Identification of Sites in Domain I of Perlecan That Regulate Heparan Sulfate Synthesis*

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Perlecan is primarily a heparan sulfate containing proteoglycan found in all basement membranes. Rotary shadowed images of perlecan show it to contain three glycosaminoglycan (GAG) side chains extending from one end of its core protein. Domain I is at the N terminus of perlecan and contains three closely spaced Ser-Gly-Asp sequences that may serve in GAG attachment. We evaluated the serines in these three sequences for GAG attachment by preparing a cDNA construct encoding for the N-terminal half (domains I, II, and III) of perlecan and then a series of constructs containing deletions and mutations within domain I of the domain I/II/III construct, expressing these constructs in COS-7 cells, and then analyzing the recombinant product for GAG side chains and GAG type. The results showed that all three serine residues in the Ser-Gly-Asp sequences in domain I can accept both chondroitin and heparan sulfate side chains but that a cluster of acidic residues N-terminal to these sequences is the primary determinant responsible for targeting these sites for heparan sulfate. Furthermore, there are two elements that can enhance heparan sulfate synthesis at a targeted site: 1) the presence of a the SEA module in the C-terminal region of domain I and 2) the presence of multiple acceptors in close proximity. These results indicate that the proportion of heparan and chondroitin sulfate at any one site in domain I of perlecan is regulated by multiple factors.

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1 The abbreviations used are: GAG, glycosaminoglycan; DI, II, and II, domains I, II, and II, respectively; DW, distilled water; SEA, sperm enterokinase agrin.
MATERIALS AND METHODS
Preparation of cDNA Constructs—cDNA constructs encoding for perlecan were prepared from cDNA clones 16 and 54 (3) and a cDNA clone encoding domain III (23) in pBluescript II SK+ (Stratagene) by using restriction enzyme sites in the multicloning region of the vector and the inserts. HindIII linkers (New England Biolabs) were used to add HindIII sites at different locations but in the same reading frame to provide for interchangeability of some constructs and facilitate construction. Short cDNA segments (35–65 base pairs), used to mutagenize specific amino acids in perlecan’s sequence, were prepared by synthesizing (Oligos, Etc.) upper and lower strands that, when annealed together, would produce a cDNA encoding for the required amino acid sequence and containing the desired overhang at the ends for in-frame ligation into unique restriction sites in the insert. Ntot and XbaI sites at the 5’ and 3’ ends, respectively, of constructs used for ligation were used to fuse the vector into the multicloning site of the pRc/CMV expression vector.

All constructs contained 31 bases of untranslated sequence 5’ to the methionine start codon of perlecan’s signal peptide to initiate protein synthesis and sequence encoding perlecan’s signal peptide to target the product for secretion. Designating the start methionine in the signal peptide as amino acid 1, the domain I construct (D I) encoded amino acids 1–195, the domain I/II construct (D I/II) encoded amino acids 1–484, and the domain I/II/III construct (D I/II/III) encoded amino acids 1–1680 (Fig. 1). A previously (23) prepared 140 base pairs of cDNA encoding perlecan’s signal peptide followed by the first 14 amino acids of domain I and containing a HindIII site at its 3’ end for in-frame ligation was used to add a signal peptide to domain II. The domain II/III construct encoded for amino acids 1–55 and 195–1680 with the addition of six acidic amino acids (Pro-Ser-Leu) between amino acids 35 and 52 and 52–1680 because of the construction of the HindIII sites. A number of constructs were made with a deletion or with a deletion and mutations in domain I but containing all of domains II and III. The ALT 1 construct encoded amino acids 1–88 and 195–1680 with the addition of Ser-Leu between amino acids 88 and 195. This construct deleted 110 amino acids from the C terminus of domain I but retained the signal peptide and 63 amino acids of the N-terminal sequence of domain I that contain the three Ser-Gly-Asp sequences (Fig. 1). The ALT 2 construct was identical to ALT 1, except that the serines in the three Ser-Gly-Asp sequences at amino acids 65, 71, and 76 were mutated to threonine (Fig. 1). The SER 1, SER 2, and SER 3 constructs were also identical to the ALT 1 construct except that the serines at residues 71 and 76 were mutated to threonine for SER 1, the serines at residues 65 and 76 were mutated to threonine for SER 2, and the serines at residues 65 and 71 were mutated to threonine for SER 3. The ALT 3 construct encoded amino acids 1–35 and 52–1680 with Asp-Pro-Ser-Leu between amino acids 35 and 52 and 52–1680 because of the construction of the three Ser-Gly-Asp sequences (Fig. 1). The ALT 2 construct was identical to ALT 1, except that the serines in the three Ser-Gly-Asp sequences at amino acids 65, 71, and 76 were mutated to threonine (Fig. 1). The ALT 5, 6, and 7 constructs were also identical to the ALT 3 construct except that they contained either 2, 4, or 6 additional amino acids (selected from residues 58–63 of ALT 4) inserted between amino acids 63 and 64 of the ALT 3 construct (Fig. 1).

Transfection of Western Blot Analysis—COS-7 cells cultured in Dulbecco’s modified Eagle’s medium containing high glucose, 10% fetal bovine serum, and 1.85 g of sodium bicarbonate/liter were used for transfection. Constructs in the pRc/CMV vector were transfected into bovine serum, and 1.85 g of sodium bicarbonate/liter were used for becco’s modified Eagle’s medium containing high glucose, 10% fetal...
Transfection for $^{35}$SO$_4$ Radiolabeling and GAG Characterization—

COS-7 cells in T-75 flasks were transfected with 20 μg of DNA/120 μl of LipofectAMINE/14 ml of serum-free medium for 6 h. After overnight culture in regular medium, the cells are cultured for 40 h in 12 ml of regular medium containing 600 μCi of $^{35}$SO$_4$ to radiolabel the GAG side chains on the recombinant product. The radiolabeled medium was removed from the flask and any debris removed by low speed centrifugation. The radiolabeled recombinant protein was captured by overnight mixing at 4 °C with 125 μl of packed protein G-Sepharose beads (Pharmacia Biotech Inc.) that had been previously absorbed with 100 μl of rabbit antiserum to perlecan (25). The material bound to the beads was eluted with 2.0 ml of buffered (0.05 M Tris, pH 6.8) 4 M guanidine HCl at 50 °C for 1 h, and any remaining unincorporated $^{35}$SO$_4$ was removed by chromatography on PD-10 columns (Pharmacia) in buffered 4 M guanidine HCl. The macromolecular material from the PD-10 column was then fractionated on Superose 6 in buffered 4 M guanidine. Aliquots of each tube were taken for measurement of radioactivity by liquid scintillation counting. Each construct was used in three separate transfections and $^{35}$SO$_4$ radiolabeling. The results were similar, and the Superose 6 profile was only one transfection for each construct shown.

Fractions from Superose 6 chromatography containing radioactivity were pooled, dialyzed against DW, lyophilized, and reconstituted in — ml of DW. GAG side chains were released by treatment with 1 x sodium borohydride, 0.05 x NaOH at 45 °C for 40 h. The proportion of heparan and chondroitin sulfate was determined by digestion with nitrous acid or chondroitinase ABC, followed by precipitation of the macromolecular material (undigested) with 2 volumes of 95% ethanol containing 1% potassium acetate and measuring digested material in the supernatant by liquid scintillation counting. The proportion was also determined by chromatography of undigested and chondroitinase-digested material on Superose 6 and calculating the proportion of radioactivity shifting to low molecular weight (digested).

A construct encoding for domain I was ligated in-frame to maltose-binding protein in the pMAL C2 vector (New England Biolabs) and expressed according to the manufacturer’s instructions. Four constructs (domain I/II/III, domain I/II, domain II/III, and domain I) were also transfected into HT 1080 cells, and stably transfected cell lines were selected and analyzed for recombinant product.

RESULTS

The constructs were transfected into COS-7 cells and the media examined for expression of recombinant protein by SDS-polyacrylamide gel electrophoresis followed by Western blot using antiserum to native perlecan. The products of the domain I construct and the domain I/II construct could not be consistently or clearly detected in either COS-7 cells or HT 1080 cells, although mRNA for these constructs could be detected in Northern blots of RNA from transiently transfected cells (data not shown). A derivative of the domain I construct did, however, express in prokaryotic cells as a fusion protein with maltose-binding protein, and the antiserum to perlecan readily reacted with recombinant product (data not shown). This indicates that the inability to detect the recombinant product of the domain I and domain I/II constructs in eukaryotic cells was not due to the lack of antibodies to these domains in the antiserum to native perlecan. These constructs were considered to be poorly expressed in eukaryotic cells, and their products were not further characterized.

COS-7 cells transfected with the domain I/II/III construct did produce a immunoreactive recombinant product that appeared as a sharp band (arrowhead) just above the 213-kDa marker and a prominent broad band of higher molecular mass (Fig. 2, lane 1). Digestion with heparitinase and chondroitinase prior to electrophoresis shifted the migration position of the band broad to that of the sharp band just above the 213-kDa marker (Fig. 2, lane 2). This shift to a lower molecular mass upon digestion indicates the broad band product of the domain I/II/III construct is a proteoglycan. The sharp band (arrowhead, lane 1) did not shift upon digestion (lane 2). This indicates that a portion of the product of the domain I/II/III construct lacks GAG side chains. Digestion also revealed a sharp band of doublet of high molecular mass (vertical bar, lane 2). This band is also produced by digestion of medium from cells transfected with the pRe/CMV vector lacking any insert (Fig. 2, lane 5) and is likely the native perlecan core protein (estimated at 400 kDa) produced by the COS-7 cells. The product of the domain I/II/III construct was detected as a band at 213 kDa (Fig. 2, lane 3) that did not change in appearance when digested with heparitinase and chondroitinase (Fig. 2, lane 4). HT 1080 cells permanently transfected with the domain I/II/III construct produced a recombinant product that behaved similarly (data not shown). This indicates that product of the domain I/II/III construct lacks GAG side chains. These data indicate domain I can accept GAG side chains.

The presence or absence of GAG side chains on recombinant products was also determined by biosynthetically radiolabeling transfected COS-7 cells with $^{35}$SO$_4$ immunoprecipitating the recombinant protein from the medium with antiserum to perlecan, and then chromatographing the solubilized material on a column of Superose 6. Chromatography showed the product of domain I/II/III construct was radiolabeled with $^{35}$SO$_4$ (Fig. 3A). Cells transfected with the domain I/II/III construct, however, produced the same levels of immunoprecipitated $^{35}$SO$_4$-radiolabeled product as cells transfected with the pRe/CMV vector lacking an insert (Fig. 3A). This confirms the Western blot data obtained for these constructs (Fig. 2). Characterization of the GAG type on the $^{35}$SO$_4$-radiolabeled products of the domain I/II/III construct showed it contained 73–81% heparan sulfate (Table 1). HT 1080 cells permanently transfected with the domain I/II/III construct produced a recombinant product containing 71–80% heparan sulfate.

Domain I/II/III constructs with deletions and mutations of amino acids within domain I were prepared to identify the sites in domain I that are involved in GAG attachment. The first of these constructs (ALT 1) contained a deletion of approximately 2/3 of the C terminus of domain I (Fig. 1). Transfection of COS-7 cells with the ALT 1 construct produced an immunoreactive product as a sharp band at the 213-kDa marker and a prominent broad band of higher molecular mass (Fig. 4, lane 1). Digestion with heparitinase and chondroitinase shifted the migration position to coincide with the band at 213 kDa (Fig. 4, lane 2). This indicates that a substantial amount of the ALT 1 product was produced as a proteoglycan. Another construct (ALT 2) was prepared that was identical to the ALT 1 construct except that the serines at amino acids 65, 71, and 76, which are in the three Ser-Gly-Asp sequences in domain I, were mutated to threonines (Fig. 1). Transfection of COS-7 cells with the ALT 2 construct produced an immunoreactive product as a prominent band at 213 kDa (Fig. 4, lane 3), and digestion with heparitinase and chondroitinase did not change the migration position of the product (Fig. 4, lane 4) but did reveal the high molecular weight core protein the native perlecan produced by the COS cells as seen in lane 5. This indicates that the serines
in the three Ser-Gly-Asp sequences in domain I accept GAG chains.

The presence or absence of GAG side chains on the recombinant products of the ALT 1 and ALT 2 constructs was also determined by metabolic radiolabeling with $^{35}$SO$_4$ immunoprecipitation, and chromatography on Superose 6. The product of the ALT 1 construct contained incorporated $^{35}$SO$_4$, but transfection with the ALT 2 construct resulted in levels of incorporated $^{35}$SO$_4$ similar to that produced by transfection with the pRc/CMV vector lacking insert (Fig. 3B). These data confirm the conclusions made from the results of the Western blot experiments (Fig. 4) with the ALT 1 and ALT 2 constructs. Characterization of the GAG type on the $^{35}$SO$_4$-radiolabeled product of the ALT 1 construct indicates it contained 61–62% heparan sulfate (Table I).

The next set of constructs focused on the serine residues in each of the Ser-Gly-Asp sequences, individually. The SER constructs were identical to the ALT 1 construct except that the serine residues in two of three Ser-Gly-Asp sequences were mutated to threonine in each construct (Fig.1). The SER 1, SER 2, and SER 3 constructs each retained the serines at residue 65, 71, and 76, respectively, while mutating the other two serine residues to threonines. Transfection of COS-7 cells with these constructs produced an immunoreactive product that appeared as a doublet above the 213-kDa marker with the upper band of the doublet being the more prominent (Fig. 5, lanes 1, 3, and 5). Digestion of the product of these constructs with heparitinase and chondroitinase ABC shifted the migration positions of the upper band to that of the lower band of the doublet (Fig. 5, lanes 2, 4, and 6). This indicates that a major portion of the product of each of these three constructs was produced as a proteoglycan and that the serines in all three Ser-Gly-Asp sequences accept GAG chains. Chromatography $^{35}$SO$_4$-radiolabeled recombinant products produced from the SER 1, SER 2, and SER 3 constructs confirmed their production as a proteoglycan (Fig. 3C). Characterization of the GAG type on these recombinant products showed the SER 1 product to contain 47–59% heparan sulfate, the SER 2 product to contain 32–37% heparan sulfate, and the SER 3 product to contain 23–30% heparan sulfate (Table I). Another set of constructs focused on sequence in domain I that is N-terminal to the three Ser-Gly-Asp sequences. The ALT 3 construct encoded for all of domains II and III but contained a deletion of 16 amino acids in the N-terminal region of domain I (Fig. 1). The ALT 4 construct was identical to the ALT 3 construct except the six acidic residues at positions 55–58, 62, and 63 were changed to polar amino acids (Fig. 1). Transfection of COS-7 cells with the ALT 3 construct produced an immunoreactive product that appeared as a broad band of 213 kDa and higher molecular mass (Fig. 6, lane 1). The prod-

Fig. 3. Chromatography of $^{35}$SO$_4$-radiolabeled recombinant products on Superose 6. COS-7 cells were transfected and metabolically radiolabeled with $^{35}$SO$_4$. The recombinant product was isolated from the medium using antiserum to perlecan coupled to protein G and applied to a column of Superose 6. Cells were transfected with: A, domain I/II/III construct in pRc/CMV (solid triangles), domain I/II construct in pRc/CMV (open squares), or pRc/CMV vector (open circles); B, ALT 1 construct in pRc/CMV (solid triangles), ALT 2 construct in pRc/CMV (open squares), or pRc/CMV vector (solid circles); C, SER 1 construct in pRc/CMV (open squares), SER 2 construct in pRc/CMV (open squares), SER 3 construct in pRc/CMV (open squares), or the pRc/CMV vector (solid circles); D, ALT 3 construct in pRc/CMV (open circles), ALT 4 construct in pRc/CMV (solid squares), or the pRc/CMV vector (open triangles); E, ALT 5 construct in pRc/CMV (open squares), ALT 6 construct in pRc/CMV (solid squares), ALT 7 construct in pRc/CMV (solid triangles) or pRc/CMV vector (open triangles). Tubes 16–19 were pooled in panels A, B, D, and E, and tubes 17–20 in panel C, and were used to determine GAG composition.
ing with $^{35}$SO$_4$ followed by immunoprecipitation and chromatography on Superose 6 showed the product of all three constructs except that either 2 amino acids (ALT 5), 4 amino acids (ALT 6), or 6 amino acids (ALT 7) were added between residues 63 and 64 of the ALT 3 construct (Fig. 1) to increase the distance between the six acidic residues and the first serine.

The last set of constructs were identical to the ALT 3 construct except that either 2 amino acids (ALT 5), 4 amino acids (ALT 6), or 6 amino acids (ALT 7) were added between residues 63 and 64 of the ALT 3 construct (Fig. 1) to increase the distance between the six acidic residues and the first serine used in GAG synthesis. Western blots of transfected cells, however, the level of $^{35}$SO$_4$ incorporated into the domain of the ALT 4 construct (Fig. 6, lane 3) migrated similarly to that seen for the product of the ALT 3 construct. Digestion of the product of both the ALT 3 and ALT 4 constructs produced an immunoreactive band that was more narrowly focused at 213 kDa (Fig. 6, lanes 2 and 4, respectively). This indicates that a portion of the products of both the ALT 3 and ALT 4 constructs was produced as a proteoglycan. Chromatography of $^{35}$SO$_4$-radiolabeled recombinant products produced from ALT 3 and ALT 4 constructs confirmed their production as a proteoglycan (Fig. 3D). Characterization of the GAG type on the ALT 3 and ALT 4 constructs produced is described in the following sections.

The results of this study show that each of the serines in the three Ser-Gly-Asp sequences in domain I of perlecan accepts GAG chains. This is not unprecedented, since a portion of the native perlecan produced by colon carcinoma cells was also shown to lack GAG side chains (26). In the transfected COS-7 cells, however, the level of $^{35}$SO$_4$ incorporated into the domain I/II/III construct (this report) and the domain III construct (23) are not made as proteoglycans. This indicates that the site in domain III does not accept GAG chains.

A detectable portion of the recombinant product derived from all constructs examined did not change migration position upon digestion with heparitinase and chondroitinase ABC in the Western blots, indicating that it lacked or had very short GAG side chains. This is not unprecedented, since a portion of the native perlecan produced by colon carcinoma cells was also shown to lack GAG side chains (26). In the transfected COS-7 cells, however, the level of $^{35}$SO$_4$ incorporated into the domain I/II/III construct was calculated to be 45-fold higher than that found in native perlecan (Fig. 3A). The GAG synthesis system may be saturated to capacity under these conditions, and, as a result, some recombinant product may pass through the endoplasmic reticulum and Golgi with very little or without posttranslational modification.

The recombinant product of the domain I/II/III construct received the highest (73–81%) heparan sulfate content. Changes in the construct reduced the heparan sulfate content and correspondingly increased the chondroitin sulfate content of the resulting recombinant product. The ALT 1 construct was prepared by deleting 110 amino acids from C-terminal end of domain I (approximately 2/3 of domain I). This region of domain I has recently been shown to contain a SEA module, a certain sequence of $\sim$80 amino acids that is also found in agrin, a heparan sulfate proteoglycan with homology to perlecan, as

### DISCUSSION

The results of this study show that each of the serines in the three Ser-Gly-Asp sequences in domain I of perlecan accepts GAG side chains. Mutating all three serines to threonines abolished the GAG-accepting ability of the recombinant product. Mutating any two of the three serines, however, still allowed the recombinant product to be produced as a proteoglycan containing a single GAG chain of either heparan or chondroitin sulfate. There are no other Ser-Gly-Asp sequences in the domains tested in this report, but there is a Ser-Gly-Glu sequence in the cysteine-rich epidermal growth factor-like repeat region of domain III. The recombinant products of the domain II/III construct (this report) and the domain III construct (23) are not made as proteoglycans. This indicates that the site in domain III does not accept GAG chains.

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| Construct | GAG type | Heparan sulfate |
|-----------|----------|----------------|
|           | Chondroitin sulfate | % | % |
| D I/II/III | 19–27 | 73–81 |
| D II/III | 38–39 | 61–62 |
| ALT 1 | SER 1 | 41–53 | 47–59 |
| ALT 1 | SER 2 | 63–66 | 32–37 |
| ALT 2 | SER 3 | 70–77 | 23–30 |
| ALT 3 | ALT 4 | 29–33 | 67–71 |
| ALT 5 | ALT 6 | 100 | 0 |
| ALT 6 | 28–38 | 62–72 |
| ALT 7 | 32–50 | 50–68 |
| D I/II/III | 33–42 | 58–67 |
| D I/II/III | 32–50 | 50–68 |
| ALT 3 | ALT 4 | 100 | 0 |

### FIG. 5. Western blot of medium from COS-7 cells transfected with the ALT 1 and ALT 4 constructs both in the pRc/CMV vector and with the vector (pRc). An aliquot of medium from each construct was also digested (+) with heparitinase and chondroitinase ABC combined (Hepase & Case) before electrophoresis. The recombinant products were detected with antiserum to perlecan.
well as in other proteins (4). The SEA module is associated with regions receiving extensive O-glycosylation, but its function is not known (4). Deleting the SEA module from perlecan reduced the heparan sulfate content of the recombinant product to 61–62%. This could indicate that the SEA module enhances heparan sulfate attachment to the three serine residues. These results suggest that sequence in non-GAG-binding regions of the core protein can influence the utilization of GAG attachment sites. Sequences in the non-GAG-binding regions of syndecan I have also been proposed to influence GAG composition, although they have not been identified (27).

Mutating two of the three GAG attachment serines altered both the elution position and the GAG composition of the recombinant product. The $^{35}$SO$_4$-radiolabeled recombinant products from the SER 1, SER 2, and SER 3 constructs eluted one tube later than the $^{35}$SO$_4$-radiolabeled recombinant product of the ALT 1 construct (Fig. 3, B and C). This indicates the recombinant products of the SER constructs are smaller than the product of the ALT 1 construct. This smaller size is likely due to the presence of only one GAG chain on the recombinant product of each of the SER constructs compared to the presence of three GAG chains on the recombinant product of the ALT 1 construct. Although the size of the recombinant products of the SER constructs were similar, their GAG compositions were different; the serine at residue 65 received 47–59% heparan sulfate, the serine at residue 71 received 32–37% heparan sulfate, and the serine at residue 76 received 23–30% heparan sulfate. Averaging the GAG composition for these three recombinant products gave a value of only 38% heparan sulfate. This is substantially less than that 61–62% heparan sulfate obtained for the recombinant product of the ALT 1 construct, which has all three serine acceptor sites. This suggests that the three Ser-Gly-Asp sites act synergistically to enhance heparan sulfate addition.

Sequence in syndecan 1 was also found to have three closely spaced Ser-Gly sites that act synergistically to enhance heparan sulfate synthesis (19). Three sites in syndecan, however, only received 60% heparan sulfate, which is considerably less than the maximum of 81% we achieved with the three sites in perlecan with the domain I/II/III construct (Table I). This may be due to the different spacing of the three acceptor sites in syndecan and perlecan. Alternatively, the higher levels of heparan sulfate priming in the domain I/II/III protein may be due to the presence of heparan sulfate enhancing elements, such as the SEA module, in the recombinant product. In support of this, fusing protein A to the 25 amino acid sequence from perlecan containing only the three acceptor sites and the adjacent cluster of acidic amino acids resulted in a recombinant product that received only 64% heparan sulfate (19). These observations support our hypothesis that the SEA module enhances heparan sulfate synthesis. Another laboratory (22), however, expressed a construct containing primarily domain I of perlecan (amino acids 1–198 plus 6 histidine residues) and found the recombinant product contained only 45% heparan sulfate. Since this construct contains the SEA module but lacks domains II and III, it may be that the SEA modules acts in concert with domains II and III to enhance heparan sulfate synthesis, or that the histidine residues added to domain I act to reduce heparan sulfate priming.

The deletion of 16 amino acids from the N-terminal region of domain I in our ALT 3 construct reduced the heparan sulfate content of the recombinant product but only to 67–71%, and this may not be significant. Mutating the aspartic and glutamic acids at residues 55–58, 62, and 63 to asparagines and glutamines (ALT 4 construct) did not alter the GAG accepting capacity of the serine residues in the three Ser-Gly-Asp sequences, but changed the GAG composition of recombinant product to be 100% chondroitin sulfate. This change from 30% chondroitin sulfate to 100% chondroitin sulfate occurred in the presence of the SEA module and on multiple acceptors in close proximity. These results indicate that the acidic residues are primary determinant responsible for targeting the serine residues in the three Ser-Gly-Asp sequences for heparan sulfate and that the multiple acceptors and the SEA module act only as enhancing elements. There are no tryptophan residues near acceptor serines in perlecan that could enhance heparan sulfate synthesis as there is in betaglycan (18). The closest tryptophan to the acceptor serines is over 60 residues away, but it is located in the SEA module and that enhances heparan sulfate synthesis.

The acidic amino acids at residues 55–58 are 7, 8, 9, and 10 residues away from the first GAG priming serine (residue 65) in perlecan. Sequence in betaglycan was also shown to contain acidic amino acids 7, 8, and 10 amino acids away from serine used for heparan sulfate priming (18). The acidic residues in betaglycan, however, are C-terminal to the serine, while the acidic residues in perlecan are N-terminal to the serine. Moving the acid residues in betaglycan closer to the serine or deleting the residues reduced heparan sulfate priming (18). In perlecan, there is a correlation between the number of acidic residues, their distance from an attachment site, and the percentage of heparan sulfate synthesized at that site in the SER 1, 2, and 3 constructs. The serine at position 65 received 47–59% heparan sulfate and has acidic residues 7, 8, 9, and 10 residues away. The serine at position 71 received 32–37% heparan sulfate and has acidic residues 8 and 9 residues away. The serine at position 76 received 23–30% heparan sulfate and has one acidic residue 9 residues away (Fig. 1). Moving the acidic residues in perlecan 2, 4, or 6 amino acids away from the first serine (ALT 5, 6, and 7) resulted in only a small reduction in heparan sulfate synthesis (Table I), but this may be due to the fact that the four acidic residues at positions 55–58 were essentially replaced by the two acidic residues at 62 and 63 by the addition of the 6 amino acids in the ALT 7 construct (see Fig. 1). Nevertheless, these results taken together indicate that it is not the direction of the acidic group from the priming site but the number of residues and a minimum distance (~7 residues) that is important in regulating heparan sulfate priming. The maximum distance has yet to be determined, and this may be difficult because protein folding could possibly bring a far away cluster of acid residues closer to the serine.

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