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BIOSYNTHESIS OF PROTEOCHONDROITIN SULFATE: SUBSTRATE REQUIREMENTS FOR FORMATION OF TWO INDEPENDENT SPECIES*

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ABSTRACT

Formation of two species of [^{14}C]proteochondroitin sulfate has previously been demonstrated with UDP-D-[^{14}C]glucuronic acid and UDP-*N*-acetylgalactosamine as substrates with a microsomal preparation from chick-embryo epiphyseal cartilage. A large species of [^{14}C]proteoglycan that appeared at an earlier stage of synthesis was excluded on Sepharose CL-2B, indicating that it was larger than proteoglycans found in cartilage matrix. Another newly synthesized, smaller [^{14}C]proteoglycan species also formed was retarded on Sepharose CL-2B, and appeared to be at a later stage of synthesis. A 6-h pulse-chase experiment using UDP-[^{14}C]GlcA and UDP-GalNAc followed by cold UDP-GlcA demonstrates that there was no conversion of the large [^{14}C]proteoglycan to the small [^{14}C]proteoglycan. Sulfation of the newly formed large and small [^{14}C]proteoglycans with adenylyl sulfate 3′-phosphate also did not alter their chromatographic patterns, indicating that sulfation did not trigger any post-synthetic size modification. Synthesis with lower concentrations of the sugar nucleotides resulted in a disproportionate diminution in formation of the large proteoglycan. The apparent K_m values for UDP-GlcA for the formation of large and small proteoglycans were 0.055 and 0.015mM, respectively. Concentration requirements for UDP-GalNAc also showed a similar 4-fold difference. These results indicate that, even though the large proteoglycan was at an earlier stage of synthesis, it was not a precursor to the small proteoglycan, and that these proteoglycans represent two separately synthesized species.

INTRODUCTION

Although cartilage chondroitin sulfate proteoglycan is known to be polydisperse, characterizations in general have focused on a species capable of aggrega-

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tion[1–6]. However, there have been a number of reports (reviewed[1–4]) concerning the occurrence of other chondroitin sulfate proteoglycans that differ in size and composition. These proteoglycans, which have been found in cartilage and other tissues, are less well defined, but potentially of considerable importance. The polydispersity is due to differences in the size, number, and sulfation of glycosaminoglycan chains and apparent differences in size and structure of core protein. This heterogeneity could represent different stages of synthesis in a single or a few species of proteoglycan. Thus a smaller proteoglycan could be a product of protease degradation where the core protein has been trimmed by one or more specific proteases. Proteoglycans could also be processed by removal of glycosaminoglycan chains, but this is unlikely as free glycosaminoglycan chains or oligosaccharides have not been found in matrix. Alternatively, the heterogeneity could be a reflection of products from multiple different genes resulting in multiple entities, rather than modifications in one or a few main primary structures.

Studies on the cell-free translation of m-RNA have indicated that a core-protein product of translation is considerably larger than core proteins found in cartilage matrix[7,8], suggesting a possible precursor–product relationship. The presence of such large precursor molecules or "proforms" has been established in collagen and glycoprotein biosynthesis[9–13]. Moreover, precursor molecules for chondroitin sulfate proteoglycan and heparan sulfate proteoglycan have recently been described[14–16]. Modifications of these "proforms" is post-translational and requires the action of proteases.

Earlier work from our laboratory has indicated that a large species of [^{14}C]proteoglycan was synthesized in a cell-free chick cartilage system during incubation of a microsomal preparation with appropriate sugar nucleotides[17]. Although considerably larger than the characteristic monomer chondroitin sulfate proteoglycan of cartilage matrix, it was not an aggregate. The structure of the newly formed [^{14}C]glycosaminoglycan chains of this large proteoglycan indicated that it had been at an early stage of synthesis, since the product consisted mainly of large [^{14}C]chondroitin chains on a primer that did not already have chondroitin sulfate chains. In addition, a non-aggregatable [^{14}C]proteoglycan was found that was smaller than proteoglycan monomer of chick limb-bud extracellular matrix. The primer for this smaller [^{14}C]proteoglycan had been at a later stage of synthesis, since the product consisted of short additions of [^{14}C]chondroitin to sulfated chondroitin chains that were already of considerable size. As the two [^{14}C]proteoglycans were not related to one another by aggregation–disaggregation and were products from primers that appeared to be at different stages of synthesis, we questioned whether the larger proteoglycan might represent a precursor of the smaller proteoglycan. Alternatively, we questioned whether the two species might constitute two separate entities.

We have now examined the formation of these two species of proteoglycans to determine whether there was any conversion of the large proteoglycan to small proteoglycan in a chase-period following synthesis. We have examined sulfation of the newly formed glycosaminoglycan chains to see if this promoted modification of

the large proteoglycan to a smaller form. We have also examined the synthesis of the glycosaminoglycan portions using a range of sugar nucleotide concentrations. Our results indicate that the synthesis of these two species occurs separately and that there is no interconversion between the two. Moreover, differences in apparent K_m values for the sugar nucleotides indicated that there might be significant differences in loci for synthesis.

EXPERIMENTAL

Materials. — UDP-D-[^{14}C]Glucuronic acid (UDP-GlcA) and UDP-*N*-acetyl-[^{3}H]galactosamine (UDP-GalNAc) were purchased from New England Nuclear (Boston, MA). Unlabeled UDP-GalNAc was prepared as previously described[18]. Adenylyl sulfate 3′-phosphate (PAPS) and UDP-GlcA were obtained from Sigma Chemical Co. (St. Louis, MO). Chondroitin 4-sulfate, chondroitin 6-sulfate, chondroitin ABC lyase, and the various disaccharides produced by degradation with chondroitin ABC lyase were purchased from Miles Laboratories Inc. (Naperville, IL). Fourteen-day-old chick embryos were obtained from SPAFAS Inc. (Norwich, CT). Calf nasal proteoglycan $A_1D_1D_1$ was generously provided by Dr. L. Rosenberg (New York, NY).

Methods. — A microsomal fraction sedimenting between 10,000 and 105,000*g* was prepared from tibial and femoral epiphyses of fourteen-day-old chick embryos as previously described[17]. Typical incubation mixtures contained 0.05M 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.5, 0.015M $MnCl_2$; various concentrations of UDP-[^{14}C]GlcA (340 mCi/mmol) and UDP-GalNAc or UDP-[^{3}H]GalNAc (1070 mCi/mmol) and UDP-GlcA; and 4–10 μL of the microsomal fraction, in a total volume of 12–25 μL. In sulfation experiments, the concentrations of PAPS in the mixture was 3.0mM. Incubations were terminated by the addition of 0.108 mL of a 1.25% deoxycholate solution buffered with 0.2M Tris HCl, pH 9.0 containing 0.25mM EDTA. Reaction mixtures were vortexed and kept overnight at room temperature to assure the complete extraction of proteoglycans.

Aliquots of the extracted [^{14}C]proteoglycans were routinely mixed with 2–3 mg of $A_1D_1D_1$ calf nasal proteoglycan as a marker and then chromatographed on a column of Sepharose CL-2B (1 × 71 cm) equilibrated with 0.5M ammonium hydrogencarbonate, pH 7.8. Radioactivity was measured in a scintillation spectrometer after adding aliquots from column fractions to 10 mL of scintillant. The modified carbazole method of Bitter and Muir[19] was used to determine the position of proteoglycan and glycosaminoglycan standards.

Pooled peak fractions from chromatography on Sepharose CL-2B were lyophilized and treated overnight at 37° with 1% pancreatin (Viobin Corporation, Monticello, IL) in 0.05M Tris HCl, pH 8.5, to free the glycosaminoglycan chains. Aliquots of pancreatin-treated [^{3}H]glycosaminoglycan and [^{14}C]glycosaminoglycan were chromatographed on a column of DEAE-Cellulose (1 × 12 cm) and eluted with a logarithmic gradient of 0.05–1.0M LiCl in 0.05M sodium acetate, pH 5.5.

Labeled proteoglycans or glycosaminoglycans were mixed with chondroitin 4-sulfate and chondroitin 6-sulfate and degraded with chondroitin ABC lyase[20]. Reaction mixtures were then spotted on Whatman No. 1 paper and chromatographed in 2:3:1 butanol–acetic acid–M ammonium hydroxide for identification of the degree of sulfation. Standards were located by viewing the u.v.-absorbing spots.

RESULTS

A pulse-chase technique was employed to examine possible processing of proteoglycan subsequent to polymerization. Following incubations (2 h) of microsomal preparations with UDP-[^{14}C]GlcA (0.5mM) and UDP-GalNAc (mM), two species of [^{14}C]proteoglycans were formed (Fig. 1). The first was exluded from a Sepharose CL-2B column and was considerably larger than a proteoglycan monomer standard, while the second smaller heterogenous species eluted later from the gel. When the proteoglycan monomer standard was added to the incubation mixtures it retained its capacity for aggregation, indicating that no significant proteases were present. [^{14}C]Proteoglycan obtained after a 6-h chase with cold UDP-GlcA is also shown in Fig. 1. The patterns on chromatography were identical, indicating that there had been no modification of the large proteoglycan to the smaller species. When such protease inhibitors as phenylmethylsulfonyl fluoride were added, or when the [^{14}C]proteoglycans were eluted with 4M guanidine

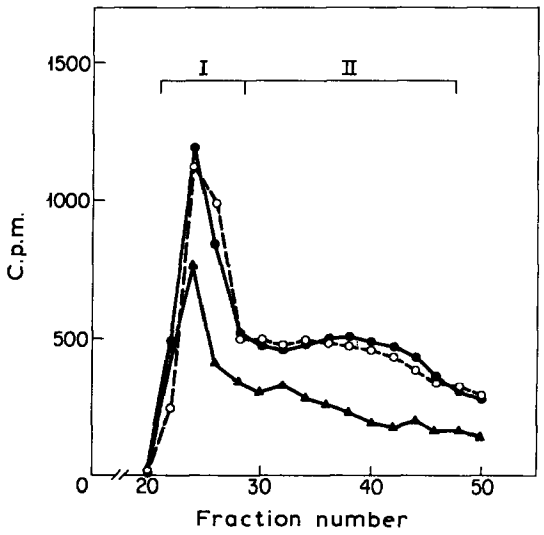

Fig. 1. Sepharose CL-2B chromatogram of [^{14}C]proteoglycan formed in absence or presence of PAPS and after a 6-h chase. Aliquots of deoxycholate-extracted [^{14}C]chondroitin proteoglycan were chromatographed on a column of CL-2B Separose (1.0 × 71.0 cm) with 0.5M ammonium hydrogencarbonate, pH 7.8, as the eluant. Fractions of 0.75 mL were collected and analyzed for radioactivity. [^{14}C]Proteoglycans formed with no PAPS ●—●; [^{14}C]proteoglycans formed after a 6 h chase period, ○--○. [^{14}C]Proteoglycans formed in the presence of PAPS, ▲—▲.

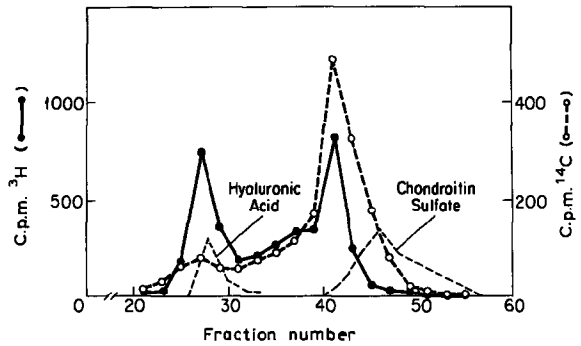

Fig. 2. DEAE-cellulose chromatogram of [^{3}H]glycosaminoglycans derived from large [^{3}H]proteoglycan and [^{14}C]glycosaminoglycans derived from small [^{14}C]proteoglycan. [^{3}H]Glycosaminoglycan derived from the large [^{3}H]proteoglycan and [^{14}C]glycosaminoglycan derived from the small [^{14}C]proteoglycan (as described in text) were chromatographed together with hyaluronic acid and chondroitin 4-sulfate standards on a DEAE-cellulose column (1 × 12 cm) equilibrated with 0.05M sodium acetate, pH 5.5, containing 0.05M LiCl. The column was eluted with a logarithmic gradient of 0.05–1.0M LiCl. Fractions of 2.0 mL were collected and assayed for radioactivity.

hydrochloride instead of 0.5M ammonium hydrogencarbonate, the patterns were identical.

A microsomal preparation was incubated with UDP-[^{14}C]GlcA and UDP-GalNAc. A duplicate preparation was incubated with UDP-GlcA and UDP-[^{3}H]GalNAc. Following 2 h of incubation, the labeled proteoglycans of each were chromatographed separately on Sepharose CL-2B as in Fig. 1. The large proteoglycan (fractions 20–28) from the chromatogram of the ^{3}H-labeled products was combined with the small proteoglycan (fraction 30–48) from the chromatogram of the ^{14}C-labeled product. The mixture was treated with pancreatin and the resulting [^{3}H]glycosaminoglycans and [^{14}C]glycosaminoglycans were chromatographed together on a column of DEAE-cellulose. Direct comparison of the glycosaminoglycan chains may be seen in Fig. 2. Approximately 50% of the [^{3}H]glycosaminoglycans derived from the large [^{3}H]proteoglycan eluted near hyaluronic acid. This 50% represents new synthesis of large non-sulfated glycosaminoglycan chains. In contrast, <10% of the [^{14}C]glycosaminoglycan derived from the smaller [^{14}C]proteoglycan was found in this area. Thus the smaller [^{14}C]proteoglycan consisted almost entirely of [^{14}C]glycosaminoglycan addition onto pre-existing chondroitin sulfate chains of substantial size. These results confirmed our earlier work, which used various fractions from microsomal preparations[17].

A microsomal preparation was incubated with PAPS in addition to the sugar nucleotides to see if sulfation of newly synthesized glycosaminoglycan affected the size of the large proteoglycan. Results are shown in Fig. 1. When PAPS was present in the mixture we consistently found slightly less synthesis of glycosaminoglycans. However, the chromatographic pattern was essentially identical, indicating that there was no apparent difference in the proportion of the large to the small proteoglycan.

Chondroitin ABC lyase was used to degrade both large and small proteoglycans formed in the presence and absence of PAPS. Chromatography of disaccharide products resulting from these degradations (Fig. 3) indicated that, when PAPS was present, 45% of the [^{14}C]GalNAc residues in the large proteoglycan had been sulfated during the course of synthesis and 25% of [^{14}C]GalNAc in the small proteoglycan had been sulfated. Similar results were found when PAPS was added to incubation mixtures subsequent to incubation with the sugar nucleotides. These findings clearly indicated that sulfation did not promote the conversion of large [^{14}C]proteoglycan to small [^{14}C]proteoglycan. Should this conversion have occurred we would have seen a shift of the pattern on Sepharose CL-2B plus a greater degree of sulfation in the small [^{14}C]proteoglycan than found in the large [^{14}C]proteoglycan.

The pulse-chase and sulfation results indicated that neither proteoglycan species was derived from the other, suggesting that the two species may have been produced in different subcellular compartments. This in turn suggested that alterations in the conditions of synthesis might affect relative proportions of these species. To examine this, microsomal preparations were incubated with varying

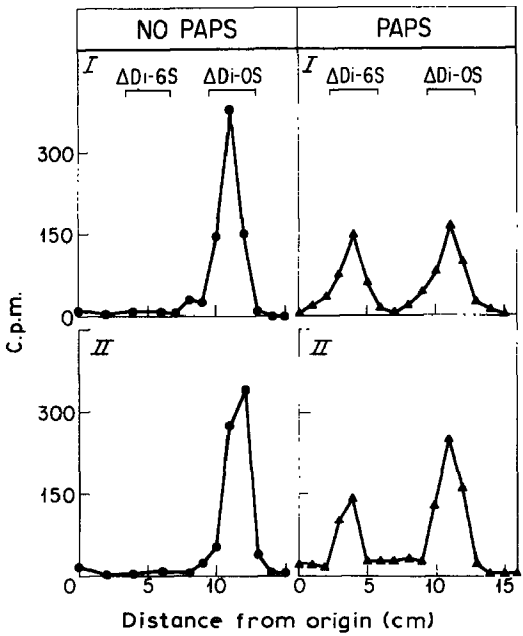

Fig. 3. Paper chromatography of products following degradation of [^{14}C]proteoglycan with chondroitin ABC lyase. Newly formed [^{14}C]chondroitin (●—●) and sulfated [^{14}C]chondroitin (▲—▲) were degraded with chondroitin ABC lyase and chromatographed as described in the Experimental section, together with disaccharide standards. Strips of 1 cm were eluted and assayed for radioactivity. (I) Products of degradation of peak I (Fig. 1, fractions 20–28) [^{14}C]chondroitin formed in the presence and absence of PAPS. (II) Products of degradation of peak II (Fig. 1, fractions 30–48) [^{14}C]chondroitin formed in the presence and absence of PAPS.

concentrations of UDP-[^{14}C]GlcA (0.002–1.0mM) while the UDP-GalNAc concentration was fixed at 1.0mM. Products of the incubation were chromatographed on Sepharose CL-2B as described in Fig. 1, and the relative amounts of large [^{14}C]proteoglycan and small [^{14}C]proteoglycan were determined. Fig. 4 shows the chromatogram for a high, a low, and an intermediate concentration of UDP-[^{14}C]GlcA. Approximately 40% of the [^{14}C]proteoglycan formed with 0.5mM UDP-[^{14}C]GlcA (high concentration) was large (fraction 20–28), whereas <10% of the [^{14}C]proteoglycan formed with 0.05mM UDP-[^{14}C]GlcA (low concentration) was of this size. Experiments were also conducted with a 500-fold concentration range of UDP-[^{3}H]GalNAc while UDP-GlcA concentration was fixed at 1.0mM. As with the varying concentrations of UDP-[^{14}C]GlcA, chromatography of products on Sepharose CL-2B indicated that relatively less of the large proteoglycan was formed at lower concentrations.

The rate of synthesis of [^{14}C]chondroitin at various concentrations of UDP-[^{14}C]GlcA in the presence of a saturating concentration of UDP-GalNAc is presented in Fig. 5. As expected, when the concentration of the UDP-[^{14}C]GlcA

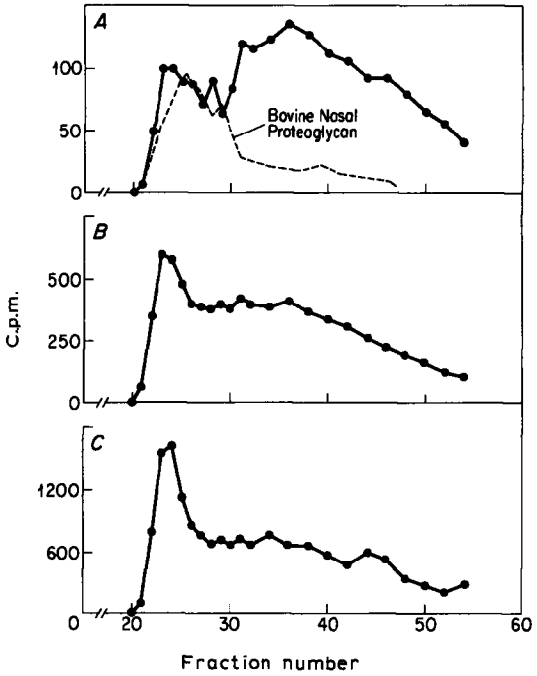

Fig. 4. Sepharose CL-2B chromatography of [^{14}C]proteoglycan formed with varying concentrations of UDP-[^{14}C]GlcA. Reaction mixtures containing (A) 0.005, (B) 0.05, or (C) 0.5mM UDP-[^{14}C]GlcA; 1.0mM UDP-GalNAc; 0.05M MES buffer, pH 6.5; 0.015M $MnCl_2$; and 4 μL of the microsomal preparation in a total volume of 12 μL were incubated for 2 h at 37°. Reactions were terminated and extracted as described under Experimental. Aliquots of 0.09 mL were then removed and chromatographed together with standard bovine proteoglycan monomer ($A_1D_1D_1$) on columns of Sepharose CL-2B as described in the Experimental section.

was increased, the number of counts incorporated into the two different size species of [^{14}C]proteoglycan increased until a plateau value (V_{max}) was reached. Incorporation was linear for the duration of all incubations. As shown in the inset, the apparent K_m value of UDP-[^{14}C]GlcA for the formation of the large and of the small proteoglycans were calculated to be 5.5 and 1.5 × 10^{-5}M, respectively, almost a 4-fold difference. The K_m determinations of UDP-[^{3}H]GalNAc were complicated by the utilization of UDP-[^{3}H]GalNAc for glycoprotein synthesis, and are not shown.

DISCUSSION

Our initial examination involved a pulse-chase of the labeled material to see if any modification in size had occurred following synthesis of [^{14}C]GlcA-labeled proteoglycan. The results presented in Fig. 1 indicated that there was no change in

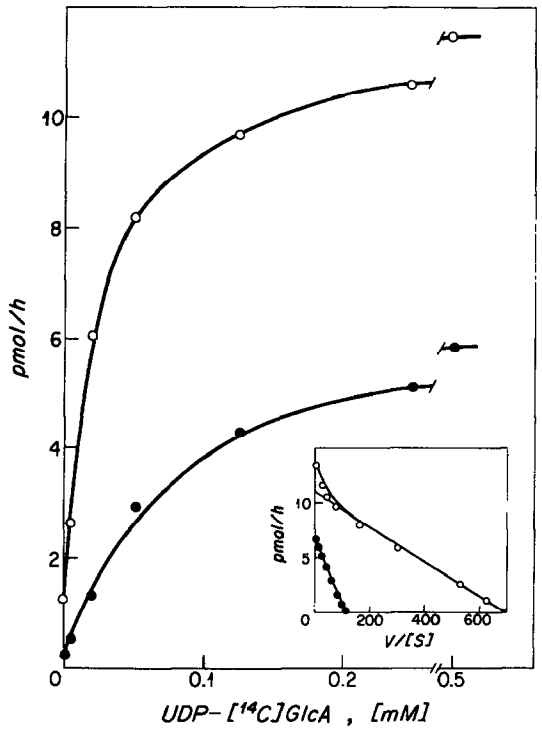

Fig. 5. Formation of large and small [^{14}C]proteoglycans with varying concentrations of UDP-[^{14}C]GlcA. Mixtures containing 4 μL of the microsomal preparation; 1.0mM UDP-GalNAc; 0.05M MES buffer, pH 6.5; 0.015M $MnCl_2$; and varying concentrations of UDP-[^{14}C]GlcA in a total volume of 12 μL were incubated for 2 h at 37°. Reactions were terminated, and aliquots were chromatographed on Sepharose CL-2B as described in Fig. 4. The amount of [^{14}C]chondroitin proteoglycan in Fig. 4, fractions 20–28 (●—●) and Fig. 4, reactions 30–48 (○—○) was quantitated and plotted as a function of UDP-[^{14}C]GlcA concentrations. Inset: Eadie Hofstee plot, of the same data.

the chromatographic pattern, even after a 6-h chase. Thus there was no conversion of larger [^{14}C]proteoglycan to the smaller form. We also questioned whether sulfation might trigger a size-modification process, and therefore we examined the chromatographic pattern of [^{14}C]proteoglycan formed in both the presence and absence of PAPS. Our results indicated that the process of sulfation, either concurrent with or subsequent to polymerization, did not affect the chromatographic pattern (Fig. 1), indicating that there was no change in size. Thus the two species did not appear to be related as precursor–product, even though they represented polymerization of chondroitin on primers at different stages of synthesis.

We found that it was possible to modify or control the relative synthesis of the large and small proteoglycans by varying the concentrations of sugar nucleotides (Figs. 4 and 5). At high UDP-sugar concentrations, the large proteoglycan was a large proportion of the total, whereas at low UDP-sugar concentrations it was much less in proportion. Thus higher substrate concentrations were necessary for the synthesis of the larger proteoglycan.

As the newly synthesized [^{14}C]glycosaminoglycans formed on both large and small proteoglycan primers contain identical disaccharide repeating-units, it is unlikely that there would be differences in glycosyltransferases for the two proteoglycans. Alternatively, the different substrate requirements could be due to differences in enzyme–substrate affinities for the primers. This would mean that the long chondroitin sulfate primers of the small species were better substrates for polymerization than were the short primers of the large species. However, this is directly contrary to previous results found with glycosaminoglycans or oligosaccharides as substrates for synthesis, where smaller glycosaminoglycans or oligosaccharides were shown to be much more effective as acceptors for GlcA and GalNAc[21]. Another possibility might be differences in the accessibility of the sugar nucleotide substrates to the respective primers. Sugar nucleotides have been shown to be actively transported in membranes during glycoprotein synthesis[22–25]. Such a transport system, perhaps mediated by carrier proteins with varying affinities for the sugar nucleotides, could be an explanation for the apparent concentration differences of sugar nucleotides for biosynthesis of proteoglycans.

The presence of several proteoglycan species in chick-embryo cartilage has been examined by others[15,26]. One report[15] has indicated that most of the chick proteoglycans were aggregatable and fast sedimenting (designated as PG-H). As such, this population was similar to the aggregatable proteoglycans of mammalian cartilage. A slower-sedimenting population (PG-L) was also described and was further subdivided (PG-Lb and PG-Lt). PG-Lb was formed from a precursor molecule and seemed to be related to proteodermatan sulfate rather than proteochondroitin sulfate. PG-Lt was shown to contain disulfide-bonded collagenous polypeptide[15,27] which was later identified[28] as type IX collagen. However, neither of our [^{14}C]proteoglycan species appeared to be similar to any of these in size or aggregability. Moreover, they were not sensitive to 1,4-dithiothreitol[17], indicating that no disulfide-bonded collagenous peptides were present.

We do not know the function of the large proteoglycan that is formed in the microsomal system. However it is at an early stage of synthesis and appears to represent a substantial portion of the primers for proteoglycan formation. As similar large proteoglycans have not been found in matrix, the possibility remains that this represents a "proform". In contrast, the smaller proteoglycan formed in the microsomal system consists of the addition of chondroitin sulfate to pre-existing proteoglycans already containing chondroitin sulfate chains of substantial size. This addition to fully formed proteoglycan could represent an artifact of the system, rather than representing a proteoglycan that would be related to matrix proteoglycan.

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