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Identification and synthesis of a recognition signal for the attachment of glycosaminoglycans to proteins

(proteoglycans/xylosyltransferase/glycosylation)

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ABSTRACT **Comparison of the amino acid sequences of three different proteoglycan core proteins reveals a 12-amino acid sequence that is about 50% homologous among these proteoglycans. In each of the proteoglycans, this sequence surrounds the serine-glycine dipeptide in which the serine is known or presumed to be substituted with a chondroitin/dermatan sulfate glycosaminoglycan chain. Peptides containing this sequence from two proteoglycans were examined for their ability to serve as acceptors for xylosyltransferase, the enzyme that begins the assembly of glycosaminoglycan chains. Those peptides corresponding to amino acid sequences known to contain glycosaminoglycan-substituted serine residues in the protein were efficient xylosyltransferase acceptors, whereas peptides from sequences with no glycosaminoglycan-substituted serine residues were not. Amino acid substitutions at four critical sites in the acceptor peptides showed that single substitutions could completely abolish acceptor activity or greatly reduce it. The results suggest that the proteoglycan recognition consensus sequence for the attachment of glycosaminoglycans to core proteins consists of acidic amino acids closely followed by the tetrapeptide Ser-Gly-Xaa-Gly, where Xaa is any amino acid. The signal appears to be contained in the primary sequence information. In this regard it resembles a number of other signals for protein processing and intracellular routing.**

Proteoglycans are abundant tissue components consisting of a core protein to which a variable number of glycosaminoglycan chains are attached (1). The glycosaminoglycan is attached to the core protein through a xyloside residue that is linked to a serine residue of the protein (2). Available information on the structure of proteoglycan core proteins suggests that the substituted serine residues are followed by a glycine residue (2–5). In some proteoglycans, such as a yolk sac tumor chondroitin/dermatan sulfate proteoglycan (6), the substituted serine residues are part of a series of repeated Ser-Gly dipeptides (4). A similar arrangement also has been suggested to be present in a heparin proteoglycan (3), and Ser-Gly or Thr-Gly repeats of a *Drosophila* biological clock gene also may carry glycosaminoglycan chains (7–9). In the fibroblast proteoglycan PG40 (10, 11), a chondroitin/dermatan sulfate chain is attached to the serine of a Ser-Gly dipeptide that occurs individually rather than in a Ser-Gly repeat (5, 10–12). The invariant chain associated with the class II molecules of the major histocompatibility complex similarly appears to have its glycosaminoglycan at an individually occurring Ser-Gly dipeptide (13–17). The significance of the Ser-Gly dipeptide in glycosaminoglycan protein linkage is also indicated by the fact that peptides containing this sequence can serve as biosynthetic acceptors for xylosyltransferase-catalyzed linkage of xyloside to peptide (18). However, additional signals must be involved in the biosynthetic recognition of the core proteins by the xylosyltransferase, since most proteins that contain Ser-Gly sequences are obviously not proteoglycans, and all Ser-Gly sequences of proteoglycan core proteins are not substituted (4, 6, 12).

We report here the identification of an amino acid sequence homology around the Ser-Gly dipeptide sites that serve as glycosaminoglycan attachment sites in the core proteins of proteoglycans. By using synthetic peptides, we demonstrate the significance of this sequence as the xylosyltransferase recognition sequence.

MATERIALS AND METHODS

Peptide Synthesis. Peptides were synthesized by use of an automatic peptide synthesizer (Applied Biosystems, Foster City, CA) equipped with the chemistry provided by the manufacturer. After cleavage from the resin with hydrogen fluoride, the peptides were washed with cold ethyl ether, precipitated from solution in trifluoroacetate with ice-cold ethyl ether, redissolved in water, and lyophilized. Amino acid analysis was performed on each peptide to verify the correct composition.

Xylosyltransferase Assay. Assays for acceptor activity were carried out with partially purified xylosyltransferase from rat chondrosarcoma as described by Rodén *et al.* (18). Reaction mixtures contained the following components in a total volume of 100 μl: 48 mM 4-morpholineethanesulfonic acid buffer (pH 6.5), 48 mM KCl, 8.4 mM $MgCl_2$, 11.4 mM $MnCl_2$, 7.5 mM KF, 1.2 nmol UDP-[^{14}C]xylose (42 nCi/nmol; 1 Ci = 37 GBq), Sephacryl S-200-purified enzyme (40 μg of protein), and various amounts of peptide. After incubation at 37°C for 1 hr, reaction products were adsorbed by AG50W-X2 (H^+ form, 200–400 mesh; Bio-Rad), washed with 3–4 bed volumes of 0.01 M HCl, and eluted with 2 M NH_4OH. Incorporated radioactivity was then measured by liquid scintillation counting. The cpm of incorporated [^{14}C]xylose was shown to be linear at least through 1 hr of incubation time at 37°C, so this was adopted as the standard duration of incubation. Likewise, the incorporation of isotope was shown to be linear through a wide range of enzyme concentrations, including the concentration routinely used for the assay (40 μg/100 μl).

Estimates of K_m and V_{max} were obtained by processing the steady-state data on a DEC-20 computer with the basic program ENZKIN. Briefly, this program fits initial velocity data to the best rectangular hyperbola by an iterative least-squares method. In cases in which the concentrations of

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peptide tested were much less than the K_m value (owing to limited peptide solubility), accurate estimates of K_m and V_{max} were not readily obtainable. In these circumstances, the equation for initial velocity degenerates to $v_0 = V_{max}[S]/K_m$ (where [S] = substrate molarity), and therefore fit of the data by linear regression was used to estimate the ratio V_{max}/K_m. The ratio V_{max}/K_m is a function of acceptor activity with increased V_{max}/K_m values reflecting higher acceptor activity because of either a high V_{max} or a low K_m.

RESULTS

Sequence Homology in Proteoglycans. Comparison of the core protein sequences of three chondroitin/dermatan sulfate proteoglycans—the fibroblast proteoglycan PG40 (11), the yolk sac tumor proteoglycan PG19 (4), and the invariant chain proteoglycan (14)—revealed a homologous 12-amino acid sequence in the three proteoglycans (Fig. 1). The core proteins are otherwise unrelated. The homology region contains a single Ser-Gly dipeptide in two of the proteoglycans (PG40 and the invariant chain). The serine residue in the dipeptide is known to carry the only glycosaminoglycan chain of PG40 (12), and a similar situation has been postulated for the invariant chain based on less direct evidence (17). In the third proteoglycan (PG19), the homology region contains the beginning of a series of alternating serine and glycine residues that is the glycosaminoglycan chain-attachment domain of that proteoglycan (4, 6). In accordance with the presumed importance of the homology around the Ser-Gly dipeptide, this region is highly conserved between human and bovine PG40 (5, 10) and human and mouse invariant chain (14, 15). Since all Ser-Gly dipeptides in proteins are not substituted with glycosaminoglycan chains, it is likely that sequence similarities around the dipeptide glycosaminoglycan attachment site in core proteins fulfill the additional sequence requirements for glycosaminoglycan substitution to occur.

Similarities within the core-protein homology include acidic amino acids on the NH_2-terminal side of the Ser-Gly dipeptide and a glycine residue on the COOH-terminal side, one residue removed from the Ser-Gly dipeptide. This latter feature, which is invariant in the core proteins, produces the sequence Ser-Gly-Xaa-Gly, where Xaa is any amino acid. To test the importance of these homologies, we synthesized peptides that duplicate various features of the homologous region and assayed them for their ability to serve as acceptors for xylosyltransferase-catalyzed transfer of UDP-xylose to the peptide. This assay was chosen because xylosyltransferase is likely to be the molecule recognizing the core-protein sequences to be substituted with a glycosaminoglycan chain (18).

Synthetic Peptides as Xylosyl Acceptors. Eleven synthetic peptides were examined in the *in vitro* xylosyltransferase acceptor assay (Table 1). Two peptides from PG40 that span the sequence substituted in the core protein were excellent xylosyltransferase acceptors with V_{max}/K_m ratios of 2×10^8 and 5×10^8 (peptides 1 and 2). An efficient acceptor is characterized by a high V_{max} or a low K_m and, therefore, a high V_{max}/K_m ratio. That the acceptor activity of these peptides is specific was shown by the complete lack of acceptor activity of a peptide that contains the sequence around one of the unsubstituted Ser-Gly dipeptides of PG40 (peptide 3). The two active peptides share the sequence Asp-Glu-Ala-Ser-Gly-Ile-Gly-Pro, which contains the two sequence elements common to the three proteoglycans: the sequence Ser-Gly-Xaa-Gly and two acidic amino acids on its NH_2-terminal side.

The importance of the Ser-Gly-Xaa-Gly sequence in the acceptor peptide sequence was examined by making substitutions in the Ser-Gly-Xaa-Gly sequence of the PG40 peptide. Substitution of threonine for serine in the PG40 peptide (peptide 4) completely removed acceptor activity, indicating that only serine can be substituted by xylosyltransferase. Substitution of alanine for glycine in the Ser-Gly dipeptide portion of the PG40 peptide (peptide 5) resulted in a 100-fold reduction in acceptor activity as compared with the native PG40 peptide, indicating that the Ser-Gly dipeptide is essential for substitution of serine by xylosyltransferase. In addition, the second glycine of the Ser-Gly-Xaa-Gly consensus sequence also contributes significantly to the acceptor activity of the PG40 peptide as shown by the 90% reduction in acceptor activity when the second glycine was substituted with alanine (peptide 6). These results show that each of the consensus residues of the Ser-Gly-Xaa-Gly acceptor sequence is important for the xylosyltransferase acceptor activity.

The significance of the acidic residues for the xylosyltransferase acceptor activity was examined in peptides derived from the homologous region of the PG19 core protein, which was deemed to be likely to yield more soluble peptides than the PG40 prototype peptide after deletion of the acidic residues (Fig. 1). The PG19 homology includes two acidic residues and is located at the NH_2 terminus of the 24-fold Ser-Gly repeat of the proteoglycan. The PG19 peptide Ser-Asp-Asp-Tyr-Ser-Gly-Ser-Gly-Ser-Gly (peptide 7) was an excellent acceptor with a V_{max}/K_m comparable to that of the PG40 prototype peptide, although its activity was lower when calculated in relation to the number of serine residues or Ser-Gly-Xaa-Gly sequences. When the PG19 peptide was modified to include two alanine residues in place of the acidic residues (peptide 8), the acceptor activity was reduced 90%. The activity of this peptide is comparable to that of a Gly-Ser-Gly-Ser-Gly-Ser-Gly-Ser (peptide 9) acceptor peptide previously described (18), which lacks the acidic residues but reproduces the Ser-Gly-Xaa-Gly feature of the PG19 peptide. Therefore, the presence of acidic residues on the NH_2-terminal side of the Ser-Gly-Xaa-Gly appears to enhance the acceptor activity of the PG19 peptide.

The relative importance of each invariant feature of the consensus sequence can be readily compared when the steady-state data are presented graphically (Fig. 2). The slope of these double reciprocal plots is K_m/V_{max}, which corre-

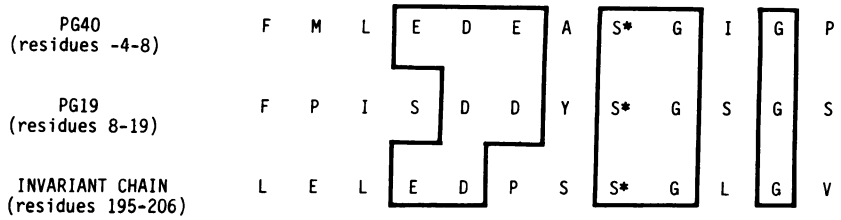

FIG. 1. Homologous sequences in PG40 and rat yolk sac tumor proteoglycans and the invariant chain of human class II major histocompatibility complex molecules. The residues thought to be important for recognition by xylosyltransferase (see text) have been boxed. The asterisks indicate the substituted serine in PG40, the presumed substituted serine in the invariant chain, and the first serine of the Ser-Gly repeat in PG19. The residue numbers refer to amino acids in the mature core proteins. The PG40 peptide is partly contained in a propeptide.

Table 1. Xylosyl acceptor activity of synthetic peptides

Peptide or protein*		K_m,	V_{max},	V_{max}/K_m,
No.	Sequence	$M \times 10^{-6}$	$cpm \cdot hr^{-1}$	$cpm \cdot M^{-1} \cdot hr^{-1}$
1	FMLEDEASGIGP	—†	—†	2.0×10^8
2	CDEASGIGPDDRD	37 ± 5.7	20,200 ± 880	$5.4 \pm 0.85 \times 10^8$
3	GTWPLKSSGIEN	0	0	0
4	FMLEDEATGIGP	0	0	0
5	FMLEDEASAIGP	—†	—†	$3.5 \pm 0.07 \times 10^6$
6	FMLEDEASGIAP	—†	—†	$2.0 \pm 0.03 \times 10^7$
7	SDDYSGSGSG	261 ± 25.0	48,600 ± 1,230	$1.9 \pm 0.02 \times 10^8$
8	SAAYSGSGSG	1,390 ± 140	28,995 ± 1,795	$2.1 \pm 0.2 \times 10^7$
9	GSGSGSGS	225 ± 11.0	6,880 ± 80	$3.1 \pm 0.2 \times 10^7$
10	FDLIAEESNVGI	0	0	0
11	AEESNVGI	0	0	0
12	Cartilage proteoglycan	2.9	32,790	1.1×10^{10}
				3.2×10^7‡
				1.1×10^8‡

*The single letter code is used for the amino acid sequences of peptides. The code is: A = Ala, C = Cys, D = Asp, E = Glu, F = Phe, G = Gly, I = Ile, K = Lys, L = Leu, M = Met, N = Asn, P = Pro, R = Arg, S = Ser, T = Thr, V = Val, W = Trp, Y = Tyr. The key sequences and individual amino acids in the peptides are shown as bold letters (see text for explanation). The proteoglycan was isolated from Swarm rat chondrosarcoma and treated with HF to remove the endogenous glycosaminoglycan chains (19).

†The regression line passes through the origin in double-reciprocal plots, precluding determination of K_m and V_{max}.

‡The 3.2×10^7 value was arrived at by calculating the activity per number of serine residues in the proteoglycan core protein (20). If the number of glycosaminoglycan chains in the core protein, which is close to 100 (18), is used, the value 1.1×10^8 is obtained. The former is probably an underestimate of the activity because all serine residues are not likely to be potential acceptors, whereas the value based on the number of glycosaminoglycan chains almost certainly overestimates the activity because all potential sites may not be substituted.

sponds to the inverse of acceptor activity. The two peptides from PG40 and PG19 that contain all of the invariant residues of the consensus sequence (i.e., acidic-acidic-Xaa-Ser-Gly-Xaa-Gly) yield relatively low slopes (Fig. 2, peptides 1 and 7) and, therefore, have high acceptor activities. Further examination of Fig. 2 shows that alteration of the first glycine residue (peptide 5) decreases the acceptor activity more than substitution of the second glycine (peptide 6) and that both of these glycines are more important for maximal acceptor activity than are the acidic residues (peptide 8). The serine is the most critical of the invariant residues, since substitution at this position by threonine completely eliminated acceptor activity. Hence, while all of the invariant elements of the consensus sequence are necessary for maximal acceptor activity, the relative importance of the various residues appears to be serine > first glycine > second glycine > acidic residues.

Peptides as Inhibitors of Xylosyltransferase. Since the acceptor assay potentially measures both recognition by the enzyme and acceptor activity, we also examined several of the inactive peptides as competitors for xylosyltransferase in peptide acceptor assays. We are particularly interested in a peptide derived from the PG19 propeptide (21) that is homologous with the 12-amino acid homology region in the core proteins. This sequence is probably not substituted with a glycosaminoglycan, since the serine is followed by an asparagine residue, not a glycine, and since peptides corresponding to this sequence were inactive as acceptors in the xylosyltransferase assay (Table 1, peptides 10 and 11). Peptide 11 (which unlike peptide 10 was soluble enough to be tested as an inhibitor) failed to inhibit xylosylation of the PG40 acceptor peptide (peptide 1), even at a 100-fold molar excess. The threonine-substituted PG40 peptide (peptide 4) also did not compete with peptide 1 for xylosyltransferase. Similarly, the unrelated Ser-Gly-containing peptide from PG40 (peptide 3) had no effect on the labeling of peptide 1 in the assay (not shown). These results suggest that any modification in acceptor peptides that reduces xylosyltransferase substitution of the peptide with xyloside also greatly diminishes binding of enzyme to the peptide.

DISCUSSION

From the results presented here, we propose that the recognition consensus amino acid sequence for the attachment of chondroitin/dermatan sulfate to protein is a sequence that has a pair or triplet of acidic amino acids closely followed by the tetrapeptide Ser-Gly-Xaa-Gly. We arrived at this sequence by comparing the amino acid sequences of known or presumed glycosaminoglycan substitution sites in three proteoglycans and by assaying synthetic peptides derived from these sequences as acceptors for xylosyltransferase.

In agreement with earlier results (18) pointing to the importance of a Ser-Gly dipeptide as the glycosaminoglycan attachment site, the serine in each of the proteoglycans is followed by glycine. Moreover, we found no or low xylosyltransferase acceptor activity in two peptides in which either the serine was substituted with a threonine or the glycine with alanine. The requirement for the glycine residue to follow the serine is different from the requirement for the attachment of O-glycosidically linked oligosaccharide units to proteins. In that case the linkage is also to serine (or threonine), but there does not appear to be any requirement for a glycine next to it (22).

From our results it is apparent that the attachment of a xyloside residue to the appropriate serine requires that the substituted serine be in the minimal protein sequence context of Ser-Gly-Xaa-Gly. Although a peptide containing the sequence Ser-Gly-Xaa-Ala within the context of the PG40 consensus sequence retained 10% of the activity of the original peptide, this represents a chemically similar substitution, and other less-similar amino acids in this position might well have a greater effect. The importance of the Ser-Gly-Xaa-Gly sequence for *in vivo* glycosaminoglycan substitution also is strongly suggested by the presence of this sequence in all of the three proteoglycans compared (4, 11, 14). Moreover, a glycosaminoglycan-carrying peptide from the large aggregating cartilage proteoglycan also contains this sequence (23). Although the significance of the Ser-Gly-Xaa-Gly sequence has not been previously recognized, earlier results obtained with $(Gly\text{-}Ser)_n$ peptides as xylosyl acceptors

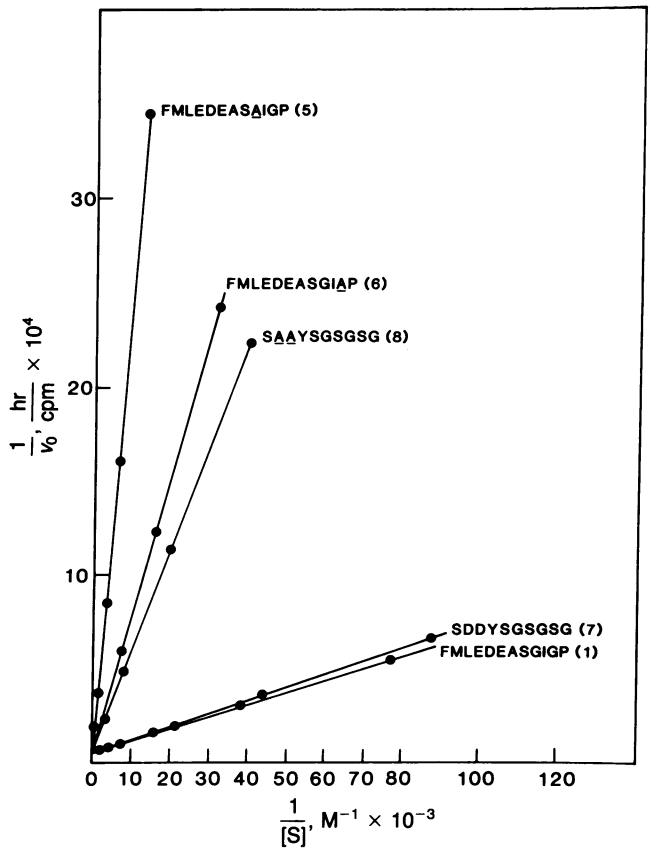

FIG. 2. Double-reciprocal plots of steady-state kinetic data obtained by testing synthetic peptides in a xylosyltransferase assay. The amino acid sequences of the peptides are shown. The number in parentheses refers to the number of each peptide in Table 1, which gives a summary of the acceptor assay data. The slope of the double-reciprocal plot is K_m/V_{max}, which represents the inverse of acceptor activity.

support the importance of this sequence (18) because such peptides contain the Ser-Gly-Xaa-Gly sequence with serine occupying the Xaa position.

An additional example of the significance of the Ser-Gly-Xaa-Gly sequence at the protein level is silk fibroin, which *in vitro* can serve as a xylosyltransferase acceptor (18, 24) but which *in vivo* is apparently not substituted with glycosaminoglycan chains. Silk fibroin is a large protein containing repeats of the amino acid sequence Gly-Ala-Gly-Ala-Gly-Ser (25), which contains the Ser-Gly-Xaa-Gly sequence, as in Gly-Ala-Gly-Ala-Gly-**Ser-Gly-Ala-Gly**-Ala-Gly-Ser.

Acidic amino acids at the NH_2-terminal side of the Ser-Gly-Xaa-Gly sequence apparently further enhance recognition and substitution of the Ser-Gly-Xaa-Gly serine by xylosyltransferase. Each of three proteoglycans (Fig. 1) has acidic amino acids adjacent to the glycosaminoglycan-substituted serine(s), and we found that peptides containing the acidic amino acids have much higher acceptor activities than do peptides lacking them. In PG19, the acidic amino acids precede the 24-fold Ser-Gly dipeptide repeat, suggesting that the xylosyltransferase first recognizes the beginning of the Ser-Gly repeat and then proceeds to substitute the 14 or so serine residues that are known to carry a glycosaminoglycan chain in this proteoglycan (6).

The PG19 precursor polypeptide contains a second site that is strongly homologous with the consensus recognition sequence; however, this sequence lacks the glycine of the Ser-Gly dipeptide, and peptides duplicating the sequence are not capable of serving as an acceptor. It may be that this peptide sequence was a glycosaminoglycan substitution site in a primordial gene for PG19 but has subsequently lost this activity through mutations resulting in replacement of the critical glycine residue.

One further sequence feature in the core protein homology is the presence of hydrophobic amino acids at the NH_2-terminal side of the pair or triplet of acidic amino acids. Our results show that these hydrophobic residues do not increase the acceptor activity at the peptide level. However, the hydrophobic residues may well be important in increasing the availability of the adjacent substitution site at the surface of the core protein.

In addition to the amino acid sequences of the chrondroitin/dermatan sulfate proteoglycans discussed here, complete sequences are available for two proteins that can, at least under some circumstances, be substituted with heparan sulfate. These proteins are fibronectin (26) and the transferrin receptor (27). Moreover, the product of the *Drosophila* per locus gene, which controls the circadian rhythm and other rhythmic functions of the fly, appears to be a heparan sulfate proteoglycan (9). In each of these proteins, there is at least one Ser-Gly-Xaa-Gly sequence, but it is not known whether these sequences serve as sites of glycosaminoglycan substitution or whether the recognition sequence we have now identified in chrondroitin sulfate proteoglycans is utilized by cells for heparan sulfate attachment. The *Drosophila* per gene product also contains numerous Thr-Gly repeats where the haparan sulfate substitution could take place. Our results suggest that threonine residues cannot be substituted by the mammalian xylosyltransferase we have been using, but it is possible that a *Drosophila* enzyme capable of substituting threonine residues exists.

The excellent activity of the synthetic peptides as xylosyltransferase acceptors relative to the core protein of the major proteoglycan from cartilage suggests that the requirements for the recognition signal can be contained in primary sequence information. In this regard, this recognition signal is similar to some other signals for intracellular protein processing and transport, such as those for N-linked glycosylation (28), routing to various organelles (29), and degradation (30, 31).

Studies with gene transfer and site-directed mutagenesis should answer questions regarding the similarity or nonsimilarity of the core-protein recognition sequences for the attachment of chondroitin/dermatan sulfate and heparan sulfate/heparin glycosaminoglycan chains. Information on the role of glycosaminoglycans in proteins might also be obtained by introducing the recognition sequence we have identified here into proteins that ordinarily would not be proteoglycans to see whether such modified proteins will become substituted with glycosaminoglycan and what effect this would have on their biosynthetic and functional properties.

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