Crystal Structure of the E2 Domain of Amyloid Precursor Protein-like Protein 1 in Complex with Sucrose Octasulfate*§

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Missense mutations in the amyloid precursor protein (APP) gene can cause familial Alzheimer disease. It is thought that APP and APP-like proteins (APLPs) may play a role in adhesion and signal transduction because their ectodomains interact with components of the extracellular matrix. Heparin binding induces dimerization of APP and APLPs. To help explain how these proteins interact with heparin, we have determined the crystal structure of the E2 domain of APLP1 in complex with sucrose octasulfate (SOS). A total of three SOS molecules are bound to the E2 dimer. Two SOSs are bound inside a narrow intersubdomain groove, and the third SOS is bound near the two-fold axis of the protein. Mutational analyses show that most residues interacting with SOS also contribute to heparin binding, although in varying degrees; a deep pocket, defined by His-376, Lys-422, and Arg-429, and an interfacial site between Lys-314 and its symmetry mate are most important in the binding of the negatively charged polysaccharide. Comparison with a lower resolution APP structure shows that all key heparin binding residues are conserved and identically positioned, suggesting that APLP1 and APP may bind heparin similarly. In transfected HEK-293 cells, mutating residues responsible for heparin binding causes little change in the proteolysis of APP by the secretases. However, mutating a pair of conserved basic residues (equivalent to Arg-414 and Arg-415 of APLP1) immediately adjacent to the heparin binding site affects both the maturation and the processing of APP.

Since the discovery of missense mutations in the gene coding for amyloid precursor protein (APP)20 years ago (e.g. Refs. 1 and 2), now more than 20 APP mutations have been documented that cause familial Alzheimer disease.3 APP is normally processed by β- and γ-secretases to give rise to a small peptide called Aβ (4). Because the senile plaque, of which Aβ is the major component (5), is one of the defining pathological features of Alzheimer disease, it has been hypothesized that Aβ is neurotoxic and can trigger biochemical changes in the brain that eventually lead to Alzheimer disease at old age (6). The disease-causing mutations in APP are all clustered around the Aβ region and appear to affect either the metabolism or the aggregation of Aβ (7).

APP and other members of its family (in mammals these include APLP1 and APLP2) are type 1 membrane proteins (8–10). The functions of APP and APLPs are not well understood. Mice lacking individual members of the family are viable and fertile (11–13). Only animals with double APP/APLP2 or APLP1/APLP2 knockouts or the triple knockouts show early postnatal lethality (12–14). The mice lacking APP/APLP2 seem to have defects in neuromuscular synapses (15), whereas the triple knock-out animals exhibit a high incidence of neuronal ectopia and other cranial abnormalities (14). These phenotypes could be related to the proposed function of APP family proteins in cell adhesion and signaling (16–18). At the cellular level, a subpopulation of APP molecules is localized to the plasma membrane, where they interact directly with components of the extracellular matrix (17). The large ectodomain of APP, which contains two heparin binding sites (19), can also be released from the membrane by proteolysis. The soluble ectodomain possesses growth-promoting activities and is often bound to the heparan sulfate (HS) present in the extracellular matrix and in circulation (20–22). Heparin and HS binding induces dimerization of APP and APLPs (23–26).

Because heparin modifies the aggregation and toxic properties of Aβ (27–29), there are interests in the medical field to use heparin as a therapeutic agent to treat Alzheimer disease (e.g. Ref. 27). Besides their direct interactions with Aβ, heparin and HS were also known to affect Aβ production itself (30–33). Some of the effects of heparin on Aβ production in cell cultures were thought to be due to its binding to β-secretase, which resulted in inhibition of the protease (33). Whether the binding of heparin and HS to APP might also influence the proteolytic processing of the precursor has not been studied before.

To help determine how APP family proteins interact with heparin and HS, we have solved the crystal structure of the E2 domain of APLP1 in complex with sucrose octasulfate (SOS) (see Fig. 1A). The E2 domain contains the higher affinity heparin binding site (19). Unlike the situation in a previous study (34), the ligand, as well as the protein side chains that interacted with it, has now been clearly defined in the electron density map. The crystal structure, combined with site-directed

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4 The abbreviations used are: APP, amyloid precursor protein; APLP, APP-like protein; Aβ, amyloid-β; HS, heparan sulfate; SOS, sucrose octasulfate; CTF, C-terminal fragment; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

5 J. Hardy and R. Crook, unpublished data.
mutagenesis, permitted the identification of all key heparin binding residues in the E2 domain. We also studied the effects of mutating some of these residues on the proteolytic processing of APP in cultured human embryonic kidney (HEK)-293 cells.

EXPERIMENTAL PROCEDURES

Reagents—The sodium salt of SOS was obtained from Toronto Research Chemicals Inc. The heparin disaccharides were obtained from Sigma–Aldrich (H9267 and H9392). Heparin was purchased from Santa Cruz Biotechnology (sc-203075).

Plasmid Construction and Protein Preparation—The APLP1285–494 fragment was amplified by PCR from human APLP1 cDNA (Open Biosystems, MHS1010-74392) and subcloned into pET28a vector (Novagen). The recombinant protein was expressed in BL21-Gold(DE3) cells (Agilent Technologies) in LB medium. The cells were induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 30°C. 0.5 and were left to grow overnight at room temperature. The cells were harvested and resuspended in the lysis buffer containing 10 mM sodium phosphate, pH 7.5, 0.5 mM NaCl, and EDTA-free protease inhibitor mixture (Roche Applied Science). The cells were lysed by freeze and thaw in the presence of lysozyme. The cell lysate was applied to a Talon metal affinity column (Clontech), and the recombinant protein was eluted with 200 mM imidazole. The N-terminal hexahistidine tag was removed by thrombin. The protein without His tag was concentrated and further purified on a Superdex S-200 column (GE Healthcare) in a buffer containing 20 mM HEPES, pH 7.5, 0.5 mM NaCl, and 5% glycerol. The recombinant protein was expressed in BL21-Gold(DE3) cells (Agilent Technologies) in LB medium. The cells were induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 30°C. 0.5 and were left to grow overnight at room temperature. The cells were harvested and resuspended in the lysis buffer containing 10 mM Tris, pH 8.0, 0.5 mM NaCl, and EDTA-free protease inhibitor mixture (Roche Applied Science). The cells were lysed by freeze and thaw in the presence of lysozyme. The cell lysate was applied to a Talon metal affinity column (Clontech), and the recombinant protein was eluted with 200 mM imidazole. The N-terminal hexahistidine tag was removed by thrombin. The protein without His tag was concentrated and further purified on a Superdex S-200 column (GE Healthcare) in a buffer containing 20 mM HEPES, pH 7.5, 0.5 mM NaCl, and 5% glycerol. The peak fraction was pooled and concentrated to 10 mg/mL and stored at −20 °C. All mutants were generated using the QuickChange® site-directed mutagenesis kit. They were expressed and purified similarly.

Crystallization and Structural Determination—APLP1285–494 was initially crystallized by the hanging-drop method. The reservoir solution contained 25% PEG 3350, 0.1 M Bis-Tris, pH 6.5, 0.2 M Li2SO4, and 25 mM ATP. Rod-shaped crystals usually grew to maximum size in 24 h and were harvested and resuspended in the lysis buffer of 20 mM sodium phosphate/acetate, pH 7.5, 0.5 M NaCl, and 5% glycerol. The peak fraction was pooled and concentrated to 10 mg/mL and stored at −20°C. All mutants were generated using the QuickChange® site-directed mutagenesis kit. They were expressed and purified similarly.

Heparin Binding Assay—After exchanging into a buffer of 10 mM sodium phosphate/acetate, pH 7.5, the protein solutions were loaded onto the 1-ml HiTrap heparin column (GE Healthcare) and eluted with a linear salt gradient (0–200 mM NaCl). The eluting NaCl concentrations were calculated based on the measured conductivity.

APP Processing in Transfected HEK-293 Cells—HEK-293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 4.5 g/liter glucose, 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin on a 6-well tissue culture dish. The human APP71 sequence was subcloned into pcDNA3.1(−) expression vector (Invitrogen) with an engineered C-terminal Myc tag. After reaching 70–80% confluency, the cells were transfected with the plasmid (1.5 μg of DNA/well) using Lipofectamine™ 2000 (5 μl/well) (Invitrogen). After 36 h, the transfected cells were harvested and lysed in a lysis buffer containing 10 mM Tris, pH 7.4, 150 mM NaCl, 0.1% sodium deoxycholate, 1% Nonidet P-40, and the Complete protease inhibitor mixture (Roche Applied Science). The cell lysates were resolved by
Crystallization solution (which contained 200 mM Li2SO4) and which contained an elongated central peak and eight peaks.

RESULTS

Overall Structure of the Complex—The new crystallization condition, which used PEG instead of phosphate salts as precipitant, was ideal for the heparin soaking experiment because the binding of heparin to E2 was inhibited at high salt concentrations. Direct soaking with a longer heparin compound unfortunately disrupted the crystal lattice. We therefore resorted to the shorter heparin disaccharides and SOS, a disaccharide that, like heparin, is heavily substituted with sulfate groups (Fig. 1B).

TABLE 1
Crystallographic statistics

|                     | Apo-APLP1 | Apo-APLP1-SOS |
|---------------------|-----------|--------------|
| Data collection     |           |              |
| Cell dimensions (Å) | a = 73.0, b = 83.0, c = 89.2 | a = 73.5, b = 82.2, c = 89.9 |
| Wavelength (Å)      | 1.071     | 1.075        |
| Resolution (Å)      | 40.0-2.2 (2.28-2.20) | 40.0-2.3 (2.38-2.30) |
| Refinements         | 205,043   | 339,192      |
| Redundancy          | 28,355    | 24,869       |
| Completeness (%)    | 99.9 (100.0) | 99.7 (99.4) |
| <I/σ>              | 13.2      | 13.6         |
| R_work, R_free     | 0.054 (0.389) | 0.084 (0.491) |

| Number of atoms     | 2,978     | 3,040        |
| Protein             | 10        | 165          |
| Sulfate ion         | 50        | 50           |
| SOS (A, B, C)       | 65        | 114, 97, 131 |
| Water               | 283       | 162          |

| B-factors           | 57        | 114, 97, 131 |
| Protein             | 50        | 50           |
| Sulfate ion         | 65        | 114, 97, 131 |
| SOS (A, B, C)       | 57        | 114, 97, 131 |
| Water               | 283       | 162          |

| r.m.s. deviations   | 0.006     | 0.012        |
| Bond lengths (Å)    | 1.04      | 1.47         |
| Bond angle (°)      | 0.7        | 0.7           |

Novex 16% Tricine gels (Invitrogen) and visualized by Western blot using mouse anti-c-Myc antibody (1:500) (Invitrogen) and goat anti-mouse alkaline phosphatase-conjugated secondary antibody (1:5,000) (Novagen). The mutants were generated using the QuikChange® site-directed mutagenesis kit and similarly expressed in HEK-293 cells.

Binding of SOS to the A and B Sites—The SOSs bound at the A and B sites were clearly defined in the electron density map (supplemental Fig. S1). Although the ligand was bound to the protein differently, the backbone of the disaccharides had similar conformations (supplemental Fig. S2A); the rotational angles around the two single bonds of the α(1→2) glycosidic linkage were similar so that the two sugar rings were superposable simultaneously. The SOS bound at the interfacial C site also appeared to adopt this conformation, although we were less certain about its modeling due to poor electron densities (supplemental Figs. S1C and S2B). The conformation of the sugar observed here had been observed before in other crystallographic studies of protein-SOS complexes (for example, see Ref. 40) (supplemental Fig. S2C).

The binding of SOS to the A site displaced the sulfate ion bound to Lys-422, Arg-425, and Arg-429 in the apo-structure (Fig. 2A). The sulfate group connected to the C2 atom of the glucose (Glc) moiety of SOS now occupied a nearby site and was hydrogen-bonded to Arg-425 and Arg-429. The same sulfate group also formed a hydrogen bond with His-426, whose side chain rotated 60° around the Cα-Cβ bond to move into the binding site. The sulfate group on C3 of the glucose was hydrogen-bonded to His-489, which was part of the C-terminal segment that was disordered in the apo-structure (Fig. 2C). The sulfate on glucose C4 was hydrogen-bonded to Arg-418, whose side chain was also disordered in the apo-structure. The sulfate on glucose C6 was hydrogen-bonded to Arg-418 and Lys-422. The fructose (Fru) moiety of SOS was raised to interact with residues from the N-terminal subdomain of E2. The sulfate on fructose C1 was hydrogen-bonded to His-426 and interacted with Lys-422 and the backbone amide group of His-307 via bridging water molecules. The sulfate on fructose C4 formed a hydrogen bond with Ser-305, and the sulfate on fructose C6 was hydrogen-bonded to Gln-387 and Lys-422. The sulfate on fructose C3 did not appear to engage in any direct interactions with the protein.

SOS was bound to the B site in a completely different mode (Fig. 2B). The disaccharide appeared to be flipped by 90°, and most of the interactions with the protein were mediated now through sulfate groups on the fructose moiety. The sulfate group on fructose C1 was hydrogen-bonded to Lys-422* and Arg-429* (the asterisk denotes the B-chain). The sulfate group on fructose C3 penetrated deep into the space between the two subdomains and formed a hydrogen bond with His-376*. The sulfate on fructose C3 also interacted with His-307* via a water molecule. The sulfate on fructose C4 interacted with the backbone amide of His-307* via a water molecule. The ester oxygen on fructose C6 was hydrogen-bonded to Lys-422*. The ring oxygen of the fructose also

around it, appeared in a nearby groove between the two subdomains of the B chain of the E2 dimer (the B site; Fig. 1D). Later refinement showed that the peripheral peaks corresponded to the eight sulfate groups of SOS. The difference density features in chain A (the A site) were more complex initially due to rearrangements in the protein structure (see below). The difference map also suggested that there was a third and weaker SOS binding site (the C site) at the interface between the two protein protomers (Fig. 1B).

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Apo-APLP1 was crystallized in space group P212121.
appeared to form a hydrogen bond with Lys-422*. The glucose moiety of SOS made only two direct interactions with the protein (between the sulfate group on C2 and Arg-429* and between the sulfate on C6 and Ser-305*). All other interactions were mediated through water molecules.

The non-crystallographic two-fold symmetry, which related the A and B sites, was not exact. In the apo-structure, it was already apparent that the A site was narrower than the B site as the result of a small “movement” of the αB helix. The binding of SOS to the A site caused αB to move further inwards (see below). Therefore, to accommodate SOS in a similar orientation at the wider B site would require a bigger movement in αB, which seemed to be restricted by crystal packing. On the other hand, the binding mode of SOS observed at the B site would cause steric clashes inside the narrower A site.

*Binding of SOS to the Interfacial C Site—Difference Fourier analysis suggested that there might be a third SOS molecule bound to the protein near the two-fold axis (Fig. 1B). Fig. 3A showed the features of the difference map in this region. Among the peaks shown, two (peaks 6 and 7) had intensities around 7σ, and three (peaks 1, 4, and 5) had intensities around 5σ. The intensities of the peaks and their proximity to each other were consistent with the possibility that they corresponded to sulfate groups on the same SOS molecule. The model shown in Fig. 3B represented the best fit that we could achieve in modeling SOS into the difference densities. The broken electron densities in the final 2Fo –Fc...
map suggested that the backbone of the sugar was probably flexible.

As evident from the distribution of the peaks in the difference map, the binding of SOS to the interfacial C site was highly asymmetrical. The center of the mass of the ligand was shifted toward the A chain and away from the two-fold axis (between the two His-433 residues in Fig. 3A). In our model, only two residues from the B chain made contacts with the ligand (Fig. 3B); Lys-314 was hydrogen-bonded to the sulfate on glucose C6, and His-433 was hydrogen-bonded to the sulfate group on fructose C6. The interactions between SOS and the protein at the C site were not as extensive as those in the A and B sites. The reason that the sugar was bound mostly to the A chain and in one orientation, instead of to the symmetry-related site in the B chain, seemed to be due to a potential clash with the SOS molecule bound at the B site; the sulfate group on fructose C3 of the B site SOS, which was also hydrogen-bonded to His-376 (Fig. 2B), would be too close to the sulfate group on glucose C4 of the C site SOS if the latter was bound to the B-chain.

Neither SOS nor heparin had an intrinsic two-fold symmetry. Therefore, the inability of the interfacial C site to simultaneously accommodate two SOS molecules (or heparin disaccharides) seemed to dictate that the binding of ligand to the E2 dimer had to be asymmetrical.

Conformational Changes Induced by SOS Binding—The binding of SOS to the protein caused little structural change in the B chain. The A chain, however, showed many subtle movements (supplemental Fig. S3A). The aB helix shifted 0.7 Å toward the C-terminal subdomain to position Ser-305 and His-307 closer to the ligand. The middle portion of the C chain that had become ordered in the crystal is shown in pink. SOS is shown in orange. Dashed lines represent hydrogen bonds.

FIGURE 3. Binding of SOS to the interfacial C site. A, a difference Fourier map (contoured at 3.5σ levels) reveals a cluster of positive peaks near the two-fold axis of the protein. B, a model of SOS bound at the C site. With the exception of the sulfate group attached to the C4 of glucose, all other sulfate groups were well defined in the difference map (peaks 1, 2, and 4–8 in A).
that the new position of αF, and possibly the ordered C-terminal segment, might represent the natural conformation of the protein in solution, which was not influenced by crystal packing and should therefore be more symmetrical.

The ordered C terminus of the protein folded back to interact mostly with residues on the αD helix (Fig. 2C). The side chain of Gln-421 was simultaneously hydrogen-bonded to the backbone amide and carbonyl groups of Leu-490; Arg-418 formed a hydrogen bond with the carbonyl oxygen of Leu-490 also, whereas Arg-414 was hydrogen-bonded to the carbonyl group of Gly-491. These interactions helped keep the main chain of the folded-back peptide by making three hydrogen bonds to its backbone; on the other hand, being positively charged, it stabilized the structure. The side chain of Arg-429 defined the left-hand side of the binding pocket directly across from His-307. It formed four hydrogen bonds with SOS at the B site. After Arg-429, mutations of Arg-418 and Arg-425 produced some moderate effects (reducing the salt concentration by 31 and 28%, respectively). The mutation of His-426, whose side chain made a rotation into the binding pocket to make two hydrogen bonds with the A site SOS, produced only a small effect (10%). Gln-387 (from αC), on the other hand, appeared to make no contribution to heparin binding.

The involvement of residues at the interfacial C site in heparin binding was confirmed by mutagenesis. Here the mutation of Lys-314 produced the second largest effect observed in our study (56%). In the SOS complex, although it only contributed one salt bridge to ligand binding, Lys-314 and its symmetry mate (Lys-314*) interacted simultaneously with the C site SOS from opposite sides of the sugar (Fig. 3B). Therefore, the large effect of the K314A mutation could be due to the loss of two side chains that interacted with the same heparin molecule. The mutations of Arg-369 and His-433, which were also close to the two-fold axis, affected heparin binding to lesser degrees (24 and 28%, respectively). The mutation of His-430 reduced heparin binding only moderately (20%).

The folded-back C terminus of the protein did not appear to make any positive contribution to heparin binding. As shown above, His-489 was the only residue from this part of the protein that made a hydrogen bond with SOS (Fig. 2C). The mutation of His-489 did not change the elution salt concentration of the protein from the heparin column. The mutation of Gln-421, whose side chain helped to anchor the C terminus on αD, actually increased the affinity of the protein for heparin slightly. Taken together, these results suggested that the folded back peptide interfered with heparin binding. Arg-414 appeared to have a dual role. On the one hand, like Gln-421, it stabilized the fold-back conformation of the peptide by making three hydrogen bonds to its backbone; on the other hand, being positively charged, it could also interact favorably with heparin after the peptide had moved away. The small reduction in the elution salt

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#### Summary of mutant data

The affinity for heparin was expressed empirically as the molar salt concentration required for eluting the protein from a heparin column. The percentages of the salt concentration relative to the wild-type (WT) value are shown in parentheses.

| Mutation          | [NaCl] m (%) |
|-------------------|--------------|
| N-terminal subdomain (A/B) |
| S305A             | 0.46 (66)    |
| H307A             | 0.45 (65)    |
| H376A             | 0.41 (58)    |
| C-terminal subdomain (A/B) |
| Q387A             | 0.66 (95)    |
| R418A             | 0.48 (69)    |
| K422A             | 0.22 (32)    |
| R425A             | 0.50 (72)    |
| H426A             | 0.63 (90)    |
| R429A*            | 0.38 (54)    |
| Dimeric interface (C) |
| K314A             | 0.31 (44)    |
| R369A*            | 0.53 (76)    |
| H430A             | 0.55 (79)    |
| H433A*            | 0.50 (72)    |
| C-terminal peptide |
| R414A             | 0.58 (83)    |
| Q421A             | 0.74 (107)   |
| H489A             | 0.68 (98)    |

* These three mutants had been described by Lee et al. (26).
concentration (17%) for the R414A mutant likely reflected a compromise between these two opposing effects.

The Heparin Binding Site Was Conserved—In Fig. 4A, the effects of the 16 single mutations on heparin binding were color-mapped onto the protein surface. Among these, 11 reduced the elution salt concentration of the protein from the heparin column by more than 20% (Fig. 4A, red to green). With the exceptions of Ser-305 and Arg-425, the other nine were conserved in APP and APLP2 (Fig. 4C, red and green). Ser-305 probably played a structural role (e.g. by capping the N terminus of aB, which could be substituted by Asn-367 of APP) instead of participating directly in ligand binding because it did not carry any positive charge. Arg-425 contributed only weakly to heparin binding and might be specific to APLP1.

A lower resolution crystal structure of APP had already been known (41). Although there were large differences between the APP and APLP1 structures, mostly in the angles between the two subdomains and between the two protein protomers (26), the region that was important for heparin binding remained similar (Fig. 4B). The conserved nine residues were similarly positioned in APP around a groove between the two subdomains and were not hindered by protein dimerization; Lys-376* and His-495* (equivalent to Lys-314* and His-433* of APLP1) from the symmetry-related molecule were also adjacent and exposed in the dimer to constitute a similar interfacial binding site.

The Effect of the Mutations on APP Processing—The complete mapping of the high affinity heparin binding site enabled us to examine whether binding of endogenous HS to the E2 domain of APP could influence APP processing. In HEK-293 and many other mammalian cell types, only a small fraction of the total pool of cellular APPs was processed by aB-secretase, which generated a membrane-bound C-terminal fragment (CTF) called C99; a larger fraction of the APP pool was processed by the competing g-secretase, which produced C83 (42). C99 and C83 were further cleaved by p-secretase to give rise to A and p3, respectively (Fig. 5A). To help visualize various APP species in intact HEK-293 cells, we engineered a Myc tag to the C terminus of APP751. Western blot analysis of the transfected cells using an anti-Myc antibody showed two sets of bands (Fig. 5B). The high molecular mass bands at 100 and 120 kDa corresponded to the immature and mature full-length APPs, respec-

FIGURE 4. Mutagenesis mapping of the heparin binding site. A, a surface representation of the heparin binding site in the A chain (for clarity, the B chain is not shown). The folded back C-terminal fragment is shown as sticks. Lys-314* and His-433* from the B chain (labeled in blue), which probably contributed to heparin binding to the A chain, are also shown as stick models. The effect of the mutations on heparin binding is represented by different colors, with red showing the largest reduction of the salt concentration required to elute the protein from the heparin column (>50%) and dark blue showing no obvious reduction. B, the CA trace of APP viewed from a similar angle (26). The nine conserved residues whose mutation reduced the affinity of APLP1 for heparin by more than 20% are shown as small spheres. The conserved Lys-476 and His-488 (equivalent to Arg-414 and His-426 of APLP1) are also shown. C, sequence alignment of the E2 domains of the three human APP family proteins. Conserved residues are highlighted. The 16 mutated APLP1 residues are highlighted in red, green, and blue, indicating their contributions to heparin binding (red being the most important).
tively (42); the dark band between the 10- and 15-kDa markers was C83, the major CTF generated by /H9251-secretase. The presence of the C-terminal Myc tag did not appear to alter the maturation and processing of APP. The effects of the well studied Swedish and Flemish disease mutations on APP processing were readily detectable by our assay (Fig. 5C). The Western blot showed that both mutations led to higher levels of C99 and that the Flemish mutation also increased the intensities of C89 (resulting from /H9252-site cleavage) and another band between C89 and C99.

We generated three double mutants inside the E2 heparin binding site (all on top of the Swedish mutation background for increased visibility of C99). The first mutant H369A/H438A eliminated two important side chains from the N-terminal subdomain (these residues corresponded to His-307 and His-376 of APLP1 in Fig. 4A). The second mutant K376A/H495A targeted the interfacial binding pocket (corresponding to Lys-314 and His-433 of APLP1). The third mutant R480A/K484A eliminated two positively charged side chains from the C-terminal subdomain (corresponding to Arg-418 and Lys-422 of APLP1). Western blot analysis showed that HEK-293 cells transfected with the double mutants accumulated similar levels of C83, C89, and C99 as the Swedish mutant alone (Fig. 5D). This result suggested that under our experimental conditions, the binding of endogenous HS to the E2 domain did not appear to affect the proteolytic processing of APP by α- and β-secretases.

Besides the three double mutants described above, we also mutated several other residues that were nearby. Like the three, most mutations (e.g. K491A) did not show any obvious effect in the cell culture experiment (Fig. 5E). The only exception was the double mutant K476A/K477A (these residues corresponded to Arg-414 and Arg-415 of APLP1). On Western blot, both immature and mature full-length APPs migrated consistently to higher molecular weights; the intensities of the CTFs were also much lower than the other mutants, suggesting greatly reduced processing by the secretases (Fig. 5E). Because in the APLP1-SOS crystal structure Arg-414 was involved in hydrogen bonding to the peptide backbone of the C terminus of the protein (Fig. 2C), we initially wondered whether the observed effect could result from a disruption of these interactions. However, mutating the equivalents of the other two residues (Arg-418 and Gln-421) that also interacted with the peptide backbone and mutating Lys-476 alone did not affect the mobilities of the full-length APP bands. Mutating Lys-477 alone also failed to produce any effect (Fig. 5E). Therefore, the observed differences in APP maturation and processing had to result from the simultaneous loss of both side chains of Lys-476 and Lys-477.
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DISCUSSION

The E2-SOS complex structure described here was more informative than a previous study in which SOS had been soaked into the crystals of the worm homolog APL-1 (34). In the worm structure, although the sugar was also found to bind between the two subdomains, the ligand was poorly defined in the electron density map. Only the glucose moiety of SOS was weakly visible; most of the sulfate substituents and the fructose moiety were disordered. Accordingly the ligand appeared to interact with only three protein side chains (they corresponded to Ser-305, His-307, and His-426 of APLP1). The crystal structure reported here suggested that at least 10 more residues could contribute to heparin binding. Furthermore, we recently showed that heparin binding induced E2 dimerization (26). The worm protein was crystallized as monomers and did not contain the binding pocket at the dimer interface, e.g., between Lys-314 and its symmetry mate. Therefore, the new crystal structure appeared to be a better mimic of the (E2)2-heparin complex. The mutagenesis data presented here also showed that residues located deep inside the intersubdomain groove (e.g., His-376) and near the two-fold symmetry axis of the protein (e.g., Lys-314), whose roles in ligand binding were not previously recognized, were some of the key players in heparin binding.

Three new conclusions about E2-heparin binding could be drawn from our current study. Firstly, heparin appeared to bind to two “hot spots” on the protein surface (Fig. 4A, red). The first hot spot was defined by His-376, Lys-422, and Arg-429 and coincided roughly with the A/B site of the E-SOS complex. His-376 and Arg-429 formed the opposing walls of a narrow groove between the two subdomains, whereas Lys-422 lay on the bottom of the groove near its opening toward solution. The second hot spot was between Lys-314 and its symmetry mate and coincided roughly with the C site of the E2-SOS complex. Secondly, the polysaccharide appeared to follow the inner contour of the groove that connected the two hot spots. One end of the sugar probably extended out from the groove and lay on top of Arg-418 and Arg-414; the other end of the sugar extended toward the two-fold symmetry axis, but the details there remained unclear. The simultaneous binding of heparin to two protein protomers near the symmetry axis was expected to strengthen dimerization. Thirdly, the E2 domain contained four histidine residues (His-307, His-376, His-426, and His-433) that were absolutely conserved in evolution. The mapped footprint of heparin covered all four histidines. Among the four, His-376 made the largest contribution to heparin binding at neutral pH. At acidic pH, protonation of the histidines was expected to increase the affinity between the protein and heparin. The conservation of the histidines suggested that this property could have important functional consequences.

Inhibition of endogenous HS synthesis caused increased Aβ secretion from cells (33). Because the binding of HS to the E2 domain could affect APP in ways that might hinder its cleavage by β-secretase (for example, the HS-induced dimerization could cause clustering of the molecule or retain the precursor at sites where local HS concentrations were high; HS was known to inhibit β-secretase activity (33)), we wanted to determine whether the loss of E2-HS interaction could contribute to the observed increase in Aβ secretion. The mapping of the E2 heparin binding site allowed us to approach this problem from a different angle by specifically mutating APP residues that were involved in heparin binding. We tested three double mutants, which covered all major regions of the protein surface that heparin contacted. Based on our Western blot analysis, none of the three mutants caused any change in APP processing in cultured HEK-293 cells. Therefore, our result seemed to be in agreement with the hypothesis that endogenous HS influenced Aβ production primarily through its direct inhibition of β-secretase (33).

We could not yet explain the effects of the K476A/K477A double mutation. For the wild-type protein, the immature 100-kDa band corresponded to the N-glycosylated full-length APP (42). The surprising shift of this band to a higher molecular weight suggested that either the mutant protein migrated abnormally on SDS gel, which seemed very unlikely, or its post-translational modification (e.g., glycosylation) was altered. Whatever the change was, it apparently did not affect the addition of O-linked sugars to the protein, which increased the molecular mass by another 20 kDa (the upper mature band) (42). However, the proteolytic processing of the mutant protein by both α-secretases and β-secretases was greatly reduced. This reduction was probably unrelated to heparin binding because the two lysines (equivalent to Arg-414 and Arg-415 of APLP1) were located on the periphery of the heparin binding site, and other heparin binding site mutants did not have this effect. It was also intriguing that the single mutants (K476A, K477A) behaved just like the wild-type protein. Further studies of the double mutant are obviously required to address the unanswered questions. These studies may reveal new mechanisms that regulate APP processing in cells.

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