Formation of two species of nascent proteochondroitin in separate loci of a microsomal preparation from chick-embryo epiphyseal cartilage

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The potential relationship of an intact membrane organization to the synthesis of chondroitin was examined before and after modification of a chick-embryo cartilage microsomal system with the non-ionic detergent Triton X-100. Incubations with labelled UDP-GlcA and UDP-GalNAc indicated that Triton X-100 had little effect on the amount of chondroitin synthesized to form one species of large proteochondroitin (Type I). However, Triton X-100 had a marked stimulatory effect on the formation of another smaller species of proteochondroitin (Type II). Presence of this detergent during chondroitin polymerization also resulted in chains that were slightly smaller. Neither of the two proteochondroitin species were collagenase-sensitive, nor did they contain dermatan-like regions. Thus in these respects they were unlike the small proteochondroitins (PG-Lb or PG-Lt) that have been found in chick-embryo cartilage. They also differed greatly in size from these small proteoglycans as well as from the large aggregatable proteochondroitin (PG-H) from the same source. Synthesis of the larger (Type I) proteochondroitin species was not affected by prior treatment of the microsomes with chondroitin ABC lyase at concentrations sufficient for elimination of synthesis of most of the smaller (Type II) proteochondroitin species. Use of chondroitin ABC lyase subsequent to synthesis of the chondroitin also resulted in preferential degradation of the smaller species. Thus there were differences in formation and limitation in access of the chondroitin ABC lyase to the two species, consistent with other differences described previously. These results indicate that there are separate loci within the microsomal membranes for synthesis of the two species.

INTRODUCTION

We have previously used UDP-[¹⁴C]GlcA and UDP-[³H]-GalNAc with and without 3'-phosphoadenylyl phosphosulphate in order to examine the biosynthesis of chondroitin/chondroitin sulphate and proteochondroitin/chondroitin sulphate by microsomal preparations from chick-embryo epiphyseal cartilage [1–6]. Two populations of proteochondroitin/chondroitin sulphate primer were observed in the formation of the chondroitin glycosaminoglycan [5,6]. The first was at an earlier stage of synthesis and consisted of glycosaminoglycan linkage regions on a protein core of undetermined size, to which large chondroitin chains (estimated by gel filtration to be an average of 35–40 kDa) were then added by the microsomal system [3]. In contrast, the second population consisted of proteochondroitin sulphate already containing glycosaminoglycan chains of substantial size (estimated by gel filtration to be an average of 20–25 kDa) to which large chondroitin oligosaccharides or short glycosaminoglycans (average of 20–30 disaccharide units) were added in these same incubations [3]. The substantial sizes of the additions were also confirmed by identification of chondroitin ABC lyase degradation products [4].

After completion of chondroitin polymerization on these primers, the first population (Type I) of nascent proteochondroitin/chondroitin sulphate (mostly excluded from Sepharose CL-2B) was larger than bovine nasal-septa proteochondroitin sulphate monomer [5] and larger than the larger proteochondroitin sulphate (PG-H) that has been found in chick-embryo cartilage matrix [7]. The second population (Type II) of nascent proteochondroitin/chondroitin sulphate was retarded somewhat by the gel [5,6], with a K_{av} indicating that it was slightly

smaller than PG-H, but considerably larger than the smaller proteochondroitin sulphate species (PG-Lb and PG-Lt) that have been found in chick-embryo cartilage matrix [8,9]. The final size of the completed chondroitin chains was similar for both of these nascent proteochondroitin/chondroitin sulphate populations, indicating that the larger proteoglycan had more chains rather than longer chains. The larger proteochondroitin/chondroitin sulphate had microsomal characteristics different from those of the smaller, in that a portion was found to be released into solution or fine suspension after synthesis, and another portion could only be completely extracted from the microsomal preparation with deoxycholate or 8 M-urea [5]. By contrast, little of the second population of smaller proteochondroitin/chondroitin sulphate was released into solution/suspension, whereas most of the remainder could be extracted with 4 M-guanidinium chloride and did not require 8 M-urea for extraction. Neither species appeared to be in an aggregated form, nor was either species aggregatable with hyaluronic acid. The first species was not a precursor of the second species, even though the primer was at an earlier stage of synthesis [6].

We also demonstrated that the relative synthesis of these two nascent species varied with the concentration of sugar nucleotide substrates utilized in the microsomal preparation [6], so that a higher concentration of sugar nucleotides was required for maximum synthesis of the larger proteochondroitin than was needed for maximum synthesis of the smaller proteochondroitin. The reason for this was not apparent, but we questioned whether access of substrates to the membrane-bound enzyme and primer for the large proteochondroitin might be more limited than was access to the membrane-bound enzyme and primer for the smaller species. If so, this might suggest that the larger proteo-

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chondroitin primer was 'buried' in a less accessible portion of the microsomes.

Since the two species of microsomal nascent proteochondroitins had detergent-extraction characteristics different from each other, one might also expect that detergent treatment of the microsomal preparation during chondroitin sulphate synthesis could result in different patterns of disruption or modification. We previously found that Triton X-100 had only a minimal effect on polymerization of chondroitin by microsomal preparations from cultured mast cells [10]. However, the synthesis of two forms of proteoglycan in the chick microsomal cartilage rather than the single form synthesized by the mast-cell microsomes [10,11] suggested that there might be considerable variation in polymerization patterns, and that Triton X-100 might affect the membrane organization in the cartilage microsomal system.

We have now shown that neither of the nascent chick-embryo cartilage proteochondroitins contain collagen-like or dermatan-like portions such as has been described for PG-Lb and PG-Lt. We have shown that the detergent, Triton X-100, markedly stimulates synthesis of the smaller proteochondroitin, but has no effect upon synthesis of the larger, and has no effect on the K_m for the sugar nucleotides in the synthesis of either species. We have also now treated microsomal preparations with small amounts of chondroitin ABC lyase to demonstrate that the larger nascent proteochondroitin is synthesized in a relatively chondroitinase-resistant compartment of the membranes, separate from the portion of the membranes where the nascent smaller proteochondroitin is synthesized.

MATERIALS AND METHODS

UDP-[^{14}C]GlcA and UDP-[^3H]GalNAc were purchased from du Pont-New England Nuclear (Boston, MA, U.S.A.). Non-radioactive UDP-GlcA, UDP-GalNAc, and 3'-phosphoadenylyl phosphosulphate were purchased from Sigma (St. Louis, MO, U.S.A.). Hyaluronic acid, chondroitin 4-sulphate, chondroitin disaccharide, chondroitin AC lyase and chondroitin ABC lyase were purchased from ICN Immunobiologicals (Lisle, IL, U.S.A.). *Clostridium histolyticum* collagenase was purchased from Worthington (Freehold, NJ, U.S.A.) and was tested for activity on a standard of 17-day-old chick-embryo calvaria [^{14}C]collagen (Type I) obtained from Dr. Barbara Smith (Boston, MA, U.S.A.). Chondroitin tetrasaccharide and hexasaccharide were prepared as previously described [12].

A microsomal fraction sedimenting between 10000 and 105000 g was prepared from the epiphyses of 100 17-day-old chick embryos as previously described [1], and was stored at -20°C until utilized.

Typical reaction mixtures containing 0.05 M-Mes buffer, pH 6.5, 0.015 M-MnCl₂, 0.2 mM-UDP-[^{14}C]GlcA (350×10^6 c.p.m./ μmol) plus 0.2 mM-UDP-GalNAc or 0.2 mM-UDP-[^3H]GalNAc (930×10^6 c.p.m./ μmol) plus 0.2 mM-UDP-GlcA and 12 μl of chick cartilage microsomal preparation (0.35 mg of protein), in a total volume of 45 μl , were incubated in the presence or absence of Triton X-100 (1%) at 37°C .

Concentrations of sugar nucleotides varying from 0.005 mM to 0.5 mM were also used in order to compare their apparent K_m (determined as described previously [6]) in the presence of Triton X-100 with their apparent K_m in its absence. In order to measure synthesis, aliquots were removed at various times, spotted on to Whatman no. 1 paper and chromatographed in ethanol/1 M-ammonium acetate (5:2, v/v), pH 7.8, overnight. After chromatography, the origins were eluted with 0.5 M-NaOH at room temperature overnight and portions were counted for radioactivity in a liquid-scintillation spectrometer. This method, followed by neutralization with acetic acid, freeze-drying and

desalting on a Sephadex G-25 column, was also used to obtain ^3H - and ^{14}C -labelled glycosaminoglycans for further characterization.

In order to obtain the proteoglycans quantitatively, the reaction mixtures were extracted with 4 M-guanidinium chloride and 2% CHAPS and then desalted over a Sephadex G-50 column. The proteoglycans were then chromatographed on a Sepharose CL-2B column with 0.5 M-NH₄HCO₃ as the running buffer, and assayed with a liquid-scintillation spectrometer. Fractions from the column were pooled; aliquots were incubated with 50 μg of *Clostridium histolyticum* collagenase at 37°C for 2 h in a final volume of 100 μl in 0.05 M-Tes buffer, pH 7.5, containing 0.36 mM-CaCl₂, and rechromatographed on the same Sepharose CL-2B column. Other aliquots of the pooled fractions were incubated with 0.2 unit of chondroitin AC lyase for 2 h at 37°C [13] and chromatographed over a Sephadex G-50 column with 0.5 M-NH₄HCO₃ along with a tetrasaccharide and a disaccharide standard.

Aliquots of [^{14}C]chondroitin obtained from incubations performed in the absence of Triton X-100 together with [^3H]chondroitin obtained from incubations performed in the presence of Triton X-100 were co-chromatographed on Sepharose CL-6B columns or DEAE-cellulose (DE-52) columns along with standards. The Sepharose CL-6B columns were eluted with 0.5 M-NH₄HCO₃. The DEAE-cellulose columns were eluted with an exponential gradient of 0.05–1.0 M-LiCl. Aliquots from the fractions were assayed for radioactivity and uronic acid [14].

Microsomes (50 μl) were treated with 0.0003–0.15 unit of chondroitin ABC lyase [12] for 1 or 5 min at room temperature and washed with 0.25 M ice-cold sucrose by centrifuging in an Eppendorf centrifuge at maximum speed for 10 min. After repeating the washing process five times, the pellets were suspended in 50 μl of 0.25 M-sucrose. Control microsomes without chondroitin ABC lyase were similarly treated. Untreated or chondroitin ABC lyase-treated microsomes (20 μl) were incubated with 0.2 mM-UDP-[^3H]GalNAc (430×10^6 c.p.m./ μmol) and 0.2 mM-UDP-GlcA, with or without 3 mM-chondroitin hexasaccharide at 37°C for 2 h in a final total volume of 50 μl . After incubation the reaction mixtures were spotted on to Whatman no. 1 paper and chromatographed with butanol/acetic acid/aq. 1 M-NH₃ (2:3:1, by vol.) for 36 h. Those origins which contained [^3H]chondroitin oligosaccharides were eluted with water to remove and assay these oligosaccharides before elution with 0.5 M-NaOH for removal of [^3H]chondroitin. The [^3H]chondroitin was isolated, assayed, and chromatographed on DEAE-cellulose as described above. Alternatively, 0.2 mM-UDP-GalNAc and 0.2 mM-UDP-[^{14}C]GlcA (350×10^6 c.p.m./ μmol) were incubated with microsomes as described above for synthesis of [^{14}C]chondroitin before treatment as described above with the chondroitin ABC lyase.

RESULTS

Synthesis of [^{14}C]chondroitin by the chick cartilage microsomal system with UDP-[^{14}C]GlcA plus UDP-GalNAc was linear for 3 h both in the presence and in the absence of Triton X-100. After 3 h the synthesis decreased slightly, but was nearly linear up to 8 h. The presence of Triton X-100 in the incubation resulted in a 3-fold increase in the overall rate of synthesis.

The size of the proteo[^{14}C]chondroitin formed in a 2 h incubation in the absence of Triton X-100 was examined by chromatography on Sepharose CL-2B (Fig. 1). As described previously [5,6], there were two peaks of radioactivity. The first of these (Type I), representing about half of the total in this experiment, was only partially retarded by the gel. The second broad peak (Type II) appeared considerably later, with a median

Fig. 1. Sepharose CL-2B chromatography of chick cartilage microsomal proteo[^{14}C]chondroitin formed in the absence or presence of Triton X-100

Aliquots of proteo[^{14}C]chondroitin formed in the absence (●) or presence (○) of Triton X-100 were chromatographed separately on a column (0.7 cm $\times$ 45 cm) of Sepharose CL-2B with 0.5 M- NH_4HCO_3 as eluant. Fractions (0.25 ml each) were collected and analysed for radioactivity.

K_{av} of approx. 0.35, as previously reported. Proteo[^{14}C]chondroitin was also synthesized in a similar, separate, incubation with Triton X-100 present in the incubation mixture. Chromatography of this proteo[^{14}C]chondroitin demonstrated a different pattern, with a 4–5-fold increase in the amount of the second peak, but little alteration of the amount of the first peak. However, Triton X-100 had no effect on the apparent K_m for the sugar nucleotides for synthesis of either of the two forms of proteochondroitin when concentrations of either UDP-[^{14}C]GlcA or UDP-[^3H]GalNAc were varied over a range from 0.005 mM to 0.5 mM.

We considered the possibility that the nascent proteoglycans might be large precursors of PG-Lt and/or PG-Lb. Therefore aliquots of the two proteo[^{14}C]chondroitins from another Sepharose CL-2B chromatogram (Fig. 2) were treated with collagenase as described in the Materials and methods section and products were then chromatographed on this same column. The collagenase treatment had no effect on either the large proteo[^{14}C]chondroitin or the smaller proteo[^{14}C]chondroitin, indicating that neither contained a collagen-like component as found in PG-Lt. Other aliquots of proteo[^{14}C]chondroitin were treated with chondroitin AC lyase and chromatographed on a Sephadex G-50 column. Only disaccharide products were obtained, indicating that there were no dermatan-like residues resistant to degradation by the chondroitin AC lyase. However, epimerization of glucuronic acid to iduronic acid in dermatan sulphate formation mainly occurs only when sulphation takes place [15,16]. For this reason we also examined the chondroitin AC lyase degradation products of chondroitin sulphate (55% sulphated) formed by microsomal incubations of the sugar nucleotides in the presence of 3 mM-3'-phosphoadenylyl phosphosulphate. All of the sulphated chondroitin was degraded by the chondroitin AC lyase, indicating that no epimerization to form dermatan or dermatan sulphate residues had taken place, even in the presence of sulphation. Thus these proteochondroitins were different in this respect from PG-Lb and PG-Lt.

The positions of the proteochondroitin peaks on elution from Sepharose CL-2B did not appear to be affected significantly by the presence of Triton X-100 during synthesis. However, the Sepharose CL-2B would not be capable of detecting small

Fig. 2. Sepharose CL-2B chromatography of chick cartilage microsomal proteo[^{14}C]chondroitin after treatment with collagenase

Aliquots of large proteo[^{14}C]chondroitin (Type I) and smaller proteo[^{14}C]chondroitin (Type II) were rechromatographed as in Fig. 1 on the same column before (●) and after (○) incubation with collagenase.

Fig. 3. Sepharose CL-6B chromatography of chick cartilage microsomal [^{14}C]chondroitin formed in the absence of Triton X-100 and [^3H]chondroitin formed in the presence of Triton X-100

Aliquots of [^{14}C]chondroitin formed in the absence of Triton X-100 (●) together with aliquots of [^3H]chondroitin formed in the presence of Triton X-100 (○) were co-chromatographed on a column (0.7 cm $\times$ 45 cm) of Sepharose CL-6B with 0.5 M- NH_4HCO_3 as eluant. Fractions (0.25 ml each) were collected and analysed for radioactivity.

changes in molecular size. For this reason [^{14}C]chondroitin and [^3H]chondroitin were derived by alkali treatment of labelled proteoglycans that had been synthesized in other experiments in the absence or presence of Triton X-100 respectively. As described in the Materials and methods section, synthesis of the [^3H]chondroitin used UDP-[^3H]GalNAc and UDP-GlcA instead of UDP-[^{14}C]GlcA and UDP-GalNAc. Co-chromatography on Sepharose CL-6B (Fig. 3) demonstrated that the [^3H]chondroitin formed in the presence of Triton X-100 was slightly smaller in molecular size than the [^{14}C]chondroitin formed in the absence of Triton X-100.

Fig. 4. DEAE-cellulose chromatography of [^{14}C]chondroitin formed in the absence of Triton X-100 and [^3H]chondroitin formed in the presence of Triton X-100

Aliquots of [^{14}C]chondroitin formed in the absence of Triton X-100 ($\bullet$) and [^3H]chondroitin formed in the presence of Triton X-100 ($\circ$), together with standards of hyaluronic acid (HA) and chondroitin 4-sulphate (Chon S), were co-chromatographed on a column (0.7 cm $\times$ 5 cm) of DEAE-cellulose with an exponential gradient of 0.05–1.0 M-LiCl as described in the Materials and methods section. In order to make valid comparisons, the amounts of the aliquots were selected to adjust for the difference in specific activities between the [^{14}C]chondroitin and the [^3H]chondroitin. Fractions (0.5 ml) were collected and assayed for radioactivity and uronic acids.

DEAE-cellulose co-chromatography of [^{14}C]chondroitin formed in the absence of Triton X-100 together with [^3H]chondroitin formed in the presence of Triton X-100 was performed to examine the relative proportions of the two types of chondroitin glycosaminoglycans synthesized in the presence or absence of Triton X-100 (Fig. 4). As previously described [3], [^{14}C]chondroitin formed by the chick cartilage microsomal system in the absence of Triton X-100 was eluted as two peaks. The first peak, which was eluted near a hyaluronic acid standard, has been shown to represent addition of a large non-sulphated polymer on to a small primer, whereas the second peak, which was eluted just before a chondroitin 4-sulphate standard, has been shown to represent addition of a smaller non-sulphated polymer on to sulphated primers that were already of substantial size [3,4]. Moreover, the first peak of chondroitin has been previously shown to be derived mainly from the large Type I proteoglycan (first peak from Sepharose CL-2B), whereas the second peak has been shown to be derived mainly from the smaller Type II proteoglycan (second peak from Sepharose CL-2B) [5,6]. Since our current results indicate that synthesis of only the smaller proteochondroitin was stimulated by Triton (Fig. 1), only the second peak of chondroitin glycosaminoglycan should show this stimulation. This is demonstrated in Fig. 4, where [^3H]chondroitin formed in the presence of Triton X-100 showed an almost 3-fold increase in the second peak, whereas there was no significant increase in the first peak.

It seemed likely from our previous work and from the above results that the two forms of nascent proteoglycan were formed in separate loci in the microsomal membranes. In order to examine this possibility further, we treated the microsomal preparation with chondroitin ABC lyase before incubation with radioactively labelled sugar nucleotides. Degradation of chondroitin or chondroitin sulphate with this enzyme leaves non-reducing glucuronic acid residues, with an unsaturated double bond, incapable of serving as acceptors for addition of GalNAc in synthesis of proteochondroitin. Even one 'clip' near the end of

Table 1. Formation of total proteo[^3H]chondroitin and [^3H]oligosaccharide after prior treatment of microsomal preparation with chondroitin ABC lyase

Chondroitinase			Radioactivity (c.p.m.) incorporated into:	
Amount (unit)	Time (min)	Hexa-saccharide	Proteo-chondroitin	Oligo-saccharide
None	—	+	12400	62000
		—	15800	—
0.003	1	+	6900	61900
		—	8760	—
0.003	5	+	2900	52400
		—	4400	—
0.03	1	+	1700	35500
		—	1850	—
0.15	1	+	0	0
		—	0	—

a chondroitin chain will eliminate that chain as a primer. Therefore pre-treatment with chondroitinase can provide a highly sensitive index of whether a chondroitin primer is at a location that is accessible to the chondroitinase (i.e. on or near the membrane outer surface) or inaccessible (i.e. intravesicular or buried in the membrane).

[^3H]Chondroitin was therefore synthesized with microsomes that had been previously treated for varying short periods with various small amounts of chondroitin ABC lyase. Chondroitin hexasaccharide was added to the synthesis reactions in order to monitor any loss of polymerizing enzyme activity due to the preincubations and to be certain that the chondroitinase had been removed before the exogenous substrates were added. [^3H]Oligosaccharide (mostly heptasaccharide) products and the [^3H]chondroitin products of the incubation were isolated as described in the Materials and methods section. Results are shown in Table 1, which indicates that there was progressively less [^3H]chondroitin formed with microsomal incubations as more chondroitinase was used. After the use of 0.15 unit of chondroitinase for 1 min, there was no incorporation. In contrast, there was no loss of [^3H]GalNAc incorporation on to hexasaccharide at the lowest concentration of chondroitinase and less loss at higher concentrations. This control indicated that the chondroitinase had been effectively removed before the incubation with labelled substrates and that the chondroitinase at these concentrations had little if any effect upon the chondroitin-synthesizing enzymes. The presence of hexasaccharide in the incubations resulted in a slight decrease in formation of [^3H]chondroitin, presumably by competitive inhibition of polymerization on the endogenous primer, as has previously been described [17].

DEAE-cellulose chromatography of the [^3H]chondroitin derived from proteo[^3H]chondroitin formed with chondroitinase-treated microsomes is shown in Fig. 5. Pretreatment of the microsomes with small amounts of chondroitin ABC lyase resulted in a loss in the synthesis of the second peak of chondroitin (short chondroitin chains added to pre-existing chondroitin sulphate), indicating that the chondroitinase had good access to the Type II proteochondroitin primer. By contrast, there was little loss in synthesis of the first peak of chondroitin (long chondroitin chains added to short primer) until much more enzyme was utilized. The results, as summarized in Table 2, indicated that the primer for formation of the Type I nascent proteochondroitin was relatively inaccessible to the chondroitinase, or alternatively contained only the GlcA-Gal-Gal-Xyl linkage region with no chondroitinase-sensitive residues.

Fig. 5. DEAE-cellulose chromatography of [^3H]chondroitin formed after pretreatment of microsomal preparation with chondroitin ABC lyase

Aliquots of [^3H]chondroitin formed after pretreatment of microsomes with chondroitin ABC lyase [(a) none; (b) 0.003 unit for 1 min; (c) 0.003 unit for 5 min; (d) 0.03 unit for 1 min], together with standards of hyaluronic acid (HA) and chondroitin 4-sulphate (Chon S), were chromatographed on a DEAE-cellulose column and assayed as in Fig. 4.

Table 2. Proteo[^3H]chondroitin formed after prior treatment of microsomal preparation with chondroitin ABC lyase

Chondroitinase		Type I proteo[^3H]chondroitin		
Amount (unit)	Time (min)	Total proteo-[^3H]chondroitin (c.p.m.)	(% of total proteo-[^3H]chondroitin)	
None	—	15800	2700	17
0.003	1	8760	3000	34
0.003	5	4400	2900	66
0.03	1	1850	1550	84

In order to confirm that the first of these two alternatives was the reason for resistance to the chondroitinase treatment, [^{14}C]chondroitin was formed by incubating the microsomal preparation with the sugar nucleotide substrates before treatment with chondroitin ABC lyase instead of subsequent to this treatment. As shown in Fig. 6, the chondroitin of nascent Type I

Fig. 6. DEAE-cellulose chromatography of [^{14}C]chondroitin treated with chondroitin ABC-lyase after synthesis with microsomal preparation

Aliquots of [^{14}C]chondroitin treated with chondroitin ABC lyase; (a) 0.003 units for 5 min; (b) 0.03 units for 1 min, following synthesis with the microsomal preparation, were chromatographed on a DEAE-cellulose column and assayed as in Fig. 4. For abbreviations, see Fig. 4.

Table 3. Preformed proteo[^{14}C]chondroitin remaining after treatment of microsomal preparation with chondroitin ABC lyase

Chondroitinase		Type I proteo-[^3H]chondroitin		
Amount (unit)	Time (min)	Total proteo-[^3H]chondroitin (c.p.m.)	(% of total proteo-[^3H]chondroitin)	
None	—	24500	3700	15
0.003	5	13900	3900	28
0.03	1	4900	2700	55

proteochondroitin was not degraded under conditions where the chondroitin of nascent Type II proteochondroitin was almost completely degraded. Results are summarized in Table 3. This demonstrated that the enzyme had much better access to the microsomal site for synthesis of the Type II proteochondroitin than to the site for synthesis of the Type I proteochondroitin subsequent to formation of chondroitin by these microsomes as well as before the formation of the chondroitin, as demonstrated in Fig. 5 and Table 2. Thus the different results for Type I proteochondroitin were independent of whether or not the Type I primer had contained chondroitinase-susceptible residues.

DISCUSSION

The chick cartilage microsomal system used for examination of chondroitin sulphate synthesis has important differences from the mast-cell microsomal system that we described previously [10,11]. By contrast with the mast-cell microsomes, where only one form of proteochondroitin primer and product was found, there are two forms [5,6]. The first has similarities to that of the mast-cell system, but generally accounts for less than 20% of the proteochondroitin that is synthesized by the cartilage microsomal system. Most of the remaining proteochondroitin that is synthesized is quite different. This second form consists of the addition of short chondroitin chains to proteochondroitin sulphate already containing chondroitin sulphate of considerable size. Sulphation of this form is unaffected by the detergents

that disrupt sulphation of the mast-cell proteochondroitin [18]. As discussed in the Introduction, the two forms differ from one another with regard to the size of the respective proteoglycans, extractability, apparent K_m for substrates, and possible localization in the microsomal membranes.

The presence of more than one form was compared with the presence of three forms of proteochondroitin sulphate (PG-H, PG-Lb and PG-Lt) reported for the extracellular matrix of chick-embryo epiphyseal cartilage [8,9]. Neither of the two intracellular nascent proteochondroitins appeared to be identical with these matrix forms. Neither of the two intracellular proteochondroitins appeared to be aggregates, and neither could be aggregated with hyaluronic acid. By contrast, it has been reported that a core protein precursor from the Swarm rat chondrosarcoma can be aggregated in this fashion [19]. It is possible that, in our system, the portion of the core protein that provides for the aggregation is masked or buried during the synthesis of the glycosaminoglycan and is then uncovered before the proteoglycan is moved out into the matrix. However, we have no information on this point. It is not clear what the relationship of the two intracellular nascent forms is to PG-H, PG-Lb and PG-Lt, but it is possible that one or both are precursors for one or more of these final cartilage matrix proteochondroitin sulphates. If so, this would imply that there is considerable modification of the nascent proteochondroitin sulphate before or during transfer from the Golgi site of synthesis to the extracellular cartilage matrix. Such information is not available at present.

There was a 3-fold stimulation of chondroitin formation when Triton X-100 was present in incubations of sugar nucleotide substrates with cartilage microsomes. This stimulation was restricted to the smaller of the two species of proteochondroitin (Fig. 1) and consequently to only one of the two species of chondroitin formed (Fig. 4). Thus the synthesis of the Type II proteoglycan that had the lower apparent K_m for sugar nucleotides, and had previously been shown to consist of additional polymerization on pre-existing proteochondroitin sulphate, was stimulated 4–5-fold. Synthesis of the Type I proteochondroitin, consisting of synthesis *de novo* of chondroitin on primer that did not already have chondroitin chains, was not stimulated appreciably. As with the mast-cell system, the chondroitin chains formed were slightly smaller when Triton X-100 was present during the polymerization (Fig. 3). These results suggest that the presence of Triton X-100 resulted in either an uncovering of endogenous primer, providing access to the polymerizing enzymes for the Type II proteochondroitin, or an increased accessibility of the sugar nucleotide substrates to the site for polymerization of this proteoglycan. However, in our experiments we used saturating concentrations of sugar nucleotides, and the apparent K_m for the sugar nucleotide substrates were not affected by Triton (Table 1). Therefore the former is more likely. Since Triton X-100 did not affect the synthesis of the Type I proteochondroitin, it apparently did not uncover additional primer for this species.

Although the apparent K_m for the sugar nucleotides in biosynthesis of these two species are different [6], we consider it to be highly unlikely that this reflects a difference in glycosyltransferases for these two proteochondroitin sulphates. It is possible that interaction of the glycosyltransferases with a proteochondroitin primer affects the affinity of these enzymes for the sugar nucleotides. Thus the differences in apparent K_m could be related to the two different proteochondroitin primers. In any case, our results suggest that the two forms were synthesized in different Golgi compartments or loci, and that Triton X-100 only affected the site for synthesis of the smaller proteoglycan. This is consistent with the differences we have previously shown [5] in extractability of the two forms from their microsomal sites of

synthesis. These results in turn indicate that there might be considerable variability in the organization of the Golgi membranes for synthesis of the glycosaminoglycan portions of various proteoglycans. Thus we now have an indication that the chondroitin synthesis of at least two different forms of proteoglycan (mast-cell microsomal proteochondroitin 4-sulphate [9] and the larger form of chick cartilage microsomal proteochondroitin 6-sulphate) are relatively unaffected by Triton X-100, whereas the chondroitin synthesis of the smaller form of chick cartilage microsomal proteochondroitin 6-sulphate is markedly stimulated.

These results all contrast with observations concerning the synthesis of heparin by mast-cell microsomes [20], where glycosaminoglycan polymerization has been reported to be abolished by detergent. This in turn suggests that there may be substantial variations from tissue to tissue and from product to product in the organization of membrane-bound glycosaminoglycan-synthesizing systems.

The microsomal localization of the two forms of nascent proteochondroitins was examined by use of chondroitin ABC lyase before and after chondroitin synthesis. We found that synthesis of the Type I form of proteochondroitin (chondroitin polymerization on a short primer) was much less susceptible to pretreatment with chondroitinase than was synthesis of the Type II form of proteochondroitin (chondroitin polymerization on a sulphated primer of substantial size) (Fig. 5, Tables 1 and 2). Moreover, similar results were found when the chondroitinase was used subsequent to chondroitin synthesis (Fig. 6; Table 3). These results are consistent with our other description of differences in the two forms of nascent proteochondroitin sulphate. Our results support the concept that the larger, Type-I-chondroitin-containing form together with its polymerizing enzymes is within microsomal (Golgi) vesicles in a relatively inaccessible location, whereas the smaller, Type-II-chondroitin-containing form and its polymerizing enzymes may be at, or near, the surface of these membranes. Although understanding the precise localization of these systems is complicated by the 'artefactual' nature of the microsomal preparation, our results indicate that there are distinct and consistent differences in localization of the two types.

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