hussain, S. M. (1996) *J. Anat.* **188,** 257–262
30. Hatten, M. E., Liem, R. K., Shelanski, M. L., and Mason, C. A. (1991) *Glia* **4,** 233–243
31. Koistinaho, M., Lin, S., Wu, X., Esterman, M., Koger, D., Hanson, J., Higgs, R., Liu, F., Malkani, S., Bales, K. R., and Paul, S. M. (2004) *Nat. Med.* **10,** 719–726
32. Matsunaga, W., Shirokawa, T., and Isobe, K. (2003) *Neurosci. Lett.* **342,** 129–131
33. Baum, L., Chen, L., Masliah, E., Chan, Y. S., Ng, H. K., and Pang, C. P. (1999) *Am. J. Med. Genet.* **88,** 136–139
34. Xian, X., Liu, T., Yu, J., Wang, Y., Miao, Y., Zhang, J., Yu, Y., Ross, C., Karasinska, J. M., Hayden, M. R., Liu, G., and Chui, D. (2009) *J. Neurosci.* **29,** 4681–4685
35. Nishida, Y., Ito, S., Ohtsuki, S., Yamamoto, N., Takahashi, T., Iwata, N., Jishage, K., Yamada, H., Sasaguri, H., Yokota, S., Piao, W., Tomimitsu, H., Saido, T. C., Yanagisawa, K., Terasaki, T., Mizusawa, H., and Yokota, T. (2009) *J. Biol. Chem.* **284,** 33400–33408
36. Bahr, B. A., and Bendiske, J. (2002) *J Neurochem.* **83,** 481–489
37. Nixon, R. A., Cataldo, A. M., and Mathews, P. M. (2000) *Neurochem. Res.* **25,** 1161–1172
38. Chu, T., Tran, T., Yang, F., Beech, W., Cole, G. M., and Frautschy, S. A. (1998) *FEBS Lett.* **436,** 439–444
39. Eisenberg, S., Sehayek, E., Olivecrona, T., and Vlodavsky, I. (1992) *J. Clin. Invest.* **90,** 2013–2021
40. Auerbach, B. J., Bisgaier, C. L., Wölle, J., and Saxena, U. (1996) *J. Biol. Chem.* **271,** 1329–1335
41. Hsueh, Y. P., and Sheng, M. (1999) *J. Neurosci.* **19,** 7415–7425
42. Laabs, T. L., Wang, H., Katagiri, Y., McCann, T., Fawcett, J. W., and Geller, H. M. (2007) *J. Neurosci.* **27,** 14494–14501
43. Tsuchida, K., Shioi, J., Yamada, S., Boghosian, G., Wu, A., Cai, H., Sugahara, K., and Robakis, N. K. (2001) *J. Biol. Chem.* **276,** 37155–37160
44. Bales, K. R., Verina, T., Dodel, R. C., Du, Y., Altstiel, L., Bender, M., Hyslop, P., Johnstone, E. M., Little, S. P., Cummins, D. J., Piccardo, P., Ghetti, B., and Paul, S. M. (1997) *Nat. Genet.* **17,** 263–264
45. Holtzman, D. M., Bales, K. R., Wu, S., Bhat, P., Parsadanian, M., Fagan, A. M., Chang, L. K., Sun, Y., and Paul, S. M. (1999) *J. Clin. Invest.* **103,** R15–R21

Supplemental Materials

LIPOPROTEIN LIPASE IS A NOVEL Aβ-BINDING PROTEIN THAT PROMOTES GLYCOSAMINOGLYCAN-DEPENDENT CELLULAR UPTAKE OF Aβ IN ASTROCYTES

Kazuchika Nishitsuji, Takashi Hosono, Kenji Uchimura, and Makoto Michikawa

Methods

Aβ oligomers were prepared as previously described (Lambert et al., Journal of Neurochemistry, 2001, 79, 595-605). In brief, Aβ42 was dissolved in hexafluoro-2-propanol (HFIP) and aliquots were placed into to microcentrifuge tubes. HFIP was removed by evaporation with traces removed under vacuum and the tubes were stored at -80 ˚C. An aliquot of Aβ42 was mixed with DMSO to a final concentration of 5 mM, which was then added to ice-cold F12 medium without phenol red to 100 μM. This solution was incubated at 4 ˚C for 24 h and then centrifuged at 14,000 x g for 10 min. The supernatant was used as the Aβ oligomer preparation.

Legend

Supplemental Fig.1. Determination of assembly state of Aβ which binds to LPL. (A, Left blot). Freshly dissolved Aβ (50 ng) was separated by SDS-PAGE and transferred to a PVDF membrane. Aβ was probed with 6E10 followed by the horseradish peroxidase-labeled anti-mouse antibody and the chemiluminescent substrate ECL Plus. **(A, Right blot).** LPL (5 μg/ml) and Aβ (500 nM) were incubated in DMEM at 37 ˚C for 3 h. Protein complexes formed were immunoprecipitated with an anti-LPL antibody (α-LPL) and the immunoprecipitates were analyzed by Western blotting using 6E10, an anti-Aβ antibody. **(B, Left blot).** Aβ oligomer preparation (1 μg) was separated by SDS-PAGE and transferred to a PVDF membrane. Aβ was probed with 6E10 followed by the horseradish peroxidase-labeled anti-mouse antibody and the chemiluminescent substrate ECL Plus. **(B, Right blot).** LPL (5 μg/ml) and Aβ oligomer (500 nM) preparation were incubated in DMEM at 37 ˚C for 3 h. Protein complexes formed were immunoprecipitated with an anti-LPL antibody (α-LPL) and the immunoprecipitates were analyzed by Western blotting using 6E10, an anti-Aβ antibody.

Supplemental Fig. 1
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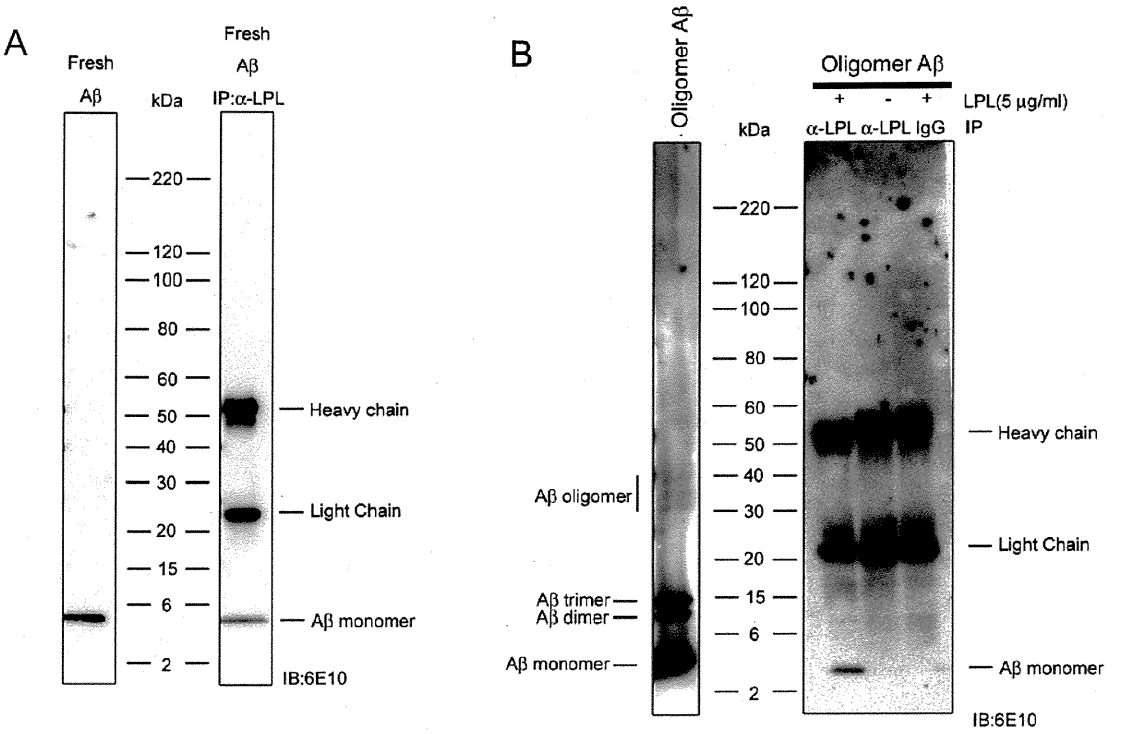

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特集：糖鎖機能の多層性と神経 sugar code

細胞外スルファターゼ Sulf によるヘパラン硫酸糖鎖機能の調節

内村健治

糖鎖は基本骨格が合成されコアタンパク質に付加された後にも酵素的修飾をうける．その修飾には，硫酸化，エピマー化，脱アセチル化，グリコリル化，脱水環状化，リン酸化等が含まれる．糖鎖の硫酸化はスルフォトランスフェラーゼというゴルジ体局在酵素により担われ，ヘパリン／ヘパラン硫酸やコンドロイチン硫酸といったプロテオグリカンの糖側鎖に多くみられる．硫酸化糖鎖は細胞表面および細胞外マトリックスにおいて発現し機能する．硫酸化糖鎖は細胞内に取り込まれた後，リソソームに運搬されスルファターゼと呼ばれる酵素により代謝分解される．近年，リソソーム局在スルファターゼとは異なる細胞外スルファターゼ Sulf-1 および Sulf-2 の存在が報告された．Sulf-1 および Sulf-2 はヘパリン／ヘパラン硫酸の6位硫酸化を細胞外で脱硫酸化するスルファターゼであることが明らかとなった．Sulf-1 および Sulf-2 は Wnt，BMP，GDNF，FGF といったヘパリン結合性因子のヘパラン硫酸糖鎖への結合を細胞外で調節し，それら因子のシグナル伝達を巧妙に制御していることが明らかになってきた．細胞外で硫酸基を遊離するという新規分解メカニズムの発見とヘパリン／ヘパラン硫酸糖鎖の細胞外における機能制御という全く新しい分野が登場した．本総説では細胞外スルファターゼ Sulf についてその分子特性，生理機能および病態病理への関与について述べる．

1. ヘパラン硫酸プロテオグリカン

ヘパラン硫酸プロテオグリカン（heparan sulfate proteoglycan：HSPG）は，ほとんどの多細胞生物において細胞表面に存在し，また細胞外マトリックス（extracellular matrix：ECM）の主成分でもある[1~3]．HSPG は多種多様な生理活性タンパク質を結合することにより多くの生物機能をもつ．これらタンパク質リガンドは成長因子，モルフォゲン，サイトカイン，ケモカイン，プロテアーゼ，マトリックス分子，接着分子，アポリポタンパク質などである（表1）[2,4]．HSPG はコアタンパク質に1本または数本のヘパラン硫酸（HS）と呼ばれるグリコサミノグリカン（GAG）糖鎖が共有結合した構造をとる（図1）[5]．HSPG の名前はコアタンパク質の種類により命名され，代表的な細胞表面 HSPG としてシンデカン（Syndecan 1–4）とグリピカン（Glypican 1–6）ファミリーが存在する．パールカン，アグリン，コラーゲン XVIII は ECM HSPG である[2,6]．

HS 糖鎖はその合成開始が厳密に制御され[7]，HSPG が持つ生物機能の本質を担う分子である．一つの例として，HS 糖鎖合成欠損によるマウス原腸胚形成異常があげられる[8]．HS 糖鎖は，ウロン酸とグルコサミンの二糖が繰返し連なった，枝分かれのない直鎖状ポリマーである．二糖繰返し単位は最大で100単位になることもある．ウロン酸残基はグルクロン酸（glucuronic acid：GlcA）またはその酵素的エピマー化により生じるイズロン酸（iduronic acid：IdoA）で，それぞれ2位が硫酸化され得る．グルコサミン残基は6位，3位が硫酸化され，さらに，*N* 位はアセチル化または硫酸化される[9]．これらウロン酸およびグルコサミン残基の硫酸化はゴルジ体局在のスルフォトランスフェラーゼ群により担われる[10]．HS 糖鎖は硫酸化の程度

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Sulfs: extracellular endosulfatases that regulate physiological functions of heparan sulfate
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表1 ヘパリン／ヘパラン硫酸糖鎖と相互作用するタンパク質

文献2, 4, 60）参照.

General class	Examples
Adhesion molecules	L-selectin, Mac-1, NCAM, PECAM-1
Chemokines	IL-8, CXCL12, CCL21, CXCL10, CCL2
Cytokines	IL-7, IFN-γ, IL-3, TNF-α, GM-CSF
Growth factors	HB-EGF, VEGF, PDGF, FGF-1, FGF-2, FGF-8, HGF, amphiregulin, midkine, pleiotrophin
Morphogens	Wnts, Shh, BMPs, TGF-β
Axon guidance molecules	Netrin-1, slit, semaphorin-5A, ephrin-A3
ECM molecules	Laminin, fibronectin, thrombospondin, fibrin, collagens, tenascin, vitronectin
Enzymes	Lipoprotein lipase, urokinase, elastase, hyaluronidase, superoxide dismutase, thrombin

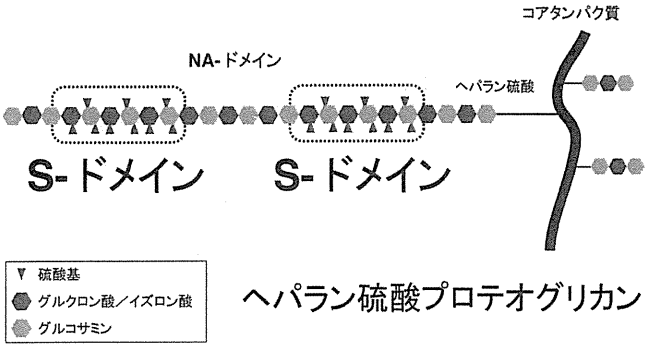

図1 ヘパラン硫酸プロテオグリカンの模式図

ヘパラン硫酸プロテオグリカン（HSPG）はコアタンパク質に直鎖状のヘパラン硫酸（HS）糖鎖が1本–数本共有結合した分子である．HS糖鎖は「S-ドメイン」と呼ばれる多硫酸化された二糖単位が2–8単位連なったドメインを含む．「S-ドメイン」は硫酸化されていないドメイン「NA-ドメイン」により分離された形をとる．ここには示さないが，S-ドメインに隣接するドメインは「transitionドメイン」と呼ばれ比較的硫酸基修飾が少ない二糖単位より成る．Sulf-1，Sulf-2はHS糖鎖内部S-ドメインの主要な構成単位であるIdoA2S-GlcNS6S二糖の6位の硫酸基を遊離するスルファターゼである．本文参照．文献5）より改変．

によりいくつかのドメインが内部で形成される．多硫酸化されたドメインは「S-ドメイン」と呼ばれ，2，6，*N*位が硫酸化されたIdoA2S-GlcNSの二糖単位を主な構成単位とする．S-ドメインに隣接するドメインは「transitionドメイン」と呼ばれ比較的硫酸化の程度が低い．これらのドメインは硫酸化がみられない*N*-アセチルグルコサミンを含む二糖単位が主体となる「NA-ドメイン」と呼ばれる領域により分離されている[5,11]．ヘパリンはその二糖単位の約80%がIdoA2S-GlcNS6Sであり，HS糖鎖「S-ドメイン」のケミカルアナログとして見なすことができる．

HS糖鎖は個体発生や組織構築に伴いその二糖単位組成，直鎖の長さおよび硫酸化のパターンに多様性をもつ[4,12,13]．すなわち，HS糖鎖の硫酸化の位置および程度によりそのタンパク質リガンドとの結合が制御されていると考えられる．さらにいえば，硫酸化のパターンによりHS糖鎖の生物機能が規定されている[3,9,14]．特に，HS糖鎖のグルコサミン残基6位の硫酸化は多くのリガンドタンパク質のHS糖鎖結合に必須であることが報告されている．

6位の硫酸化はスルフォトランスフェラーゼ（HS6ST-1, HS6ST-2, HS6ST-3）によりゴルジ体で合成される[9]．この6位の硫酸化を特異的に分解する酵素，細胞外スルファターゼSulf-1，Sulf-2が2001年から2002年にかけて報告された[15~17]．硫酸化された後に細胞外でその硫酸基を遊離するという，新規酵素的分解メカニズムの発見とHS糖鎖の細胞外における機能制御という全く新しい分野が登場した．以下，これらSulf-1，Sulf-2のクローニングおよび生物機能に関して現在までに明らかになっている事項を総説としてまとめる．

2. 新規細胞外スルファターゼSulfの発見

2-1. スルファターゼファミリー

スルファターゼは様々な分子の硫酸エステル結合を加水分解する酵素である．現在までにヒトでは17種のスルファターゼ遺伝子が確認されており，多くはリソソームに局在する[18]．リソソーム局在スルファターゼ群は，酸性条件下においてヘパラン硫酸，コンドロイチン硫酸およびケラタン硫酸といったGAGや硫酸化糖脂質などを連続的に代謝分解する．また，ヒドロキシステロール硫酸を加水分解するステロールスルファターゼは，ミクロソームに局在する膜結合型のタンパク質である．小胞体やゴルジ体に局在するスルファターゼも存在する[18]．

2-2. QSulf-1の発見

Dhootらはウズラ胚の体節よりソニックヘッジホッグ（*Shh*）応答遺伝子としてQSulf-1遺伝子をクローニングした[15]．*QSulf-1* mRNAは，ウズラ胚発生期の体節，神経底板，神経管腹側部，脊索において高レベルで検出される．アンチセンスを用いた*Shh*遺伝子発現阻害により*QSulf-1*の筋分化時期の体節および神経管における発現がブロックされた．*QSulf-1*が体節および神経管における*Shh*応答遺伝子であることが明らかにされた．また，QSulf-1遺伝子の発現阻害により筋分化調節因子MyoDの発現が選択的に阻害された．MyoD遺伝子発現がWntシグナル依存性であることから，QSulf-1はWntシグナルを正に制御する因子であると予想された．DhootらはC2C12筋芽細胞株

およびTCF（T cell factor）転写因子応答ルシフェラーゼアッセイを用いて，QSulf-1がWntシグナル制御因子であることを確認した[15]．Sulf-1タンパク質は，HS，ヘパリン，ケラタン硫酸糖鎖の非還元末端グルコサミンの6位の硫酸基に作用する酵素であるリソソーム局在グルコサミン-6-スルファターゼ（G6S）と高い相同性を有する領域をもっていた．また一連のリソソーム局在型スルファターゼとは異なり，QSulf-1はその発現細胞の細胞表面に局在した．QSulf-1が細胞表面HSPGに結合するWntを脱硫酸化により遊離させ，Wntシグナルを正に制御することが示唆された．現在においてこの生理機能は細胞外スルファターゼSulfの最も良く検証された機能の一つであり，後に述べる病態研究においても重要となる．Dhootらに続いて，ラットのQSulf-1相同遺伝子 *RSulfFP1* が報告された[16]．しかしながら，QSulf-1，RSulfFP1いずれにおいてもスルファターゼ活性を有することは示されなかった．

2-3. HSulf-1，HSulf-2の発見と細胞外スルファターゼ活性

筆者が在籍していたカリフォルニア大学サンフランシスコ校Steven Rosen研究室では上記と全く異なるアプローチによりSulf遺伝子の発見とクローニングを行った[17]．L-セレクチンと呼ばれる細胞表面分子は，レクチンの一種で糖鎖を認識する[19]．この認識糖鎖構造は，6位が硫酸化されたグルコサミンを含むシアリル6-スルフォルイスX構造である．この硫酸化を担うスルフォトランスフェラーゼはGlcNAc6ST-1，GlcNAc6ST-2である[20~22]．Rosen研究室ではこのシアリル6-スルフォルイスXの6位の硫酸基を細胞外で分解しL-セレクチンの認識を制御する機構があるのではないかという仮説を立て，その細胞外分解酵素を探索していた．その過程で従来のリソソーム局在型スルファターゼとは異なるタンパク質をコードする遺伝子を2種，ヒトとマウスにおいて同定した[17]．それぞれ，ヒトおよびマウスの脳，心臓，肺，子宮，精巣など各種臓器においてmRNAレベルで発現が確認された[17,23]．我々は2種の遺伝子にコードされるタンパク質が強制発現CHO細胞の培養上清に分泌されることを明らかにした．分泌されたタンパク質はともに4-メチルウンベリフェリル硫酸（4-MUS，細胞内局在スルファターゼの活性測定に広く用いられる基質）に対してアリルスルファターゼ活性を示した．他のほとんどのスルファターゼと異なり，酵素活性に対する至適pHが中性であることはこの酵素が細胞外で働くことを強く支持した[17]．予想とは異なりL-セレクチン認識糖鎖はこれらの酵素により分解されなかったが，陰イオン交換カラム高速液体クロマトグラフィーおよび各種GAG糖鎖を用いたアッセイ法から，これら2種のタンパク質が細胞外に分泌されるヘパリン／HS糖鎖を基質とするエンド型（糖鎖内部の硫酸基に働く）スルファターゼであることを突き止めた．さらに詳しく解析した結果，HS糖鎖「S-ドメイン」のIdoA2S-GlcNS6S単位の6位硫酸基を遊離する活性をもつことを明らかにした（図2）．我々及び他の

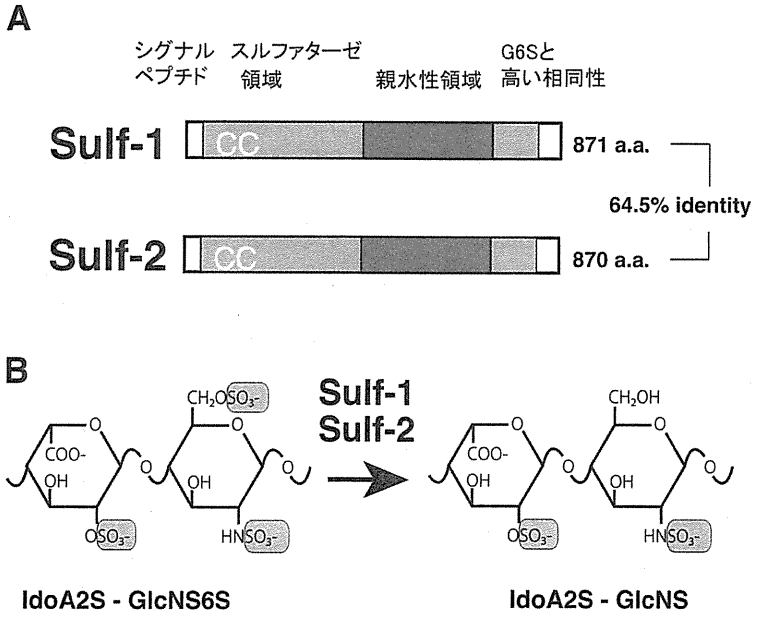

図2 細胞外スルファターゼSulfの構造と酵素反応様式

A．ヒトSulf-1，Sulf-2を示す．スルファターゼ活性に必須であるシステイン残基（CC）はSulf-1およびSulf-2で保存されている．G6S：リソソーム局在グルコサミン6スルファターゼ．B．Sulf-1，Sulf-2はHS糖鎖内部「S-ドメイン」のIdoA2S-GlcNS6S二糖の6位硫酸基を遊離する[17,36,63]．

グループのその後の解析から，GlcA/IdoA-GlcNS6S単位の6位硫酸基を遊離する活性も保持することが明らかにされた[24~26]．2種のうち一つは *QSulf-1* の相同遺伝子としてヒト遺伝子を *HSulf-1*，マウス遺伝子を *MSulf-1*，と名付けた．また，もう一つは全く新しいファミリー遺伝子としてそれぞれ *HSulf-2*，*MSulf-2* と命名した[17]．

2-4. Sulfの構造とプロセシング：pre-pro-protein

QSulf-1やRSulfFP1の構造と同様に，予想されたヒトおよびマウスのSulf-1，Sulf-2は870-875アミノ酸（a.a.）であった（図2）．両SulfはN末端に22-27 a.a.のシグナルペプチド，リソソーム局在スルファターゼ群と相同性をもつ約370 aaのスルファターゼ領域，約320 aaの親水性領域（以下，HD領域）およびヒトG6Sと高い相同性をもつ約100 aaのC末端領域を有していた（図2）．Sulf-1，Sulf-2のアミノ酸配列はそれぞれ，ヒト及びマウス種間で非常に高い相同性があった（93-94%）．Sulf-1およびSulf-2の間では63-65%のアミノ酸配列が一致している．両Sulfのスルファターゼ領域には真核生物の全スルファターゼに共通するシステイン残基が含まれていた[15,17]．このシステイン残基はsulfatase modifying factor 1によりα-ホルミルグリシンに変換されスルファターゼ活性に必須である[27,28]．Sulf-1およびSulf-2は「pre-pro-protein」として生合成された後，シグナルペプチドが切断されて，約125 kDaの「pro-protein」となる（図3）．その後，furinプロテアーゼ[29]によりHD領域内でプロセシングされ75 kDaと50 kDaのフラグメントとなる[17,30,31]．さらに，これらのフラグメントはジスルフィド結合により結合する．ヒトおよびマウス両Sulfはこれらプロセシングを受け，細胞表面または細胞外に分泌される[17,30,32]．一方，ウズラのSulfは細胞表面に局在し，細胞外に分泌されない[15,33]．この種間の違いがなぜ起きるのかは明らかになっていない．

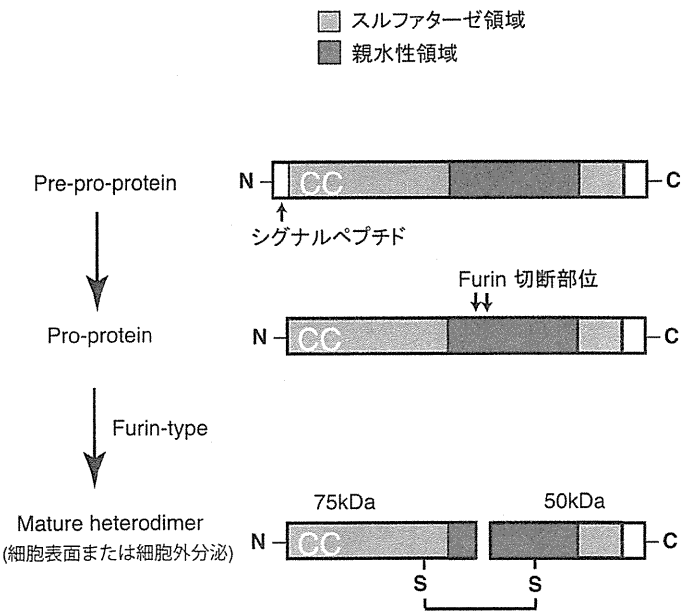

図3 細胞外スルファターゼSulfのプロセシング
ヒトSulf-1，Sulf-2（HSulf-1，HSulf-2）はシグナルペプチドをもつ「pre-pro-protein」として生合成される．小胞体においてシグナルペプチドが切断され「pro-protein」となる．親水性（HD）領域内に存在するfurinプロテアーゼ部位においてプロテアーゼ切断を受け，75 kDa，50 kDaのフラグメントが生成される．これらはジスルフィド結合によりヘテロ二量体となり細胞外へ分泌されるか細胞表面に局在する．プロテアーゼ切断はSulfのスルファターゼ活性に影響を与えないが，Sulfの脂質ラフトへの局在およびWntシグナル伝達促進作用には必須である[30]．本文参照．文献60）より改変．

Sulfの HD領域はSulfタンパク質の細胞表面局在においても重要な働きをする[30,33,34]．この細胞表面相互作用は塩濃度により可逆的である[17,30]．ヒトSulf-1，Sulf-2は脂質ラフトに濃縮されており[30]，細胞外に分泌された活性型Sulfは超高速遠心によりペレットとして回収できることが明らかにされた[32]．大変興味深いことに，furinによるプロセシングはSulfの4-MUSを基質とするアリルスルファターゼ活性，ヘパリン／HS糖鎖を基質とするエンドスルファターゼ活性のいずれにも必須ではないが脂質ラフトへの局在には必要である[30]．脂質ラフトにおける各種タンパク質の会合が，多くのシグナル伝達において重要なことから，Sulfプロセシングがヘパリン結合性因子シグナル調節機能に深く関わることが示された．

3. 新規細胞外スルファターゼSulfの生物機能

3-1. Sulfによるリガンドタンパク質-HS分子間相互作用の調節：bioavailabilityの制御

現在までに，Sulfが多くのタンパク質リガンドのヘパリンまたはHS糖鎖への結合を調節していることが明らかにされている[25,26,35~37]．中でもSulf-2に関して詳しく調べられている[35,36]．血管内皮細胞増殖因子（VEGF）165，線維芽細胞増殖因子（FGF）-1，SDF-1/CXCL12，SLC/CCL21の固層化ヘパリンへの結合が，リコンビナントまたはネイティブのHSulf-2前処理により，消失または減少する[35]．VEGF165，FGF-1に対する作用はグルコサミン6位の硫酸基がそれら因子の結合に重要であるという以前の報告によく合致した．他のリガンド分子に関しては，6位硫酸基の重要性が新たな知見となった．さらに，HSulf-2が固層化ヘパリンに結合したこれらリガンド分子を結合複合体より遊離する作用をもつことが示された[35]．すなわち，細胞表面や細胞外マトリックスのHSPGに隔離又は貯留（sequestration）されているリガンド分子をSulfが遊離させ，その受容体を発現する細胞への作用を促進するメカニズムの存在が示唆された．実際，HSulf-2は *in vivo* で血管新生を促す[38]．HSPGに貯留された血管新生因子（例えばVEGF165）を遊離させ，その生物学的利用率（bioavailability）を増加させた結果であることが予想された．

3-2. Sulfによる細胞レベルでのHS結合性因子シグナルの正の制御：Wnt，BMP，GDNF

QSulf-1 遺伝子発見の項で述べたように，Wnt応答性細胞にQSulf-1を発現させると，Wnt1リガンドによるシグナル伝達が当該細胞で増強される[15]．また，QSulf-1はWnt8のHSPGへの結合を調節する[25]．QSulf-1で観察された結果はHSulf-1，HSulf-2のWntリガンド（Wnt1，Wnt3，Wnt3a，Wnt4）に対する作用においても確認された[30,39]．Aiらは図4に示すモデルを提唱している．Sulf-1を発現していない細胞では，Wntリガンドは細胞表面HSPG上のHS鎖と強固に結合し捕捉されているため，そのFrizzled受容体との機能的な相互作用が困難である．そのためWntシグナル伝達が抑えられていると考えられる．一方，Sulf-1を発現する細胞ではSulf-1によりHS鎖の6-硫酸が分解され，HS鎖に結合しているWntとHS鎖との親和性が低下する．その結果，WntのFrizzledへの結合が促され，Wnt-HS-Frizzledの三量体が構成されると考えられる．最終的にWnt下流のシグナルが活性化され，Wnt標的遺伝子の転写が誘導される（図4）．同じようなアプローチにより，Sulfの骨形成因子（BMP）-4[26]およびグリア細胞由来神経栄養因子（GDNF）のシグナル伝達促進作用が明らかとなった[37,40]．BMPの機能を阻害するアンタゴニストであるNogginはBMPと結合し，BMPとその受容体との相互作用を阻害する．また，Nogginの細胞表面からの放出および拡散は，HS鎖により調節されており，NogginはHS糖鎖S-ドメインの硫酸基を介してHS鎖に結合する[26]．SulfはHS糖鎖SドメインのIdoA2S-GlcNS6S単位の6位硫酸基を分解することから，SulfによりNogginがHS鎖から遊離される可能性について培養細胞を用いて詳細に検討された．QSulf-1の過剰発現により細胞表面に結合しているNoggin量が減少し，BMPシグナルの下流に存在するSMADのリン酸化が促進されることが明らかとなった[26]．すなわち，HS糖鎖との結合によりNogginが細胞表面に限局されている場合は，BMPは細胞表面のNogginに強固に捕捉され，その受容体との相互作用が阻害されると示唆された．一方，SulfによりNogginの細胞表面での限局が崩れると，BMPのその受容体への到達（accessibility）が容易となり，BMPシグナルの活性化が起こると考えられた．Sulf-1がNogginの放出および拡散をコントロールすることにより，BMPシグナルの受容を細胞レベルで選択している可能性が強く示唆された．

3-3. Sulfによる細胞レベルでのHS結合性因子シグナルの負の制御：HB-EGF，FGF-2，HGF

Sulfにより正に制御される上記リガンド分子とは対照的に，ヘパリン結合性上皮成長因子様増殖因子（HB-EGF），FGF-2，肝細胞増殖因子（HGF）のシグナル伝達はSulf発現細胞で負に制御される[41~47]．Sulf-1強制発現細胞をヘパリン結合性増殖因子であるHB-EGFで処理すると，HB-EGF受容体のリン酸化およびそのシグナルカスケードの下流に存在するMAPK/ERK（mitogen activated protein kinase/extracellular signaling regulated protein kinase）のリン酸化レベルが，対照細胞に比べて減少する[41]．同じくヘパリン結合性増殖因子であるFGF-2やHGFで処理した場合も，HB-EGFで処理した場合と同様にそのシグナル伝達の下流に存在するMAPK/ERKのリン酸化レベルは減少する[43,45,46]．これに対し，ヘパリンと結合しない上皮成長因子（EGF）で細胞を処理してもEGF受容体のリン酸化やMAPK/ERKのリン酸化レベルは，Sulf-1強制発現細胞と対照細胞で同じである[41]．細胞表面におけるリガンド-HS-受容体の三量体形成（FGF2-HS-FGFR1）を困難にするこ

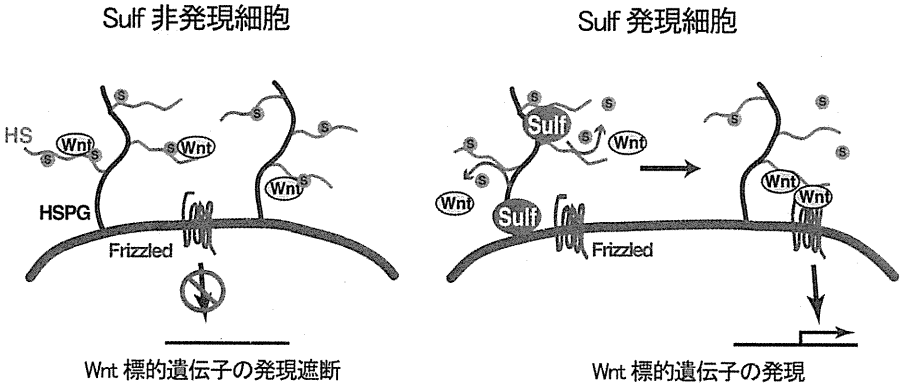

図4 SulfによるWntシグナルの制御モデル
Sulf発現細胞では，SulfがHS鎖S-ドメインの6位硫酸基を遊離し，HS鎖S-ドメインを介して結合しているWntのHS鎖への親和性を低下させる．WntのFrizzledへの結合が促され，Wnt-HS-Frizzledの三量体が構成されると推測される．Wnt下流のシグナルが活性化され，Wnt標的遺伝子の転写が誘導される．HSPG（グリピカン）とその硫酸化がWntのシグナル伝達に関わるという以前の報告はこのモデルを支持する[64,65]．本文参照．文献25）より改変．

とにより，Sulf-1は細胞内への増殖シグナルの減少を誘導している可能性が示唆された．グルコサミンの6位硫酸化はFGF-2-HS糖鎖ではなくFGFR1-HS糖鎖の結合に必要であるため，Sulfは後者の分子間相互作用を調節する可能性がある．Sulf-1がHS糖鎖リモデリングにより受容体自身による阻害を促進する可能性も検討しなければいけない[48]．*MSulf-1*，*MSulf-2* 遺伝子欠損マウスより調製した胚線維芽細胞においてFGF-2によるシグナル伝達と細胞分裂が野生型に比べて増加する結果は，上記仮説をよく支持した[49,50]．

3-4. Sulfの生体内における役割：Sulfノックアウトマウスの表現型

既に述べたように，Sulf-1はWnt依存的な筋組織の発生に関わる[15]．しかしながら，両Sulf-1，Sulf-2は胚発生時のWntシグナルに必須とはいえない．このことはマウスにおいて明らかである．Wntリガンド分子の遺伝子欠損では胎生致死または出生直後の死亡が観察される（http://www.stanford.edu/group/nusselab/cgi-bin/wnt/）．しかし，*Sulf-1* または *Sulf-2* 単独の遺伝子ノックアウトでは，ほとんどの場合わずかな異常が発生時に観察されるのみである[37,49~51]．ジーントラップ法による *Sulf-2* 遺伝子ノックアウトでは，胎仔マウスの生育が遅れ，出生後の体重の減少と肺の異常が一部のマウスで観察される[51]．胎生致死に至った *Sulf-2* 遺伝子ノックアウトマウスは脳機能不全と関連する[52]．*Sulf-1* および *Sulf-2* の両遺伝子欠損では，約50%の出生直後の死亡が観察されるが残りのマウスは成体まで成長する[37,49,50]．胎生致死でない *Sulf-1*，*Sulf-2* 両遺伝子欠損マウスは野生型に比べて体格が小さいが，いずれの臓器も組織レベルでの異常はみられない[50]．一方，*Sulf* 両遺伝子欠損マウスのわずかな骨格形成異常が報告された[53,54]．*Sulf-1*，*Sulf-2* 両遺伝子欠損マウスの表現型で最も詳しく解析されているのが摂食障害である[37]．Aiらは *Sulf* 両遺伝子欠損マウスの食道における平滑筋への神経分布の異常を観察し，そのことが筋収縮能の障害を引き起こしていると結論づけた．すなわち，両Sulfが筋組織への神経分布におけるGDNFを介したシグナル伝達を増加させていることを明らかにした[37]．さらにAiらは，精巣セルトリ細胞が発現するSulf-1およびSulf-2が，GDNFシグナル伝達で制御される精子形成幹細胞自己複製を量的に規定していることを明らかにした[40]（図5）．また，Sulf-1およびSulf-2が筋サテライト細胞のFGF-2依存性増殖を抑え，筋細胞への分化を誘導し筋再生を促していることが，*Sulf* 両遺伝子欠損マウスより明らかとなった[55]．いずれの場合も *Sulf-1* または *Sulf-2* 単独の遺伝子ノックアウトでは異常がみられない．どちらか一方のSulfが，HS糖鎖スルファターゼ酵素活性レベルで補償作用を示すのか，または機能的な補償作用を示すのか明らかにする必要がある．

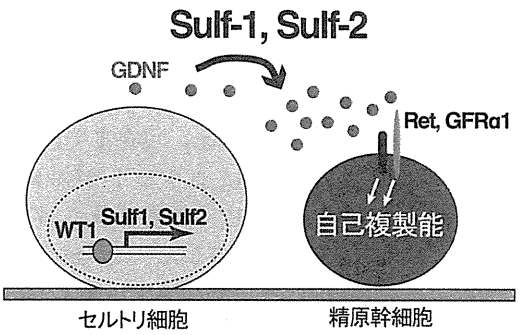

図5 SulfによるGDNFシグナルの制御モデル
精巣セルトリ細胞（Sertoli cell）より発現されるSulf-1，Sulf-2がHS鎖S-ドメインを介し細胞表面や細胞外マトリックスに貯留，隔離（sequestration）されているGDNFをHS鎖S-ドメイン分解によりリリースさせる．結果的に精子形成幹細胞（spermatogonial stem cell）へのGDNF生物学的利用率（bioavailability）を増大させる．精子形成幹細胞が発現するRetおよびGFR α1を介したシグナル伝達が正に制御される．精巣セルトリ細胞におけるSulf-1，Sulf-2の遺伝子発現はWT1転写因子により調節される[40,66]．本文参照．文献40）より改変．

さらに，ヘパラン硫酸6-硫酸転移酵素[10]のグルコサミン6位の硫酸化亢進による代償作用の有無も検討しなければならない．また，もう一つの解釈として，Sulfは *in vitro* では多くのシグナル伝達系を制御する潜在的な作用を示すが，正常な個体発生においてはSulfの作用は根本的に不可欠なものではなく，シグナル伝達の強度を微調整するファインチューナーの役割を担っているのかもしれない．さらに詳しい解析が必要である．

4. 新規細胞外スルファターゼSulfの病態への関与：Sulfのがんにおける発現調節不全

HSulf-1，*HSulf-2* のクローニング後，我々はがんにおけるSulfの関与を明らかにするためSAGE（serial analysis of gene expression，連続的遺伝子発現解析）法を行った．SAGE法とは，それぞれのmRNAから10-11bpの遺伝子配列（タグ）を抽出し作成したライブラリーをもとに，組織におけるmRNA発現量を定量する方法である．ライブラリーにおける特定遺伝子に対応するタグの出現数は，組織でのその遺伝子の発現量を表している．ライブラリーのタグの総数に対する出現した特定遺伝子タグ数の割合を計算することによって，発現頻度を求めることができる．*HSulf-2* 遺伝子（*SULF2*）に関して，タグの発現頻度は3種のがん，すなわち乳がん，中枢神経系がん，大腸がんにおいてその正常組織と比較した場合著しく高かった．そのタグの発現頻度は腫瘍組織において6-8倍増加している．*HSulf-1* 遺伝子（*SULF1*）に関しては，より少ないタグ数ではあったが乳がんと中枢神経系がんにおいてその発現頻度は高くなっていた[23]．これらの結果はSulfのがんへの関与の最初のヒントとなった．その後，ヒト乳がんにおける *SULF1*，*SULF2* の発現上昇が確認された[38]．引き続きマ

ウスの乳がんモデルであるMMTV-Neuマウスおよび MMTV-Wnt1マウスを解析した結果，Sulf-2は正常乳腺組織では検出されないが，過形成乳腺および乳腺腫瘍ではその発現が観察された[38]．上記に加え，現在までに定量PCRまたはマイクロアレイ解析によりヒトがんにおける *SULF1*，*SULF2* の発現上昇が広く報告されている．例えば，*SULF1* は肝細胞がん[43]，膵臓がん[45]，頭頸部扁平上皮がん[56]，胃がん[57]，肺腺がん[58]，肺扁平上皮がん[58]で発現が増加している．*SULF2* は，肝細胞がん[59]，肺腺がん[58]，肺扁平上皮がん[58]で発現が増加している．公開されているマイクロアレイ解析データベースOncomine（www.oncomine.com）を用いて，正常組織と腫瘍組織における発現レベルを解析すると，有為差（$p<0.0001$）を伴う変動のうち，*SULF1* は30の比較例で3–60倍の発現増加が確認される．2例の比較においてのみ発現減少がみられる．*SULF2* は有為差（$p<0.0001$）を伴う変動9例全てにおいて2–8倍の発現増加が確認された．比較例の詳細は文献60）に記載されている．また，レトロウイルスを用いたマウス挿入変異誘発システムの解析から，*Sulf-2* が神経膠腫において発がん性遺伝子の一つであることが明らかにされた[61]．大変興味深いことに，上述のOncomineの *SULF2* 解析結果9例のうち5例はヒト神経膠腫である[60]．

5. おわりに

Wnt，BMP，GDNF，FGFなどのシグナル伝達が中心となる疾患において，Sulf-1とSulf-2の疾患の発症や進行への関与が明らかになってきている．Otsukiらは，ヒト変形性骨関節炎における関節軟骨でSulf-1，Sulf-2のmRNA及びタンパク質が正常軟骨に比べて発現増加することを示した．その後，ヒト軟骨細胞および *Sulf-1* または *Sulf-2* 遺伝子欠損マウスを用いて，変形性骨関節症におけるSulfの役割を明確に示した．すなわち，Sulf-1，Sulf-2はBMP7シグナルを増強させ，FGF2シグナルを減弱させることにより，関節軟骨における恒常性維持を担っている[54]．このバランスが破綻すると軟骨の変性が誘発されると示唆された．さらに，加齢に伴って関節軟骨におけるSulf-1，Sulf-2の発現が増加することを示した[62]．加齢は多くのヒト疾患においてリスクファクターとなっており，Sulfの加齢に伴い発症する他の疾患（例えばアルツハイマー型認知症）への関与があるかもしれない．我々はある種の神経変性疾患におけるSulf-2とHS糖鎖S-ドメインの発現調節不全を見出しており，今後この分野におけるHS糖鎖とSulfの機能解明に貢献できればと願っている．

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文献

1) Lindahl, U., Kusche-Gullberg, M., & Kjellen, L. (1998) *J. Biol. Chem.*, **273**, 24979–24982.
2) Bernfield, M., Gotte, M., Park, P.W., Reizes, O., Fitzgerald, M. L., Lincecum, J., & Zako, M. (1999) *Annu. Rev. Biochem.*, **68**, 729–777.
3) Bishop, J.R., Schuksz, M., & Esko, J.D. (2007) *Nature*, **446**, 1030–1037.
4) Esko, J.D. & Selleck, S.B. (2002) *Annu. Rev. Biochem.*, **71**, 435–471.
5) Gallagher, J.T. (2001) *J. Clin. Invest.*, **108**, 357–361.
6) Iozzo, R.V. (2001) *J. Clin. Invest.*, **108**, 165–167.
7) Sugahara, K. & Kitagawa, H. (2000) *Curr. Opin. Struct. Biol.*, **10**, 518–527.
8) Yan, D. & Lin, X. (2009) *Cold Spring Harb. Perspect. Biol.*, **1**, a002493.
9) Habuchi, H., Habuchi, O., & Kimata, K. (2004) *Glycoconj. J.*, **21**, 47–52.
10) Habuchi, O. (2000) *Biochim. Biophys. Acta*, **1474**, 115–127.
11) Esko, J.D. & Lindahl, U. (2001) *J. Clin. Invest.*, **108**, 169–173.
12) Nakato, H. & Kimata, K. (2002) *Biochim. Biophys. Acta*, **1573**, 312–318.
13) Lamanna, W.C., Kalus, I., Padva, M., Baldwin, R.J., Merry, C. L., & Dierks, T. (2007) *J. Biotechnol.*, **129**, 290–307.
14) Lee, J.S. & Chien, C.B. (2004) *Nat. Rev. Genet.*, **5**, 923–935.
15) Dhoot, G.K., Gustafsson, M.K., Ai, X., Sun, W., Standiford, D. M., & Emerson, C.P., Jr. (2001) *Science*, **293**, 1663–1666.
16) Ohto, T., Uchida, H., Yamazaki, H., Keino-Masu, K., Matsui, A., & Masu, M. (2002) *Genes Cells*, **7**, 173–185.
17) Morimoto-Tomita, M., Uchimura, K., Werb, Z., Hemmerich, S., & Rosen, S.D. (2002) *J. Biol. Chem.*, **277**, 49175–49185.
18) Diez-Roux, G. & Ballabio, A. (2005) *Annu. Rev. Genomics Hum. Genet.*, **6**, 355–379.
19) Rosen, S.D. (2004) *Annu. Rev. Immunol.*, **22**, 129–156.
20) Uchimura, K., Gauguet, J.M., Singer, M.S., Tsay, D., Kannagi, R., Muramatsu, T., von Andrian, U.H., & Rosen, S.D. (2005) *Nat. Immunol.*, **6**, 1105–1113.
21) Uchimura, K. & Rosen, S.D. (2006) *Trends Immunol.*, **27**, 559–565.
22) Kawashima, H., Petryniak, B., Hiraoka, N., Mitoma, J., Huckaby, V., Nakayama, J., Uchimura, K., Kadomatsu, K., Muramatsu, T., Lowe, J.B., & Fukuda, M. (2005) *Nat. Immunol.*, **6**, 1096–1104.
23) Morimoto-Tomita, M., Uchimura, K., & Rosen, S.D. (2003) *Trends Glycosci. Glycotechnol.*, **15**, 159–164.
24) Saad, O.M., Ebel, H., Uchimura, K., Rosen, S.D., Bertozzi, C. R., & Leary, J.A. (2005) *Glycobiology*, **15**, 818–826.
25) Ai, X., Do, A.T., Lozynska, O., Kusche-Gullberg, M., Lindahl, U., & Emerson, C.P., Jr. (2003) *J. Cell. Biol.*, **162**, 341–351.
26) Viviano, B.L., Paine-Saunders, S., Gasiunas, N., Gallagher, J., & Saunders, S. (2004) *J. Biol. Chem.*, **279**, 5604–5611.
27) Cosma, M.P., Pepe, S., Annunziata, I., Newbold, R.F., Grompe, M., Parenti, G., & Ballabio, A. (2003) *Cell*, **113**, 445–456.
28) Dierks, T., Schmidt, B., Borissenko, L.V., Peng, J., Preusser, A., Mariappan, M., & von Figura, K. (2003) *Cell*, **113**, 435–444.
29) Nakayama, K. (1997) *Biochem. J.*, **327**, 625–635.

30) Tang, R. & Rosen, S.D. (2009) *J. Biol. Chem.*, 284, 21505–21514.
31) Nagamine, S., Keino-Masu, K., Shiomi, K., & Masu, M. (2010) *Biochem. Biophys. Res. Commun.*, 391, 107–112.
32) Hossain, M.M., Hosono-Fukao, T., Tang, R., Sugaya, N., van Kuppevelt, T.H., Jenniskens, G.J., Kimata, K., Rosen, S.D., & Uchimura, K. (2010) *Glycobiology*, 20, 175–186.
33) Ai, X., Do, A.T., Kusche-Gullberg, M., Lindahl, U., Lu, K., & Emerson, C.P., Jr. (2006) *J. Biol. Chem.*, 281, 4969–4976.
34) Frese, M.A., Milz, F., Dick, M., Lamanna, W.C., & Dierks, T. (2009) *J. Biol. Chem.*, 284, 28033–28044.
35) Uchimura, K., Morimoto-Tomita, M., Bistrup, A., Li, J., Lyon, M., Gallagher, J., Werb, Z., & Rosen, S.D. (2006) *BMC Biochem.*, 7, 2.
36) Uchimura, K., Morimoto-Tomita, M., & Rosen, S.D. (2006) *Methods Enzymol.*, 416, 243–253.
37) Ai, X., Kitazawa, T., Do, A.T., Kusche-Gullberg, M., Labosky, P.A., & Emerson, C.P., Jr. (2007) *Development*, 134, 3327–3338.
38) Morimoto-Tomita, M., Uchimura, K., Bistrup, A., Lum, D.H., Egeblad, M., Boudreau, N., Werb, Z., & Rosen, S.D. (2005) *Neoplasia*, 7, 1001–1010.
39) Nawroth, R., van Zante, A., Cervantes, S., McManus, M., Hebrok, M., & Rosen, S.D. (2007) *PLoS ONE*, 2, e392.
40) Langsdorf, A., Schumacher, V., Shi, X., Tran, T., Zaia, J., Jain, S., Taglienti, M., Kreidberg, J.A., Fine, A., & Ai, X. (2010) *Glycobiology*, 21, 152–161.
41) Lai, J., Chien, J., Staub, J., Avula, R., Greene, E.L., Matthews, T.A., Smith, D. I., Kaufmann, S.H., Roberts, L.R., & Shridhar, V. (2003) *J. Biol. Chem.*, 278, 23107–23117.
42) Lai, J.P., Chien, J., Strome, S.E., Staub, J., Montoya, D.P., Greene, E.L., Smith, D.I., Roberts, L.R., & Shridhar, V. (2004) *Oncogene*, 23, 1439–1447.
43) Lai, J.P., Chien, J.R., Moser, D.R., Staub, J.K., Aderca, I., Montoya, D.P., Matthews, T.A., Nagorney, D.M., Cunningham, J.M., Smith, D.I., Greene, E.L., Shridhar, V., & Roberts, L.R. (2004) *Gastroenterology*, 126, 231–248.
44) Dai, Y., Yang, Y., MacLeod, V., Yue, X., Rapraeger, A.C., Shriver, Z., Venkataraman, G., Sasisekharan, R., & Sanderson, R.D. (2005) *J. Biol. Chem.*, 280, 40066–40073.
45) Li, J., Kleeff, J., Abiatari, I., Kayed, H., Giese, N.A., Felix, K., Giese, T., Buchler, M.W., & Friess, H. (2005) *Mol. Cancer*, 4, 14.
46) Wang, S., Ai, X., Freeman, S.D., Pownall, M.E., Lu, Q., Kessler, D.S., & Emerson, C.P., Jr. (2004) *Proc. Natl. Acad. Sci. USA*, 101, 4833–4838.
47) Narita, K., Staub, J., Chien, J., Meyer, K., Bauer, M., Friedl, A., Ramakrishnan, S., & Shridhar, V. (2006) *Cancer Res.*, 66, 6025–6032.
48) Schlessinger, J. (2003) *Science*, 300, 750–752.
49) Lamanna, W.C., Baldwin, R.J., Padva, M., Kalus, I., Ten Dam, G., van Kuppevelt, T.H., Gallagher, J.T., von Figura, K., Dierks, T., & Merry, C.L. (2006) *Biochem. J.*, 400, 63–73.
50) Holst, C.R., Bou-Reslan, H., Gore, B.B., Wong, K., Grant, D., Chalasani, S., Carano, R.A., Frantz, G.D., Tessier-Lavigne, M., Bolon, B., French, D.M., & Ashkenazi, A. (2007) *PLoS ONE*, 2, e575.
51) Lum, D.H., Tan, J., Rosen, S.D., & Werb, Z. (2007) *Mol. Cell Biol.*, 27, 678–688.
52) Kalus, I., Salmen, B., Viebahn, C., von Figura, K., Schmitz, D., D'Hooge, R., & Dierks, T. (2008) *J. Cell. Mol. Med.*, 13, 4505–4521.
53) Ratzka, A., Kalus, I., Moser, M., Dierks, T., Mundlos, S., & Vortkamp, A. (2008) *Dev. Dyn.*, 237, 339–353.
54) Otsuki, S., Hanson, S.R., Miyaki, S., Grogan, S.P., Kinoshita, M., Asahara, H., Wong, C.H., & Lotz, M.K. (2010) *Proc. Natl. Acad. Sci. USA*, 107, 10202–10207.
55) Langsdorf, A., Do, A.T., Kusche-Gullberg, M., Emerson, C.P., Jr., & Ai, X. (2007) *Dev. Biol.*, 311, 464–477.
56) Kudo, Y., Ogawa, I., Kitajima, S., Kitagawa, M., Kawai, H., Gaffney, P.M., Miyauchi, M., & Takata, T. (2006) *Cancer Res.*, 66, 6928–6935.
57) Junnila, S., Kokkola, A., Mizuguchi, T., Hirata, K., Karjalainen-Lindsberg, M.L., Puolakkainen, P., & Monni, O. (2010) *Genes Chromosomes Cancer*, 49, 28–39.
58) Lemjabbar-Alaoui, H., van Zante, A., Singer, M.S., Xue, Q., Wang, Y.Q., Tsay, D., He, B., Jablons, D.M., & Rosen, S.D. (2010) *Oncogene*, 29, 635–646.
59) Lai, J.P., Sandhu, D.S., Yu, C., Han, T., Moser, C.D., Jackson, K.K., Guerrero, R.B., Aderca, I., Isomoto, H., Garrity-Park, M. M., Zou, H., Shire, A.M., Nagorney, D.M., Sanderson, S.O., Adjei, A.A., Lee, J.S., Thorgeirsson, S.S., & Roberts, L.R. (2008) *Hepatology*, 47, 1211–1222.
60) Rosen, S.D. & Lemjabbar-Alaoui, H. (2010) *Expert Opin. Ther. Targets*, 14, 935–949.
61) Johansson, F.K., Brodd, J., Eklof, C., Ferletta, M., Hesselager, G., Tiger, C.F., Uhrbom, L., & Westermark, B. (2004) *Proc. Natl. Acad. Sci. USA*, 101, 11334–11337.
62) Otsuki, S., Taniguchi, N., Grogan, S.P., D' Lima, D., Kinoshita, M., & Lotz, M. (2008) *Arthritis Res. Ther.*, 10, R61.
63) Uchimura, K., Lemjabbar-Alaoui, H., van Kuppevelt, T.H., & Rosen, S.D. (2010) *Methods Enzymol.*, 480, 51–64.
64) Tsuda, M., Kamimura, K., Nakato, H., Archer, M., Staatz, W., Fox, B., Humphrey, M., Olson, S., Futch, T., Kaluza, V., Siegfried, E., Stam, L., & Selleck, S.B. (1999) *Nature*, 400, 276–280.
65) Lin, X. & Perrimon, N. (1999) *Nature*, 400, 281–284.
66) Ratelade, J., Arrondel, C., Hamard, G., Garbay, S., Harvey, S., Biebuyck, N., Schulz, H., Hastie, N., Pontoglio, M., Gubler, M. C., Antignac, C., & Heidet, L. (2010) *Hum. Mol. Genet.*, 19, 1–15.

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Matrix Pathobiology

Heparan Sulfate Subdomains that are Degraded by Sulf Accumulate in Cerebral Amyloid ß Plaques of Alzheimer's Disease

Evidence from Mouse Models and Patients

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Alzheimer's disease (AD) is characterized by extracellular cerebral accumulation of amyloid β peptide (Aβ). Heparan sulfate (HS) is a glycosaminoglycan that is abundant in the extracellular space. The state of sulfation within the HS chain influences its ability to interact with a variety of proteins. Highly sulfated domains within HS are crucial for Aβ aggregation *in vitro*. Here, we investigated the expression of the sulfated domains and HS disaccharide composition in the brains of Tg2576, J20, and T41 transgenic AD mouse models, and patients with AD. RB4CD12, a phage display antibody, recognizes highly sulfated domains of HS. The RB4CD12 epitope is abundant in the basement membrane of brain vessels under physiological conditions. In the cortex and hippocampus of the mice and patients with AD, RB4CD12 strongly stained both diffuse and neuritic amyloid plaques. Interestingly, RB4CD12 also stained the intracellular granules of certain hippocampal neurons in AD brains. Disaccharide compositions in vessel-enriched and nonvasculature fractions of Tg2576 mice and AD patients were found to be comparable to those of non-transgenic and non-demented controls, respectively. The RB4CD12 epitope in amyloid plaques was substantially degraded *ex vivo* by Sulf-1 and Sulf-2, extracellular HS endosulfatases. These results indicate that formation of highly sulfated HS domains may be upregulated in conjunction with AD pathogenesis, and that these domains can be enzymatically remodeled in AD brains. *(Am J Pathol 2012, 180: 2056–2067; DOI: 10.1016/j.ajpath.2012.01.015)*

Heparan sulfate (HS) is a linear polysaccharide that exists in large quantities in the extracellular space. One or more HS chains are covalently bound to a core protein comprising heparan sulfate proteoglycan (HSPG).[1,2] HS chains and heparins, structural analogues of HS chains, are a family of glycosaminoglycans consisting of repeating disaccharide units of glucuronic/iduronic acid and glucosamine. Modification with sulfation as well as elongation of these disaccharides is enzymatic,[3] bestowing on the chains structural diversity.[4–6] HS contains highly

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sulfated domains and partially sulfated or non-sulfated domains, which are transitional.[3] Highly sulfated domains are formed by consecutive clusters of sulfated disaccharides. It has been shown that a trisulfated disaccharide structure [-iduronic acid(2S)-Glucosamine(NS,6S)-] occurs within highly sulfated domains. RB4CD12, a phage display anti-HS antibody, has been shown to recognize trisulfated disaccharide-containing HS subdomains[7–9] Trisulfated disaccharides are considered to be key elements in molecular interactions between HS/heparin and many ligands, including growth factors and morphogens.[1,10] Trisulfated disaccharides, as well as the RB4CD12 epitope, are degraded by extracellular sulfatases, Sulf-1, and Sulf-2.[8,11,12] In the brain, we have shown that the RB4CD12 HS domains are abundantly present in the vasculature[9] and that these domains can be degraded by the Sulfs *ex vivo*.[8] However, the roles of the RB4CD12 HS domains in pathological and physiological processes in brain vasculature are not known.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder. One of the pathological hallmarks of AD is the presence of extracellular amyloid plaques in brain areas that are responsible for cognition and memory functions. The predominant composition of amyloid plaques is fibrils made of amyloid β peptide (Aβ). A great deal of biochemical and genetic evidence has indicated that aggregation and accumulation of Aβ in toxic forms within the extracellular space play a central role in AD pathogenesis. One of the authors previously reported that certain structures of HS chains exist in amyloid plaques of AD brains,[13] and that structural variation of HSPG correlates with amyloid plaque formation in the brains of AD patients.[14] HSPG is also known to facilitate cerebral amyloid deposition induced exogenously in a rat model *in vivo*.[15] Functional roles of HS and HSPG in AD pathology are proposed to be acceleration of Aβ fibril formation and protection of the fibril against microglial phagocytosis.[16] It was reported that the aggregation state of Aβ requires its binding properties to heparin.[17] Pathological correlations between the RB4CD12 HS domains, which are rich in heparin and AD have not been established. Here we present evidence that the RB4CD12 HS domains are accumulated in cerebral amyloid plaques of transgenic AD mouse models and patients with AD, and that these HS epitopes can be degraded by Sulf-1 and Sulf-2 *ex vivo*.

Materials and Methods

Materials

The RB4CD12 phage display-derived anti-heparan sulfate antibody was produced in a vesicular stomatitis virus (VSV)-tag version and purified as previously described.[7] Alternative nomenclature of RB4CD12 is HS3A8. The following materials were commercially obtained from the sources indicated. Heparinases (I, II and III), polyclonal rabbit anti-laminin antibody (Ab), horseradish peroxidase-conjugated monoclonal anti-VSV Ab, and Cy3-conjugated monoclonal anti-VSV Ab were from Sigma (St. Louis, MO); biotinylated monoclonal anti-amyloid β (N-terminus) Ab (82E1) was from IBL (Gunma, Japan); polyclonal rabbit anti-VSV Ab was from Bethyl Laboratories (Montgomery, TX); Cy2-conjugated goat anti-mouse IgG (H+L), Cy2-conjugated goat anti-rabbit IgG (H+L), Cy2-conjugated goat anti-rat IgG (H+L) Abs, and Cy2-conjugated streptavidin were from Jackson ImmunoResearch Laboratories (West Grove, PA); rabbit anti-Iba1 Ab was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); rabbit anti-glial fibrillary acidic protein and monoclonal anti-phospho-PHF-tau pThr231 (AT180) Abs were from Thermo Scientific (Rockford, IL); goat anti-mouse syndecan-3 Ab was from R&D Systems, Inc (Minneapolis, MN); rabbit anti-glypican-1 (M-95) Ab was from Santa Cruz Biotechnology, Inc (Santa Cruz, CA); polyclonal goat anti-rabbit IgG Nanogold, ϕ1.4 nm, was from Nanoprobes (Yaphank, NY); and horseradish peroxidase-conjugated goat anti-rabbit IgG was from Cell Signaling Technology, Inc. (Beverly, MA).

Animals

C57BL/6 mice were from Japan SLC Inc. (Hamamatsu, Japan). Heterozygotic transgenic mice that expressed the human amyloid precursor protein bearing the Swedish (K670N, M671L) mutation (Tg2576 strain),[18] the Swedish and Indiana (V717F) mutations (J20 strain),[19] or the Swedish and London (V717I) mutations (T41 strain)[20] were maintained in barrier facilities. Tg2576 mice were purchased from Taconic Farms, Inc., Hudson, NY. J20 mice were from the Jackson Laboratory (Bar Harbor, ME). The National Center of Geriatrics and Gerontology Institutional Animal Care and Use Committee approved the animal studies.

Human Postmortem Brain Tissues

Patients with sporadic AD received a pathological diagnosis according to the criteria of the Consortium to Establish a Registry for Alzheimer's Disease and the Braak stage. Non-demented controls were elderly patients who were age-matched and without significant neurological disorders. Patients were also cognitively evaluated by neuropsychological tests using the Mini-Mental State Examination and Hasegawa's dementia scale, which is commonly used in Japan. Entorhinal cortex and hippocampus postmortem tissue samples from neurologically unimpaired subjects (non-demented controls [NDCs]) and from subjects with AD were obtained under Committees on Human Research approval of National Center for Geriatrics and Gerontology and Choju Medical Institute of Fukushimura Hospital. Diagnosis of AD was confirmed by pathological and clinical criteria (Table 1). The incidence of vascular risk factors (eg, atherosclerosis, myocardial infarction, and so forth), the sex ratio, age, and the postmortem interval were comparable between NDC and AD (Table 1). Tissue was cut and frozen or fixed with formalin, and then embedded with paraffin. Frozen tissues were subjected to structural analysis of HS. The embedded tissues were cut using a microtome.

Table 1. Clinical and Neuropathological Characteristics of Alzheimer's Disease and Non-Demented Control Donor Patients used in the Disaccharide Composition Analysis of Heparan Sulfate

Patient number	Age (years)	Sex	Stage of amyloid deposits (0, A, B, C)*	NFT stage (I–VI)	Cerebral amyloid angiopathy	Vascular risk factors	PMI (hr)
Alzheimer's disease patients							
0508	94	F	C	V	+	CI	43
0512	83	F	C	VI	+	ATH	2
0604	91	F	C	V	–	CI	8
0805	93	F	C	VI	+	CI	27
0810	80	M	C	V	–	CI	15
0811	81	M	C	VI	–	–	8
0814	91	M	C	V	+	–	5
0824	87	F	B	VI	–	–	9
Age-matched non-demented controls							
0707	95	F	A	II	–	MI	4
0710	83	F	A	II	–	CH/CI	24
0601	90	F	B	II	–	MI	4
0802	93	F	A	III	–	CH/CI	20
0704	84	M	B	II	–	CI	3
0807	82	M	0	I	–	CH	8
0908	91	M	A	II	–	–	NA
0903	87	F	0	II	–	CI	7

*0 = none, A = rare or a few, B = mild or moderate, C = numerous or marked.
ATH, atherosclerosis; CH, cerebral hemorrhage; CI, cerebral infarction; F, female; M, male; MI, myocardial infarction; NA, not applicable; NFT, neurofibrillary tangle; PMI, postmortem interval.

Fractionation of Brain Samples

A snap-frozen mouse cortex (~25 mg) was placed in a tube containing 600 μL (30 volume of the tissue weight) of ice-cold Tris-buffered saline (TBS) (20 mmol/L Tris and 137 mmol/L NaCl, pH 7.6) and protease inhibitors (complete protease inhibitor cocktail; Roche Diagnostics, Mannheim, Germany). The tube was placed in a water bath of the Bioruptor ultrasonic vibration (CosmoBio, Tokyo). The tissue was fragmented by sonicating the tube for 15 seconds with the maximum ultrasonic wave output power 4 to 5 times until solid materials in the tube became invisible. The material was ultracentrifuged at 100,000 × *g* for 20 minutes at 4°C. The supernatant was collected and stored frozen as TBS or "TBS soluble fraction." The resulting precipitate was suspended in 600 μL (the same volume as previously described) of TBS containing 1% SDS. The suspension was centrifuged at 12,000 rpm for 20 minutes at room temperature. The resulting supernatant was collected and stored frozen as TBS or "TBS-insoluble/1% SDS-soluble fraction." The protein concentrations of both fractions were measured with a BCA Protein Assay Reagent Kit (Thermo Scientific). Brain cortices were dissected out from 3 Non-Tg or 3 Tg2576 18-month-old mice and then snap frozen. Brain samples were put together, placed on a glass Petri dish, and minced with a blade. The tissues were transferred into a tube containing 1 mL of ice-cold TBS. The tissues were homogenized with a Dounce homogenizer. The homogenate was filtered with a 100-μm nylon mesh. The filtered materials on the mesh were collected and then subjected to the structural analysis described as follows ("vessel-enriched fractions"). Materials filtered through the 100-μm nylon mesh were collected and then analyzed ("non-vasculature fractions"). Methylene blue staining and bright field microscopy confirmed cerebral blood vessels on the filters.

Immunohistochemistry

Fresh mouse brains were embedded in O.C.T. compound (Sakura Finetek, Torrance, CA) and frozen in liquid nitrogen. The brains were stored at −80°C until analysis. Cryostat-cut sections (10-μm thick) were prepared on MAS-coated glass slides (Matsunami, Osaka, Japan), fixed in ice-cold acetone for 15 minutes, and then air-dried for 30 minutes. Sections were incubated with blocking solution (3% bovine serum albumin in PBS) for 15 minutes at RT. Sections were washed twice with PBS and then incubated with a mixture of RB4CD12 (1:100 dilution), rabbit anti-laminin antibody (1:100 dilution, Sigma), and biotinylated 82E1 (1:50 dilution) overnight at 4°C. Then, primary antibodies were detected with Cy3-conjugated monoclonal anti-VSV-G (4 μg/mL), Cy2-conjugated polyclonal goat anti-rabbit IgG (3 μg/mL), and aminomethylcoumarin acetate-conjugated streptavidin (6.8 μg/mL, Jackson ImmunoResearch, West Grove, PA). Sections were mounted in FluorSave Reagent (Merck, Darmstadt, Germany). Digital images were captured by fluorescent microscopy (model BX50, Olympus, Tokyo, Japan) at the same setting for each antibody. The fluorescently stained area was quantitatively determined using Image-Pro Plus software (Media Cybernetics, Bethesda, MD). To determine the effects of the Sulfs and heparinases, 3% bovine serum albumin-blocked sections were pre-treated with 100 μL of a reaction mixture containing 5 μmol HEPES, pH 7.5, 1 μmol $MgCl_2$, and enzymes at 37°C overnight. Recombinant human Sulf-1 (0.4 μg) and human Sulf-2 (0.4 μg) were prepared from conditioned medium of transfected HEK293 cells and used as previously described.[8] For pretreatment with heparitinases, a mixture of 1 mU heparinase I, 0.25 mU heparinase II, and 0.1 mU heparinase III were added to the