Repellative Ser-Gly Sequences Enhance Heparan Sulfate Assembly in Proteoglycans*

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We showed previously that the synthesis of heparan sulfate on betaglycan occurs at a Ser-Gly dipeptide flanked by a cluster of acidic residues and an adjacent tryptophan (Zhang, L., and Esko, J. D. (1994) J. Biol. Chem. 269, 19295–19299). A survey of the protein data base revealed that most heparan sulfate proteoglycans contain repetitive (Ser-Gly)2 segments (n = 2) and a nearby cluster of acidic residues. To study the role of these amino acid sequences in controlling heparan sulfate synthesis, we have examined the assembly of glycosaminoglycans on Chinese hamster ovary (CHO) cell syndecan-1. The glycosylation sites were mapped by making chimeric proteoglycans containing segments of CHO syndecan-1 cDNA fused to Protein A. Two sites near the transmembrane domain (EGS205GEN- and ETS215GEN-) were used solely for chondroitin sulfate synthesis, whereas three sites near the N terminus (DGS39GDSDNF55GS57GTG-) supported both heparan sulfate and chondroitin sulfate synthesis. The strongest sites for heparan sulfate synthesis consisted of the repeat unit, -S45GS47G-. An unusual coupling phenomenon occurred across the adjacent SG dipeptides, leading to a greater proportion of heparan sulfate than predicted by the behavior of each site acting independently. The clusters of acidic residues adjacent to the heparan sulfate sites play important roles as well. These sequence motifs suggest a set of rules for predicting heparan sulfate assembly at glycosylation sites in proteoglycan core proteins.

Chondroitin sulfate and heparan sulfate proteoglycans contain glycosaminoglycan (GAG)2 chains attached to specific serine residues of core proteins. Studies of hybrid proteoglycans2 such as betaglycan, rydocan, and syndecan-1 showed that two types of GAG attachment sites exist (1-4). One type carries heparan sulfate and chondroitin sulfate chains, whereas the other type carries only chondroitin sulfate. In the proteoglycan, betaglycan, a specific amino acid sequence drives heparan sulfate synthesis (2). This site consists of a Ser-Gly dipeptide, a nearby cluster of acidic residues, and an adjacent tryptophan that augments the proportion of heparan sulfate made (2). These structural elements may enhance the interaction of glycosylated core protein intermediates with a key α-GlcNAc transferase that initiates the formation of heparan sulfate (5, 6).

Almost all cloned heparan sulfate proteoglycans contain a cluster of acidic residues near one or more putative heparan sulfate attachment sites, but only a few contain nearby tryptophan. To study whether other amino acid sequences surrounding GAG attachment sites might enhance heparan sulfate assembly, we have examined the assembly of GAGs on syndecan-1, a hybrid proteoglycan containing both heparan sulfate and chondroitin sulfate (7). Kokenyesi and Bernfield (4) showed recently that the N-terminal half of mouse syndecan-1 contains heparan sulfate and chondroitin sulfate chains, whereas the C-terminal half contains only chondroitin sulfate. Five conserved Ser-Gly dipeptides may act as the sites for GAG addition, but the identity of the sites that prime heparan sulfate was not determined. Our study reveals that most of the heparan sulfate occurs at an attachment site containing (Ser-Gly)2 through an unusual coupling mechanism. This sequence motif occurs frequently in heparan sulfate proteoglycans and always includes a cluster of acidic amino acids flanking the site. We also show that the biosynthetic capacity of the cell to make heparan sulfate and the amount of core protein expression affect the proportion of heparan sulfate assembled.

EXPERIMENTAL PROCEDURES

Cell Culture—Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (CCL-61, Rockville, MD). Cells were grown in Ham’s F12 medium supplemented with 7.5% (v/v) fetal bovine serum (HyClone, Salt Lake City, UT), penicillin G (100 units/ml), and streptomycin sulfate (100 μg/ml) under an atmosphere of 5% CO2, 95% air, and 100% relative humidity. Cells were passaged with trypsin every 3–4 days and revived periodically from frozen stocks. Sulfate-free F12 medium was prepared with chloride salts instead of sulfate (8), penicillin G (100 units/ml), and 10% (v/v) fetal bovine serum, or with sodium sulfate that had been exhaustively dialyzed against phosphate-buffered saline (PBS) (9). Radioiodination Studies—Na235SO4 (25–40 Ci/mg; 1 Ci = 37 GBq) was purchased from Amersham Corp. Syndecan-1 was labeled biosynthetically by incubating cells in sulfate-deficient medium containing 35SO4 (100 μCi/ml) for 12 h. The medium was discarded, the radiolabeled monolayer was rinsed five times with cold PBS, and the proteoglycans were extracted with Triton X-100 buffer (1% (w/v) Triton X-100, 150 mM NaCl, 10 mM NaH2PO4, 2 mM KH2PO4, pH 7.4) containing protease inhibitors (5 mM N-ethylmaleimide, 5 mM benzamidine-HCl, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 1 μg/ml of pepstatin A). The extract was clarified by centrifugation (10,000 × g, 60 min), and the pellet was re-extracted with 5 ml of Triton X-100 buffer for 1 h at room temperature. The supernatants were combined, and solid urea was added to achieve a final concentration of 6 M. Bovine tracheal chondroitin sulfate A (1 mg, Calbiochem) was added as carrier, and the samples were applied to a 0.5-ml column of DEAE-Sepharose (Pharmacia Biotech Inc.) prepared in a disposable pipette tip and equil-
lbated in urea buffer (6 M urea, 0.2 M NaCl, 0.5% (w/v) Triton X-100, 25 mM Tris-HCl, pH 7.0, and protease inhibitors). The columns were washed with urea buffer (15 ml), and radiolabeled proteoglycans were eluted with 2.5 ml of urea buffer containing 1 M NaCl. The proteoglycans were desalted over a PD-10 column (Pharmacia) equilibrated in 10% (v/v) ethanol in water containing protease inhibitors. After lyophilization to the same volume, they were dissolved in PBS containing protease inhibitors (10 ml).

Syndecan-1 Purification and Characterization—The proteoglycan extract was passed over an affinity column (0.5 ml) of anti-syndecan-1 2E9 monoconal antibody3 coupled to Sepharose CL-4B (10). The column was washed with 8 ml of PBS, followed by 2 ml of PBS supplemented with 0.5 M NaCl. Syndecan-1 was eluted from the resin with 5 ml of a solution containing 4 M guanidine hydrochloride, 50 mM sodium acetate (pH 5.8), and protease inhibitors. Salt and guanidine were removed from purified syndecan-1 by chromatography over a PD-10 column before further analysis. More than 95% of purified syndecan-1 was recovered from the affinity column, and binding was independent of GAG content (10, 11).

A portion of affinity-purified syndecan-1 was resuspended in 0.5 M NaOH containing 1 M sodium borohydride and inactivated at 4°C for 2 h to β-eliminate the chains. The sample was neutralized by adding 10-µl aliquots of 1 M acetic acid until bubble formation ceased. Radiolabeled syndecan-1 and the released GAG chains were analyzed by gel filtration HPLC (TSK G4000SW, 30 cm × 7.5 mm inner diameter, Pharmacia). Samples were eluted with 0.5 M NaCl in 1.0 ml of KH2PO4 (pH 6.0) containing 0.2% Zwittergent 3–12 at a flow rate of 0.5 ml/min, and radioactivity in the effluent was determined by in-line liquid scintillation spectrometry using Ultima Gold XR scintillant (Packard Instrument Company, Downers Grove, IL). GAGs were digested with 20 million units of chondroitinase ABC (Seikagaku) in 50 mM Tris-HCl and 50 mM NaCl containing 0.2% Zwittergent 3–12 at a flow rate of 0.5 ml/min, and radioactivity in the effluent was determined by in-line liquid scintillation spectrometry using Ultima Gold XR scintillant (Packard Instrument Company, Downers Grove, IL). GAGs were digested with 20 million units of chondroitinase ABC (Seikagaku) in 50 mM Tris-HCl and 50 mM NaCl containing 0.2% Zwittergent 3–12 at a flow rate of 0.5 ml/min, and radioactivity in the effluent was determined by in-line liquid scintillation spectrometry using Ultima Gold XR scintillant (Packard Instrument Company, Downers Grove, IL). 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The plasmid, pc17, containing the full-length fibroglycan cDNA sequence (M81687, a gift from Dr. J. John Gallagher, Manchester, United Kingdom) was used as a template for PCR of the fibroglycan fragment (DMYLDSS1E5AESSLYP14DDDDYYSSAGGYAEYDKGSPLDT5Q).

3 Recent studies suggest that 2E9 also recognizes syndecan-3.

The wild-type betaglycan construct, SPGDSSGWPDGYEDEL, and the mutant, SPDGDSSAPGDYEDLE, were made using primers described previously (2). Overlapping PCR primers were designed to generate the following betaglycan fragments (M77809).

SPGDSSGPDPGYEDEL
N: 5'-GGCGAATTCCTCGGATGAGCAGACTGGGACCTAGGAC-3'
C: 5'-GGCGAATTCATCCGTCCTCTCGAGAGAGG-3'

SPGDSSGAPGDYEDLE
N: 5'-GGCGAATTCCTCGGATGAGCAGACTGGGACCTAGGAC-3'
C: 5'-GGCGAATTCATCCGTCCTCTCGAGAGAGG-3'

PDGYEDLE
N: 5'-GGCGAATTCCTCGGATGAGCAGACTGGGACCTAGGAC-3'
C: 5'-GGCGAATTCATCCGTCCTCTCGAGAGAGG-3'

PDGDSSAPGYEDLE
N: 5'-GGCGAATTCCTCGGATGAGCAGACTGGGACCTAGGAC-3'
C: 5'-GGCGAATTCATCCGTCCTCTCGAGAGAGG-3'

Overlapping PCR primers were designed for the following peptide sequences.

Perlecan (M71777, D4FELDLDPSAGDLSGSDGDSVSVGDF)
N: 5'-GGCGAATTCCTGGAATGATGGCAAGATCCTGGGCAGTAGATGGCAGATGGC-3'
C: 5'-GGCGAATTCATCCGATGACTGACATCTGGGCAGTAGATGGCAGATGGC-3'

Syndecan-1, fragment 1 (M84910, DDELDDIYSGSGSGYFEQESGLE)
N: 5'-GGCGAATTCCTCGGATGAGCAGACTGGGACCTAGGAC-3'
C: 5'-GGCGAATTCATCCGTCCTCTCGAGAGAGG-3'

Syndecan-3, fragment 1 (M84910, DDELDDIYSGSGSGYFEQESGLE)
N: 5'-GGCGAATTCCTCGGATGAGCAGACTGGGACCTAGGAC-3'
C: 5'-GGCGAATTCATCCGTCCTCTCGAGAGAGG-3'

Yrosine kinase receptor (M51596, YFVNVTDALSDDGDEDGDED)
N: 5'-GGCGAATTCATCCGATGACTGACATCTGGGCAGTAGATGGCAGATGGC-3'
C: 5'-GGCGAATTCATCCGTCCTCTCGAGAGAGG-3'

Thrombomodulin (M16552, DSGKDGDSSPSPPFTPTTSGT)
N: 5'-GGCGAATTCCTCGGATGAGCAGACTGGGACCTAGGAC-3'
C: 5'-GGCGAATTCATCCGTCCTCTCGAGAGAGG-3'
Proline-rich proteoglycan (L17318, DENGSDONDDGGSDGDDVN)
N: 5'-GGCGAAATCTCGAAGAAATGGAAGGTTGGATGTCGATAGATGAGG-3'
C: 5'-GGCGAAATCTCAGTTCATTGATCAGGTTGGATGTCGATAGATGAGG-3'

Glypican (X54232, DVPQDA5DGGSGSGDGDGGCLDDLGC)
N: 5'-GGCGAATCCAGCGGCTGCTGAGCAAGGTGATGAGGCTGAGG-3'
C: 5'-GGCGAATCCAGCGGCTGCTGAGCAAGGTGATGAGGCTGAGG-3'

Epican (S54674, EDEROHLSFSGSSIDDDDEDIS)
N: 5'-GGCGAAATCTCGAAGAAATGGAAGGTTGGATGTCGATAGATGAGG-3'
C: 5'-GGCGAAATCTCAGTTCATTGATCAGGTTGGATGTCGATAGATGAGG-3'

Decorin (M14219, EDEASGIGPEVPDDRD)
N: 5'-GGCGAAATCTCGAAGAAATGGAAGGTTGGATGTCGATAGATGAGG-3'
C: 5'-GGCGAAATCTCAGTTCATTGATCAGGTTGGATGTCGATAGATGAGG-3'

PCRs fragments were extracted with phenol/chloroform, precipitated with ethanol, digested with EcoRI, purified by agarose gel electrophoresis, and transferred to DEAE paper (Schleicher & Schuell). After elution, the fragments were ligated into a derivative of the eukaryotic expression vector pPROTA (16) that had been treated with EcoRI and calf intestinal alkaline phosphatase. All constructs were sequenced to confirm their identity.

Transfection—For transient transfection experiments, each well of a 12-well plate was seeded with 2 x 10^4 cells in F12 medium containing 10% (v/v) NuSerum (Collaborative Research, Inc., Bedford, MA). After one day, the medium was removed, and 0.5 ml of F12 medium containing 0.25 mg/ml of DEAE-Dextran (Sigma D-9885), 50 mM Tris-HCl (pH 7.4), 50 g/ml of chloroquine, and 10 g/ml of plasmid DNA was added to each well. Approximately 3 days after the solution was aspirated, and 0.5 ml of F12 medium without serum. Cells were then labeled at 37°C for 2 days with 50 mCi/ml of 35SO4 in sulfate-deficient growth medium or with 50 mCi/ml of TRAN35S-LABEL, a mixture of 35S-methionine and 35S-cysteine (ICN, Irvine, CA).

Stable transformants were created by transfecting a monolayer of wild-type CHO cells (50% confluence) with 16 mCi of pPROTA containing CHO syndecan-1 cDNA coding for amino acid residues 26–240. The vector pHAMNEO was included (2). Stable transformants were created by transfecting a monolayer of wild-type CHO cells (50% confluence) with 16 mCi of pPROTA containing CHO syndecan-1 cDNA coding for amino acid residues 26–240. The vector pHAMNEO was included (2).

RESULTS

CHO Syndecan-1—Syndecan-1 represents 15–20% of CHO cell 35S-proteoglycans based on binding to an affinity column composed of the 2E9 monoclonal antibody against human syndecan-1 described by Lories et al. (10). All of the affinity-purified material behaved like proteoglycan since the 35S-counts eluted in the Vn of a gel filtration column and shifted to included fractions after β-elimination (Fig. 1). Treatment purified syndecan-1 with low nitrogen acid, which depolymerizes heparan sulfate chains by degrading N-sulfated glucosamine residues (12), converted 80–85% of the counts to small 35S-oligosaccharides and free 35SO4. A residual core protein containing heparan sulfate chains and chondroitin sulfate emerged at a Kav of 0.27. Treating a sample with chondroitinase ABC, which depolymerizes chondroitin sulfate chains to 35S-disaccharides, caused 15–20% of the 35S-counts to elute in the Vn of the column (Fig. 1A). Thus, CHO syndecan-1 consisted of 80–85% heparan sulfate and 15–20% chondroitin sulfate. The same composition was obtained when the released GAG chains were analyzed by enzymatic digestion (Fig. 1B).

There are five possible GAG attachment sites in mouse, human, and rat syndecan-1 based on the presence of Ser-Gly dipeptides (7). The same five sites were present in CHO syndecan-1, which was cloned by PCR using the mouse cDNA sequence to design appropriate primers (Fig. 1B). The CHO syndecan-1 amino acid sequence showed 94, 87, 86, and 77% homology to the Syrian golden hamster, rat, mouse, and human sequences, respectively (Fig. 2B). More importantly, the amino acid sequences near the putative GAG attachment sites are highly conserved among all the different species (7). Kokenyesi and Bernfield (4) reported that both heparan sul-
fate and chondroitin sulfate chains were present on the N-terminal half of murine syndecan-1, but only chondroitin sulfate was found on the C-terminal half. Of the five potential GAG attachment sites, three reside in the N-terminal half and two in the C-terminal half. We showed previously that the synthesis of heparan sulfate on betaglycan requires a cluster of acidic residues located near a specific Ser-Gly attachment site (2). Two clusters of acidic amino acids border the three Ser-Gly sites on the N-terminal part of the syndecan-1 (EDQDGSG37EDQDGSG47, Fig. 2B), consistent with the idea that one or more of these sites might support heparan sulfate assembly.

To examine how GAGs assemble at the individual sites, we prepared chimeras composed of CHO syndecan-1 segments fused to the IgG binding domain of Staphylococcal Protein A with a signal peptide from the secreted metalloprotease, transthyretin, attached to the N terminus (16). The chimeras were introduced into wild-type CHO cells by transient transfection, and the attachment of GAG chains was assessed by 35SO4 incorporation into secreted chimeras (“Experimental Procedures”). The activity of each Ser-Gly site was measured independently by mutating other potential sites in the constructs (Table I). All three Ser-Gly dipeptides toward the N terminus (EDQDGS37GDDSDNFS45GS47GTG-) were capable of priming heparan sulfate chains, but their relative ability varied from 9% at Ser 37 to 23% at Ser 47. In contrast, the two sites near the transmembrane domain (-PTGEGS205GEQDFTFETS215GENTA-) primed only chondroitin sulfate. A sixth Ser-Gly dipeptide exists in both CHO and Syrian golden hamster syndecan-1 (Ser181), but not in mouse, rat, and human syndecan-1. Expression of a 156-amino acid chimera containing residues 49–204 did not result in incorporation of 35SO4 into GAGs (Table I), suggesting that Ser181 does not act as a glycosylation site.

The extent of substitution of the chimeras with heparan sulfate was less than expected from the study of endogenous syndecan-1, which contained 80–85% heparan sulfate (Fig. 1). The difference was not due to variation in the extent of sulfation of heparan sulfate and chondroitin sulfate chains, since comparable results were found when the cells were labeled with [6-3H]GlcN and chain length did not vary significantly (Fig. 3). The difference also was not due to poor expression of the chimera since only 20% of material labeled with TRAN35S-LABEL was converted to high molecular weight proteoglycan.

We also tested the chimeras in CHO idlD cells, which cannot obtain UDP-GalNAc from UDP-GlcNAc due to a deficiency in the 4'-epimerase that interconverts the nucleotide sugars (18). This strain makes less chondroitin sulfate when deprived of exogenous GalNAc (19). When the chimeras were introduced into idlD cells, the amount of heparan sulfate was enhanced (up to 79%), but the relative amount made at each N-terminal site remained about the same (Table I, values in parentheses). Interestingly, Ser205 (-TGEGS205GEQDF-) , which primed only chondroitin sulfate in wild-type cells, generated about 28% heparan sulfate in idlD cells. The other chondroitin sulfate site at Ser215 (-TFETS215GENTA-) remained ineffective.

Coupling across Adjacent Ser-Gly Attachment Sites—A key factor controlling the extent of heparan sulfate substitution emerged from studies of chimeras containing more than one GAG attachment site. A 39-amino acid chimera covering residues 26–64 and containing the two adjacent sites at Ser45 and Ser47 (SGH, M29967) (36), mouse (Mur, X15487) (13), rat (Rat, M81785) (37), and human (Hum, X60306) (10), identical amino acids; *, a gap to improve alignment; boldface letters, potential GAG attachment sites.

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**Fig. 2.** Sequence of CHO syndecan-1 and comparison with sequences from other species. A, nucleotide sequence (upper line) and predicted amino acid sequence (lower line) of CHO syndecan-1 cDNA. CHO syndecan-1 was amplified from the CHO-K1 quick-done cDNA library by PCR. The sequence of the PCR primers, indicated in lower-case letters, was derived from mouse syndecan-1 (13). The underlined sequences define the fragments for generating the chimeras shown in the tables (GenBank™ accession number L38991). B, comparison of predicted protein sequence of syndecan-1 from CHO, Syrian golden hamster (SGH, M29967) (36), mouse (Mur, X15487) (13), rat (Rat, M81785) (37), and human (Hum, X60306) (10).
Repetitive Ser-Gly Enhances Heparan Sulfate

Five GAG attachment sites exist in CHO syndecan-1

Chimeras containing syndecan-1 segments were introduced into wild-type CHO cells and labeled biosynthetically with TRAN35S-LABEL or 35SO4 for 2 days (see “Experimental Procedures”). The numeric designations in the segments indicate the first and last amino acid residue of CHO syndecan-1 (Fig. 2) and the position of each potential GAG attachment site. The dashes (-) indicate that some of the residues are not shown. The boldface letters indicate mutated residues. The chimeras were affinity-purified (see “Experimental Procedures”), and the 35S-counts were quantitated. The amount of 35SO4 in nontransfected cells or in cells transfected with the constructs in the wrong orientation was 200-fold less than the amount incorporated with the correct orientation. The amount of TRAN35S-LABEL incorporation into constructs in the wrong orientation was comparable with the values shown since most of the Cys and Met was present in the Protein A portion of the chimera (2). The GAG composition was determined by chondroitinase ABC digestion of a portion of the affinity-purified proteoglycan labeled with 35SO4. The amount of resistant heparan sulfate was quantitated and expressed relative to the total amount of 35S-proteoglycan treated. The values in parentheses reflect the proportion of heparan sulfate produced when Id D cells were transfected.

| Syndecan-1 Segments | TRAN35S-LABEL | | |
|---------------------|----------------|-----------------|
| 26–EDQDGSG2GDDSDNF37GAGTG–64 | 5.2 | 41 | 7.9 | 9 (47) |
| 26–EDQDGAGDDSDNF45GAGTG–64 | 5.2 | 43 | 8.3 | 15 (75) |
| 26–EDQDGAGDDSDNF45GS47GTG–64 | 4.3 | 49 | 11 | 23 (79) |
| 187–ATAQLPTGEGDQEDQFETE–214 | 4.5 | 63 | 14 | 3 (28) |
| 49–GDPDQGPS14GTGSIKEVAYE–204 | 8.4 | 30 | 14 | 3 (14) |
| 207GEQDFTFETS215GENTAVAAIE–240 | 6.4 | 0.2 | <0.1 | 2 |
| 26–EDQDGSG2GDDSDNF37GTSV–64 | 8.4 | 36 | 4.3 | 23 |
| 26–EDQDGSG2GDDSDNF37GS–64 | 8.5 | 63 | 4.3 | 35 |
| 26–EDQDGAGDDSDNF14GS22GTG–64 | 7.0 | 37 | 5.3 | 48 |
| 26–EDQDGSG2GDDSDNF37GS–64 | 6.1 | 41 | 6.7 | 60 |

Table II

Adjacent Ser-Gly dipeptides stimulate heparan sulfate assembly in betaglycan chimeras

Protein A chimeras containing wild-type or mutated betaglycan segments (see “Experimental Procedures”) were introduced into wild-type CHO cells and labeled biosynthetically with 35SO4 for 2 days. The numeric designations in the constructs indicate the first and last amino acid residue of rat betaglycan (30). The GAG composition was determined by chondroitinase ABC digestion. The amount of resistant heparan sulfate was quantitated and expressed relative to the total amount of 35S-proteoglycan treated.

| Construct | [35S]GAG | Heparan sulfate |
|-----------|----------|----------------|
| 530SPGDSGWDGYEDLE445 | 22 | 54 |
| 530SPGDSGAFGYEDLE445 | 18 | 21 |
| 530SPGDSGSAFEDLE545 | 15 | 51 |
| 530SPGDSGWDGYEDLE445 | 17 | 58 |
| 530SPGDSGAFGYEDLE445 | 16 | 15 |
| 530SPGDSGSAFEDLE545 | 12 | 10 |
| 530SPGDSGAFGYEDLE445 | 19 | 17 |
| 530SPGDSGAFGYEDLE445 | 18 | 6 |

Fig. 3. Gel filtration chromatography of [35S]syndecan-1 chimeras. Syndecan-1 chimeras containing the sequences 26–EDQDGAGDDSDNF37GAGTG–64 and 26–EDQDGAGDDSDNF37GS47GTG–64 were affinity-purified from 35SO4-labeled cells (see “Experimental Procedures”). A portion of material was β-eliminated to liberate the [35S]GAGs from the chimeras, and an aliquot was digested with chondroitinase ABC or nitrous acid (see “Experimental Procedures”). The samples were then analyzed by gel filtration HPLC (see “Experimental Procedures”). A, intact chimeras; B, β-eliminated GAG chains. C, heparan sulfate and chondroitin sulfate chains. D, chimeras with a single acceptor site (26–EDQDGAGDDSDNF37GAGTG–64); E, chimeras with two acceptor sites (26–EDQDGAGDDSDNF37GS47GTG–64); F, β-eliminated chains from the single site chimera; G, β-eliminated chains from the two-site chimera; H, heparan sulfate chains from the two-site chimera; I, chondroitin sulfate chains from the two-site chimera.

Ser47 yielded about 48% heparan sulfate (Table I). An identical construct containing all three N-terminal sites gave 60% heparan sulfate. Enhanced synthesis also occurred in constructs containing either Ser37 and Ser45 or Ser37 and Ser-47 (Table I). These findings suggested that some type of coupling occurs across nearby attachment sites that increases the proportion of heparan sulfate.

To test whether coupling was dependent on flanking amino acid sequences, a repetitive SGSG sequence was introduced into betaglycan. In betaglycan, the site that supports heparan sulfate synthesis contains Ser-Gly-Trp flanked by a cluster of acidic residues (Table I). We showed previously that converting the Trp residue to Ala reduced the level of heparan sulfate from 54 to 21% (2). When a second Ser-Gly dipeptide was substituted for the Ala residue, the normal level of heparan sulfate synthesis was restored (51%, Table II). Thus, the coupling across adjacent Ser-Gly sites appeared to be independent of surrounding sequence. Separating the Ser-Gly repeats by one or more residues reduced the proportion of heparan sulfate, suggesting that the coupling depended on proximity (Table I). Inserting a Trp residue next to the repeat Ser-Gly segment gave a slight enhancement of heparan sulfate (58 versus 51%).

A stable transfectant expressing a chimera with all five sites (residues 26–240) was analyzed (Table III). Unlike the behavior of the transient transfectants, the stably transfected cell line converted all of the chimera to high molecular weight proteoglycan (data not shown) and produced about 3-fold more total 35S-proteoglycan than nontransfected control cells (Table III). The proportion of heparan sulfate was reduced in the chimera (55%) compared with the proteoglycans found in non-
transfected parental cells (72%). Interestingly, the endogenous CHO cell proteoglycans also contained less heparan sulfate in the stable transfectant (54–62%). These findings suggested that the reduced level of heparan sulfate may have been due in part to enhanced core protein expression. Similar effects have been observed when full-length syndecan and betaglycan constructs were expressed in cells (1, 4).

To gain insight into the mechanism that results in a higher proportion of heparan sulfate at adjacent attachment sites, we analyzed the GAG substitution pattern of syndecan-1 chimeras containing one site (26–EDQDGAGGDSDNSF45GTG–64) and two sites (26–EDQDGAGGDSDNSFS47GTG64). As expected, the two-site chimera migrated with a greater hydrodynamic volume during gel filtration than the chimera containing one site (Fig. 3A). β-elimination shifted the [35S]GAG to a more included position, but the mixture of GAG chains on both chimeras had essentially the same elution position (Fig. 3B). Analysis of the heparan sulfate and chondroitin sulfate chains on the two-site chimeras showed that they were comparable in size as well (Fig. 3C).

The two-site chimera presumably consisted of glycoforms containing one or two GAG chains, with various combinations of heparan sulfate and chondroitin sulfate chains. Analysis of [35S]-labeled material by SDS-PAGE revealed smeared bands characteristic of proteoglycans (Fig. 4, lane 3). The material of faster mobility migrated in the same position as a chimera containing both Ser45 and Ser47, then the proportion of chimeras containing one and two GAG chains are indicated to the right of the gel, and the protein molecular weight standards are indicated to the left of the gel. Each lane contained the following insert derived from syndecan-1: lane 1, 26–EDQDGAGGDSDNSF45GTG–G64; lane 2, 26–EDQDGAGGDSDNSFTGS46GTG64; lane 3, 26–EDQDGAGGDSDNSFS47GTG–G64; lane 4, 26–EDQDGAGGDSDNSFS47GTG64 after treatment with chondroitinase ABC; lane 5, 26–EDQDGAGGDSDNSFS47GTG64 after treatment with heparin lyase III; lane 6, 26–EDQDGAGGDSDNSFS47GTG64 after treatment with both chondroitinase ABC and heparin lyase III. Equal portions of the samples were analyzed to show the reduction in signal due to enzymatic digestion.

**FIG. 4. Electrophoresis of [35S]syndecan-1 chimeras.** Chimeras were purified from [35SO4]-labeled cells by IgG affinity chromatography and dissolved in SDS-PAGE protein sample buffer. The samples were analyzed on SDS-polyacrylamide (5–16%) gradient gels, and the dried gel was imaged for data collection (see "Experimental Procedures"). The positions of chimeras containing one and two GAG chains are indicated to the right of the gel, and the protein molecular weight standards are indicated to the left of the gel. Each lane contained the following insert derived from syndecan-1: lane 1, 26–EDQDGAGGDSDNSF45GTG–G64; lane 2, 26–EDQDGAGGDSDNSFTGS46GTG64; lane 3, 26–EDQDGAGGDSDNSFS47GTG–G64; lane 4, 26–EDQDGAGGDSDNSFS47GTG64 after treatment with chondroitinase ABC; lane 5, 26–EDQDGAGGDSDNSFS47GTG64 after treatment with heparin lyase III; lane 6, 26–EDQDGAGGDSDNSFS47GTG64 after treatment with both chondroitinase ABC and heparin lyase III. Equal portions of the samples were analyzed to show the reduction in signal due to enzymatic digestion.

**TABLE III**

GAG composition of proteoglycans in wild-type CHO cells and a stable transfectant

| Source of material | Total [35S]GAG cpm x 10^5 | Cell-associated | Chimeras | Medium after removing chimeras |
|--------------------|---------------------------|-----------------|----------|-------------------------------|
| Wild-type CHO cells| 44 (72%)                  | 23 (73%)        | <0.1     | 15 (65%)                      |
| Stable transfectant| 150 (54%)                 | 14 (62%)        | 114 (55%)| 20 (56%)                      |

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Table IV

| Acidity | Number of chains (%) | Method of analysis |
|---------|----------------------|-------------------|
|         |                      | Experimental      |
|         |                      | Theoretical       |
| 1       | 1.1%                 | 22%               |
| 1/2     | 9%                   | 10%               |
| 2       | 14%                  | 1.1%              |

preparing. Mutating the Asp and Glu residues in the chimeras containing Asn and Gln, respectively, reduced the proportion of heparan sulfate from 38% in the control construct to 8% and 13%, depending on the cluster (Table V). Mutating both clusters diminished heparan sulfate synthesis to background (4%). Thus, the clusters of acidic residues are important elements in both syndecan-1 and betaglycan.

Discussion

This report describes the use of protein chimeras to determine the assembly of heparan sulfate chains on proteoglycans. The results suggest a set of rules for predicting whether heparan sulfate will assemble at glycosylation sites. These rules include features of the core protein, the cellular capacity to produce individual GAGs, and the relative abundance of core proteins. Together, they support a model for GAG chain assembly in which specific core protein determinants interact with a key biosynthetic enzyme involved in heparan sulfate biosynthesis.

Clusters of Acidic Residues Are Necessary but Not Sufficient—Studies of betaglycan suggested that one type of heparan sulfate attachment site consists of a Ser-Gly dipeptide located near a cluster of acidic residues (2). Mutation of the acidic residues diminished heparan sulfate synthesis in cultured cells. The enhanced synthesis of heparan sulfate across adjacent Ser-Gly attachment sites is due to the presence of Ser-Gly dipeptide at the indicated sites (1–23). However, it is not known how these clusters of acidic residues drive heparan sulfate synthesis. Thus, clusters of acidic residues appear to be necessary but not sufficient determinants for heparan sulfate assembly.

Hydrophobic Amino Acids Can Act as Enhancer Elements—In betaglycan, the site that supports heparan sulfate synthesis contains a Trp residue adjacent to the Ser-Gly unit (2). Changing this residue to Ala reduced heparan sulfate synthesis. More importantly, inserting Trp next to a chondroitin sulfate site with a nearby cluster of acidic residues enhanced heparan sulfate formation. Although Trp occurs relatively rarely in proteoglycans (Table VI), aromatic and aliphatic residues are quite common, enhancing the overall hydrophobicity of the immediate region by the GAG attachment site (4). The hydrophobicity of chondroitin sulfate sites tends to be less pronounced (Table VI).

Earlier studies of β-D-xylosides in CHO cells showed that priming of chondroitin sulfate most likely occurs by default (24, 25). Simple xylosides containing alkyl chains as an aglycone prime chondroitin sulfate but not heparan sulfate. Compounds containing fused aromatic rings, which may simulate the structure of the indole side chain of tryptophan, activate heparan sulfate as well as chondroitin sulfate synthesis (25). Thus, aromatic β-D-xylosides may mimic the hydrophobic patch found near heparan sulfate attachment sites. Synthesis of heparan sulfate by aromatic β-D-xylosides requires a relatively high concentration of primer (30 μM) compared with endogenous intermediates, possibly because they lack negative charges imparted by the acidic residues in natural core proteins.

Adjacent Ser-Gly Attachment Sites Are Coupled—Most heparan sulfate proteoglycans contain (Ser-Gly), segments, where $n \geq 2$ (Table VI). Experiments with peptides derived from a number of proteoglycans that contain repeating Ser-Gly units primed heparan sulfate, albeit to different extents: fibroglycan, 60% (QDG-D-xylosides); syndecan-3, 56% (QDG-D-xylosides); syndecan-3, 56% (QDG-D-xylosides); syndecan-3, 56% (QDG-D-xylosides); syndecan-3, 56% (QDG-D-xylosides); syndecan-3, 56% (QDG-D-xylosides). These variation may reflect differences in primary sequence immediately adjacent to the site or secondary and tertiary structure in the segments. This possibility may explain why natural glypicans contains only heparan sulfate (26), whereas the glypicans chimeras primed heparan sulfate relatively poorly compared with other constructs (Table VI).

The enhanced synthesis of heparan sulfate occurs at adjacent Ser-Gly sites. The upstream site in syndecan-1 (Ser37) couples to the downstream sites at Ser45 and Ser47 (Table I). Shworak et al. (3) showed that stable
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Protein A chimeras containing the following proteoglycan segments were introduced into wild-type CHO cells and analyzed for heparan sulfate as described in Table I: decorin, EDAEASGIGPEVPDDR; thrombomodulin, DSGKVDGDSSGSGEPSSPTGPST; fibroglycan, DMYLDSSSEEASYGLYPIDDDYSSASGGAYEKGSPDLTTSQ; epican, EDERDHLFSGSGIDDEDFIS; glypican, DFQDA5DGGGSGSDDCLDDLGC; syndecan-3, DDELDDYDSSGSGYGEQQESGL; syndecan-3, DLEVPTSSSGPSGDFFDEEETT; N-syndecan, TFTQDEPEVPVSAGPSGDFELQEE; perlec, DDELDAADSGDGLGSGDVGSG; proline-rich-proteoglycan, DENGDGDDEDDGSGDSGDN; tyrosine kinase receptor, YIFNVTDLASSGDDEDDNNGS; betaglycan, SPGDSGGPDPYEDLE and DSIGNPDPYEDLESGDNGFPGDGDEG. Data for syndecan-1 were taken from Table I. The sequences have been arranged to align an SG residue, * sites proven to contain a GAG chain either by direct protein sequencing or by expressing cDNAs in cells. ND, not determined.

| Attachment site | Sequence | Heparan sulfate |
|-----------------|----------|-----------------|
| Syndecan-1 (L38991) | GATNQLPTEGGSGEQDFFETTSGE | 3 |
| *Decorin (M14219) | GLDFPLDEDASGGIGEPVPDPDF | 4 |
| *Thrombomodulin (M16552) | DSGKVDGDSSGSGEPSSPTGPST | 3 |
| *Betaglycan (M77809) | GMDDPGVEDLESGDNGFPGDGDEG | 3 |
| *Biglycan (J04599) | DGPMMNDEEASADTSGVLDPDS | ND |
| *Invariant chain (A93981) | PKESLELDPSGGLVTRQDLGPV | ND |
| *Type IIX collagen (M16715) | GPPGHIQVQGAGAQLFCTNCP | ND |
| *APLP2 (U15571) | HFFPSLSNEQGAEQDDGLGA | ND |
| *M-CSF (M70877) | AMGTVNPEEASAEISIPFQST | ND |
| *H3-30 (Bikunin) (X04494) | RRAVLPQEEEGGGQLVTEVKK | ND |
| Syndecan-3 (M84910) | SDLVPTSSGFGDFEIQEEETT | 7 |
| N-Syndecan (X63143) | TTQDEPEVPVSAGPSGDFELQEE | 8 |

| Heparan sulfate sites | |
|-----------------------|-----------------|
| *Perlecan (M777174) | SDDEDLLPDDASDGLGSGGVGS | 64 |
| *Fibroglycan (M18667) | YFIDEVSASGAYEGDGPDD | 60 |
| Syndecan-3 (M84910) | PFDGDSIVGSYSGTEQGSG | 56 |
| *Betaglycan (M77809) | IVQVPSGFDSGWNPDYEDLESG | 54 |
| Syndecan-3 (L38991) | GSGSBDNSFSGSTGAPELTSRSQ | 38 |
| Tyrosine kinase receptor (M35196) | YFMVNVTDAISGSEDGDOTSED | 27 |
| Epican (S45674) | NEQDRHRLFGSGGDGDEDS | 24 |
| Glycan (B49142) | DFOQARDDQSGSCGLDLC | 20 |
| Proline-rich proteoglycan (L17318) | GDNNDDDDGDGDSDDNRERPO | 17 |
| *Ryudocan (M81786) | LEQDSDFEQLSSGDLDTEEPTF | ND |
| *Ryudocan (M81786) | DQPDLEQLRFYPSGLPDDLADDG | ND |
| Syndecan-3D (U03282) | DLGGYGGGSGPGDDAEPO | ND |
| Cerebroglycan (L20468) | DNSDADGDSSGGQYADWKA | ND |
| Bone marrow (X65787) | CEPREEEREXGSGEDASKKDGAV | ND |
| Serygcan (M12393) | DFISNYDGVGSGFPG | ND |
| Agrin (X67480) | PCEPAENGSSGGEDQSCQEL | ND |
| *Testican (X73608) | SERSQDGPGGGGSVVLLNLV | ND |

transfectants expressing ryudocan contain more heparan sulfate when multiple attachment sites were present. These findings may indicate that coupling can occur at a distance, possibly by juxtaposing the sites through secondary and tertiary structures. Interestingly, chimeras containing the sites in syndecan-1 (SDLEVPTSSGFGPSGDFFDEEETT) and N-syndecan (TTQDEPEVPVSAGPSGDFELQEE) do not prime much heparan sulfate (7 and 8, respectively). Both sequences contain a proline residue between the Ser-Gly units, which may act as an inhibitor. The low degree of coupling between closely spaced Ser-Gly sites in betaglycan also may have been due to an intervening proline residue (2), which may have altered the conformation of the peptide (Table III). If correct, this idea is reminiscent of the inhibitory effect of Pro on the attachment of Glc3Man, GlcNAc2 oligosaccharides from dolichol-P intermediates to Asn-Xaa-Ser/Thr sites in glycoproteins (27). Perhaps negative regulatory sequences/amino acids play a role in proteoglycan assembly as well.

As shown in Table VI, repetitive Ser-Gly dipeptides with a flanking cluster of acidic residues represent a common motif in a variety of heparan sulfate proteoglycans. Thrombomodulin also contains adjacent Ser-Gly dipeptides, but lacks a cluster of acidic residues and therefore does not prime heparan sulfate. Searching the Wisconsin gene bank for sequences consisting of (Ser-Gly)₁₋₃ and a nearby cluster of acidic amino acids (ID/E)₁₋₃ within 6 residues yielded 15 out of 16 heparan sulfate proteoglycans cloned to date. The search also yielded proteins from bacteria, viruses, parasites, and eukaryotic subcellular organelles. Cell surface and secreted proteins, including agrin, a tyrosine kinase receptor, prostatic spermine-binding protein, and sporozoite surface antigen have a high chance of bearing heparan sulfate chains based on their location on the surface or outside the cell. Among them, agrin was shown recently to bear heparan sulfate chains (28). Interestingly, a chimera prepared from the tyrosine kinase receptor sequence (M35196) primed heparan sulfate, suggesting that the native protein might contain a heparan sulfate chain.

Biosynthetic Capacity Affects GAG Composition—A few heparan sulfate proteoglycans lack the repetitive Ser-Gly sequences of syndecan-1 or the aromatic residue found in betaglycan (e.g. proline-rich proteoglycan), suggesting that other enhancing factors may exist. One possibility is that the proportion of chains depends on the relative capacity of cells to produce GAG chains. In IdlD cells, which have reduced ability to make chondroitin sulfate, the relative proportion of heparan sulfate increases at the expense of the chondroitin sulfate chains (Table I). Some sites that do not make much heparan sulfate will even become active when expressed in IdlD cells (Table I). Conversely, the proportion of chondroitin sulfate increases dramatically in pgsD mutants, defective in heparan sulfate synthesis (6, 29).

Proteoglycans vary in composition in different tissues and cells. For example, serglycin contains chondroitin sulfate chains when expressed in various myeloid cells (30) and a mixture of heparin and chondroitin sulfate chains when expressed.
pressed in connective tissue mast cells. Syndecan-1 also varies in composition in different cells (31) and in response to growth factors (32). No systematic study of the biosynthetic enzymes has been undertaken in these tissues, but it is reasonable to assume that changes in enzyme expression could alter GAG composition. Thus, the actual complement of chains borne by a proteoglycan can be determined by biosynthetic capacity as well as permissive elements embedded in the core protein sequence.

The Amount of Core Protein Can Affect GAG Composition—The amount of core protein substrates passing through the biosynthetic compartments may also affect GAG composition. Stable transfectants expressing a syndecan chimera overproduce GAG and cause a decline in the proportion of heparan sulfate (Table II). The effect was not limited to the chimera, since endogenous proteoglycans also contained less heparan sulfate chains. Stable transfectants expressing syndecan-1 and betaglycan show similar effects (1, 2, 4). Recent studies suggest that core protein sequences outside the GAG attachment sites may also affect GAG composition. Thus, the actual complement of chains borne by a proteoglycan may be determined by biosynthetic capacity as well as permissive elements embedded in the core protein sequence.

Although the proportion of heparan sulfate declines, the actual amount of material increases after transfection. The data presented in Table II show that wild-type cells produced about 3.2 × 10^6 cpm of [35S]heparan sulfate (4.4 × 10^6 cpm × 72%). The stable transfectant produced 8.1 × 10^6 cpm of [35S]heparan sulfate (1.5 × 10^7 × 54%). This 2–3-fold increase in heparan sulfate synthesis is offset by a much larger increase in chondroitin sulfate synthesis, causing the relative composition to change. The compositional difference mimics the effect of β-oxidosides, which depress the proportion of heparan sulfate because the primers preferentially stimulate chondroitin sulfate assembly (25).

A Model for Heparan Sulfate Biosynthesis—The above information suggests a model for controlling GAG composition, in which a key enzyme in the biosynthetic pathway senses permissive elements of the core and controls if heparan sulfate assembles. The identity of this enzyme emerged from earlier enzymatic studies showing that a unique α-GlcNAC transferase (α-GlcNAC Ti) acts on the tetrasaccharide intermediate, -GlcAlβ1-3Galβ1-3Galβ1-4Xyl-protein (5, 6). This unique transferase may bind to structural elements in the core protein and select specific linkage tetrasaccharides for heparan sulfate assembly. In betaglycan and related proteoglycans, the recognition element consists of the cluster of acidic residues and the adjacent Trp, which together may determine the affinity of the substrate for the transferase. Analogous interactions between distal glycosyltransferases and nascent glycoproteins occur during the addition of GalNAc to GlcNAc on the termini of N-linked oligosaccharides of glycoproteins (34) and mannose 6-phosphate to the termini of chains found on lysosomal glycoproteins (35). In syndecan-1, the mechanism involves a cluster of acidic residues and perhaps a hydrophobic pocket defined by aromatic and aliphatic residues. In addition, a coupling phenomenon occurs across nearby Ser-Gly attachment sites. The juxtaposition of two or more sites may raise the probability that an α-GlcNAC Ti will act on adjacent linkage fragments. Thus, initiation of the heparan sulfate chains may involve both sequence recognition and a quasi-processive mechanism with regard to the carbohydrate acceptors.

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