Subcellular co-localization and potential interaction of glucuronosyl-transferases with nascent proteochondroitin sulphate at Golgi sites of chondroitin synthesis

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Microsomal membranes from chick embryo epiphyseal cartilage were fractionated by equilibrium sucrose-density-gradient centrifugation and assayed for GlcA (glucuronic acid) transferase I (the enzyme that transfers GlcA from UDP-GlcA to Gal-Gal-Xyl of proteochondroitin linkage region), for comparison with GlcA transferase II (the GlcA transferase of chondroitin polymerization). Gal(β 1-3)Gal β 1-methyl (disaccharide) and GalNAc(β 1-4)GlcA(β 1-3)GalNAc(β 1-4)GlcA(β 1-3)GalNAc (pentasaccharide) were used respectively as acceptors of [¹⁴C]GlcA from UDP-[¹⁴C]GlcA. Distributions of the two GlcA transferase activities in the sucrose-density-gradient fractions were compared with each other and with the previously reported distribution of the activities of Gal transferases (UDP-Gal to ovalbumin, and to xylose of the proteochondroitin linkage region) and GalNAc (*N*-acetylgalactosamine) transferase II of chondroitin polymerization. The linkage-region GlcA transferase I had a dual Golgi distribution similar to that of chondroitin-polymerizing GlcA transferase II and distinctly different from the distribution of linkage-region Gal transferases I and II, which were found exclusively in the heavier fractions. Solubilized GlcA

transferase I was partly purified by sequential use of Q-Sepharose, heparin-Sepharose and wheatgerm agglutinin-agarose and was accompanied at each step by some of the GlcA transferase II activity. Both GlcA transferase I and II bound to the Q-Sepharose as though they were highly anionic. However, treatment with chondroitin ABC lyase eliminated the binding while markedly decreasing enzyme stability. The enzyme activities could not be reconstituted by adding chondroitin or chondroitin pentasaccharide to the chondroitin ABC lyase-treated enzymes. Incubation of the partly purified enzymes with both UDP-GlcA and UDP-GalNAc resulted in a 40-fold greater incorporation than with just one sugar nucleotide, indicating the presence of bound, nascent proteochondroitin serving as the acceptor for chondroitin polymerization. These results, together with the membrane co-localization, indicate that GlcA transferase I and GlcA transferase II occur closely together with nascent proteochondroitin at the site of synthesis and that this complex with the nascent proteochondroitin stabilizes both enzymes during purification.

INTRODUCTION

The formation of proteochondroitin sulphate involves the synthesis of core protein followed by stepwise transfer of individual sugars from nucleotide sugar precursors to form GalNAc-GlcA-Gal-Gal-Xyl [1] oligosaccharide linkages to which GlcA and GalNAc are then added sequentially [2,3], with sulphation taking place [3,4] while chondroitin chains are growing in length [5]. Substrate competition experiments have indicated that the first GlcA (glucuronic acid) added to the Gal-Gal-Xyl of the linkage region involves an enzyme (GlcA transferase I) distinct from the enzyme (GlcA transferase II) involved in the formation of the chondroitin polymer [6,7], and enzyme separation techniques have indicated that the enzyme involved in adding the first GalNAc (*N*-acetylgalactosamine) is distinct from the enzyme (GalNAc transferase II) for chondroitin synthesis [8].

Use of membrane fractions and/or immunocytochemistry [9–11] has shown that the initiation of the oligosaccharide linkage region occurs in endoplasmic reticulum or early Golgi fractions with transfer of Xyl to specific Ser residues. We have utilized a sucrose-density-gradient fractionation technique with chick em-

bryo epiphyseal cartilage microsomes to demonstrate that the subsequent addition of the two Gal residues then occurs sequentially in separate *cis*- or medial Golgi membrane fractions [12,13].

We had previously shown with the chick microsomal preparation that there was synthesis of the chondroitin sulphate portions of two distinct size populations of proteochondroitin 6-sulphate [14,15]. The chondroitin-polymerizing enzymes for each population were found in separate Golgi locations [12], with a larger proteochondroitin sulphate being formed in earlier medial portions of Golgi in density-gradient fractions peaking slightly later but overlapping the Gal transferase I and Gal transferase II activities, whereas a smaller proteochondroitin was formed in lighter well-separated *trans*-Golgi/*trans*-Golgi network fractions. There was no Gal transferase I or II activity in these lighter fractions. We also found that the membrane localization for enzymes involved in the addition of both Gal residues and for synthesis of the larger of the two proteochondroitin species were not affected by the fungal metabolite, brefeldin A, whereas transport of proteoglycan to the site for synthesis of the smaller species was eliminated [13]. Furthermore microsomal membranes

Abbreviation used: WGA, wheatgerm agglutinin. Nomenclature: the enzymes adding the first GlcA (glucuronic acid) and GalNAc (*N*-acetylgalactosamine) to the Gal-Gal-Xyl at the linkage region have been termed GlcA transferase I and GalNAc transferase I, whereas the enzymes involved in polymerization have been termed GlcA transferase II and GalNAc transferase II [1].

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involved in the synthesis of the larger proteochondroitin were less affected by detergent and were less accessible to bacterial chondroitin lyase [16] than were the microsomal membranes involved in the synthesis of the smaller proteochondroitin. In all cases, membrane-bound proteochondroitin was not easily solubilized from the microsomes [14] either before or after polymerization had occurred on the microsomal nascent proteochondroitin acceptors after incubation with sugar nucleotide substrates. It is of note that the addition of nascent proteochondroitin attached to inactivated microsomal fractions did not act as an additional acceptor when added to fresh microsomal incubations, even if detergent was present. Thus exogenous nascent proteochondroitin was not able to reach the site of active microsomal enzymes.

No information has been available to indicate the sites of formation of the putative final GalNAc-GlcA of the linkage region. Thus one or both of the two activities for completion of the linkage region could be located in Golgi compartments near the compartments for Gal-Gal-Xyl formation, or alternatively these activities could occur in later Golgi compartments such as those that contain chondroitin-polymerizing and chondroitin-sulphating enzymes but no Gal transferase I or II. We have now compared the sucrose-density-gradient distribution of GlcA transferase I activity with that of other enzymes of chondroitin sulphate synthesis including GlcA transferase II. Our results demonstrate that GlcA transferase I has a dual distribution similar to that of GlcA transferase II in both medial and *trans*-Golgi/*trans*-Golgi networks, rather than having a distribution solely localized to the earlier Golgi linkage region where both Gal transferases were found. Furthermore partial purification of GlcA transferase I as well as GlcA transferase II followed by incubation with chondroitin ABC lyase indicates an interaction with and protection by membrane-bound nascent proteochondroitin sulphate at the site of synthesis.

MATERIALS AND METHODS

Materials and enzyme preparation

UDP-[¹⁴C]GlcA (325 mCi/mmol), UDP-[³H]GalNAc (17.8 Ci/mmol) and [³H]glucosamine were purchased from DuPont-New England Nuclear (Boston, MA, U.S.A.). Gal(β1-3)Galβ1-methyl (disaccharide) and wheatgerm agglutinin (WGA)-agarose were purchased from Sigma (St. Louis, MO, U.S.A.). Chondroitin ABC lyase and 2-acetamido-2-deoxy-3-*O*-(β-D-glucose-4-enopyranosyluronic acid)-D-galactose disaccharide were purchased from Seikagaku America (Rockville, MD, U.S.A.). Chondroitin and GalNAc(β1-4)GlcA(β1-3)GalNAc(β1-4)GlcA(β1-3)-GalNAc (pentasaccharide) were prepared as described previously [17]. Heparin-Sepharose and Q-Sepharose were purchased from Pharmacia-LKB Biotechnology Products (Piscataway, NJ, U.S.A.). Chondroitin and chondroitin 6-sulphate were coupled to EAH-Sepharose with the aid of 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide [18].

Microsomal enzymes [2,19] and sucrose-density-gradient fractions [13] were prepared as described previously. The GlcA transferase I and II activities, as well as the Gal transferase I and GalNAc transferase II activities, remained particulate, with no significant activities being found in supernatants after high-speed centrifugation.

Purification and treatment of enzymes

All operations were performed at 4 °C. GlcA transferases from homogenates of chick embryo epiphyseal cartilage microsomes were solubilized as previously described [20] by suspending

12000–105000 g pellets in 10 mM Hepes buffer, pH 7.2, containing 1 % (v/v) Triton X-100, 1.0 M NaCl, 10 mM MgCl₂, 2 mM CaCl₂, 1 mM dithiothreitol, 20 % (v/v) glycerol and protease inhibitors (1 mM PMSF, 10 mM EDTA, 1 mM *N*-ethyl maleimide, 0.5 μg/ml leupeptin and 0.1 μg/ml pepstatin). After adjustment of the NaCl concentration to 0.05 M, the solubilized enzyme was applied as described previously [20] to a 1.5 cm × 14 cm Q-Sepharose column previously equilibrated with the same buffer containing only 0.02 % Triton X-100. After being washed with 10 vol. of the buffer, bound proteins were eluted with a linear NaCl gradient of 0.05–1.0 M. Pooled fractions that contained both GlcA transferase I and GlcA transferase II activities were adjusted to 0.15 M NaCl and applied to a heparin-Sepharose column (1.5 cm × 20 cm) equilibrated with the same buffer containing 0.15 M NaCl. After washing with 5 vol. of the buffer, bound enzymes were eluted with the same buffer containing 1.0 M NaCl. Pooled heparin-Sepharose fractions containing both GlcA transferases were dialysed against buffer containing 0.15 M NaCl and mixed for 3 h with 10 ml of WGA-agarose in a sealed column. The column was then washed with 10 column vol. of the buffer, and enzymes were eluted with the same buffer containing 0.5 M GlcNAc.

Aliquots of active fractions from the Q-Sepharose column were incubated with 1.5 units of chondroitin ABC lyase in 0.5 M Tris/HCl (pH 7.6)/0.1 M NaCl/0.1 % BSA at room temperature for 2 h; aliquots were then reappplied and eluted from the Q-Sepharose, heparin-Sepharose and WGA-agarose columns as described above.

Enzyme assays

Gal transferase I, Gal ovalbumin transferase and GalNAc transferase II were assayed as described previously with linear activities over the period of assay incubation [4,12,13,17]. Protein contents of density gradient fractions were reported previously [12].

GlcA transferases: (1) UDP-[¹⁴C]GlcA was incubated with Gal(β1-3)Galβ1-methyl for assay of GlcA transferase I [6,7], whereas (2) endogenous acceptor and/or chondroitin pentasaccharide was utilized similarly for assay of GlcA transferase II. Reaction mixtures, with 12 μl of microsomal preparation, 12 μl from the sucrose-density-gradient fractions or 12 μl of solubilized enzyme in a total volume of 15 μl, contained 0.05 M Mes buffer, pH 6.5, 0.015 M MnCl₂, 0.05 % Triton X-100 and 0.07 mM UDP-[¹⁴C]GlcA (325 mCi/mmol). To this was added 10–100 μg of disaccharide (final concentration 0.2–2.0 mM) and/or 5–60 μg of pentasaccharide (final concentration 0.04–0.5 mM). After a 2 h incubation at 37 °C, the reaction mixtures were spotted on Whatman No.1 paper for the isolation of products.

(1) [¹⁴C]GlcA-labelled trisaccharide: paper electrophoresis was performed in 0.08 M pyridine/0.046 M acetic acid (pH 5.3) at 70 V/cm for 45 min [6]; the new radioactive spot corresponding to the trisaccharide product was eluted with water and chromatographed on Sephadex G-15 to separate it from small amounts of [¹⁴C]-labelled degradation products of UDP-[¹⁴C]GlcA.

(2) [¹⁴C]GlcA-labelled hexasaccharide and endogenous chondroitin: paper chromatography was performed in butanol/acetic acid/1 M NH₄OH (2:3:1, by vol.) for 48 h. [¹⁴C]GlcA-labelled hexasaccharide, which does not move from the origin in this system, was separated from [¹⁴C]GlcA-labelled endogenous material, which remains fixed at the origin, by elution with water. The origins were then treated with 0.5 M NaOH at room temperature to remove the [¹⁴C]GlcA-labelled endogenous chondroitin acceptor from the fixed proteoglycan.

Figure 1 Sephadex G-25 chromatography of products formed by GlcA transferase I

Products formed by incubation of UDP-[^{14}C]GlcA with disaccharide (●) and the products formed after treatment with β -glucuronidase (○) were chromatographed separately on a 1 cm $\times$ 45 cm column of Sephadex G-25 with 2-acetamido-2-deoxy-3-*O*-(β -D-glucose-4-enepyranosyluronic acid)-D-galactose (Δ Di-OS) disaccharide and [^3H]glucosamine standards as described in the Materials and methods section.

Polymerization on endogenous acceptors

Reaction mixtures in a total volume of 15 μl containing 0.05 M Mes buffer, pH 6.5, 0.015 M MnCl_2 , 0.05% Triton X-100 and 0.07 mM UDP-[^{14}C]GlcA (325 mCi/mmol) with or without 0.2 mM UDP-GalNAc or 0.07 mM UDP-[^3H]GalNAc (2000 mCi/mmol) with or without 0.2 mM UDP-GlcA were incubated with 12 μl of partly purified enzyme from the WGA-agarose step at 37 $^\circ\text{C}$ for 2 h. After incubation the reaction mixtures were spotted on Whatman No. 1 paper and chromatographed in ethanol/ammonium acetate (5:2, v/v) as described previously [19]. The origins were treated with 0.5 M NaOH at room temperature overnight to elute the labelled chondroitin sulphate chains; aliquots were then counted.

Identification of products

Incubations of UDP-[^{14}C]GlcA and disaccharide together with active GlcA transferase I resulted in the appearance, after electrophoresis, of a new [^{14}C]GlcA-labelled substance ($R_{\text{UDP-GlcA}}$ 0.5) that was well separated from UDP-[^{14}C]GlcA. However, quantitative analysis was somewhat limited in accuracy by the need for additional chromatography on Sephadex G-15 to separate the product from small amounts of [^{14}C]labelled degradation products of UDP-[^{14}C]GlcA. Chromatography of the isolated product on a 1 cm $\times$ 45 cm column of Sephadex G-25 with 0.5 M ammonium bicarbonate, pH 7.8, as eluent demonstrated a [^{14}C]GlcA-labelled trisaccharide (Figure 1). Incubation at 37 $^\circ\text{C}$ with 100 μg of β -glucuronidase in 50 μl of 0.05 M acetate buffer, pH 5.0, for 2 h released the [^{14}C]labelled material, which co-chromatographed with a [^3H]glucosamine standard. Similarly, on electrophoresis, the migration of [^{14}C]labelled material after β -glucuronidase treatment was identical with that of standard [^{14}C]GlcA (results not shown).

As shown previously [21], incubation of microsomal enzyme with UDP-[^{14}C]GlcA in the absence of pentasaccharide or other exogenous acceptor resulted in the incorporation of [^{14}C]GlcA on the non-reducing terminal ends of nascent proteochondroitin sulphate, whereas similar incubations containing pentasaccharide

resulted in the additional formation of [^{14}C]labelled hexasaccharide.

RESULTS

The formation of [^{14}C]labelled products after incubation of microsomes and UDP-[^{14}C]GlcA with and without added acceptor substrates is shown in Table 1. Reactions were linear for the course of these incubations, and sugar nucleotide substrates were found to be essentially undegraded. Reactions that contained neither the pentasaccharide nor the disaccharide resulted in the incorporation of [^{14}C]GlcA only on endogenous microsomal proteochondroitin sulphate that remained bound to the origin after electrophoresis or after paper chromatography, as described in the Materials and methods section. When microsomal preparations were incubated with UDP-[^{14}C]GlcA plus various concentrations of disaccharide, there was increasing incorporation on the terminal Gal to form trisaccharide but no diminution in the incorporation on the endogenous material. Similar experiments with various concentrations of pentasaccharide also showed increasing incorporation of [^{14}C]GlcA on the terminal GalNAc to form hexasaccharide. However, in contrast with the results with the disaccharide, there was proportional diminution of [^{14}C]GlcA incorporation on microsomal endogenous nascent proteochondroitin sulphate. These results conform with our previous experiments showing that chondroitin polymerization on nascent proteochondroitin was inhibited by the presence of the pentasaccharide or hexasaccharide [17] and demonstrate that there was little endogenous material with linkage-region Gal that had not already been replaced by GlcA and probably some chondroitin. This is in keeping with our previous work, which has shown that much of the chondroitin formed in microsomal systems when both UDP-GlcA and UDP-GalNAc are present consists of the addition of chondroitin to pre-existing proteochondroitin already containing chondroitin sulphate chains of substantial length [4,14].

In concurrence with earlier work [6], incorporation of [^{14}C]GlcA on the disaccharide was not inhibited by the presence of chondroitin pentasaccharide, nor was incorporation on pentasaccharide inhibited by the presence of the disaccharide. Thus our results support the work establishing that the enzyme involved in the addition of GlcA to Gal of the linkage region is different from that involved in the addition of GlcA to GalNAc of chondroitin.

Subcellular localization of the two GlcA transferase activities was examined by use of sucrose density gradients to separate Golgi vesicles as previously demonstrated for Gal transferase I [12,13], Gal transferase II [13], GalNAc transferase II [13] and

Table 1 Effect of varying the concentration of chondroitin pentasaccharide and Gal(β 1-3)Gal β 1-methyl on the incorporation of [^{14}C]GlcA

Substrate added (μg)		Incorporation of [^{14}C]GlcA (c.p.m.) to form		
Disaccharide	Pentasaccharide	Trisaccharide	Hexasaccharide	Endogenous
—	—	—	—	2800
10	—	2200	—	3060
50	—	7350	—	2650
100	—	14500	—	2700
—	5	—	2700	1950
—	30	—	5250	790
—	60	—	13900	520
50	30	8200	5500	820

Figure 2 Golgi distribution of GlcA transferase I and GlcA transferase II activities

Fractions from the gradient were analysed as described in the Materials and methods section: (A) Gal transferase I (●, solid line), Gal-ovalbumin transferase (●, broken line) and percentage sucrose (△); (B) GlcA transferase I; (C) GlcA transferase II; (D) GalNAc transferase II.

chondroitin polymerization [12,13] and sulphation [12,13] with the same chick embryo cartilage system. Assays for Gal transferase I and GalNAc transferase II activities as previously described [12,13] are shown in Figure 2(A) as a point of reference together with the assay for Gal-ovalbumin transferase activity. The Gal transferase I had been found [12] at a slightly lower sucrose concentration range than we reported for NADH:cytochrome *c* reductase (a *cis*-Golgi marker), which in turn was at a still lower sucrose concentration range than that of glucose-6-phosphatase (an endoplasmic reticulum marker). Distributions of GlcA transferase I, GlcA transferase II and GalNAc transferase II are shown in Figures 2B, 2C and 2D. All three enzymes demonstrated a dual distribution distinct from the single distribution of the Gal transferase I, but similar to the distribution of chondroitin-polymerizing and chondroitin-sulphating activities and similar to the distribution of Gal-ovalbumin transferase as previously described [12]. Thus the distribution of GlcA transferase I was correlated with the distribution of the enzymes involved in the formation of the chondroitin sulphate polymer, with approximately half of the activity differing completely from the distributions of the enzymes involved in the

Figure 3 Elution of GlcA transferase I and GlcA transferase II from a Q-Sepharose column

Solubilized enzyme material was applied to a 1.5 cm × 14 cm Q-Sepharose column as described in the Materials and methods section and eluted with a linear gradient of 0.05–1.0 M NaCl, which was started at fraction 51. Fractions of 3 ml each were collected and assayed for GlcA transferase I (●) and GlcA transferase II (○). Protein was monitored by absorbance at 280 nm (△).

formation of the Gal-Gal portions of the oligosaccharide linkage region.

Both microsomal GlcA transferases were stable at -20°C for many months and retained good activity even after freezing and thawing several times. After solubilization, both enzymes retained this stability. The apparent K_m values of solubilized GlcA transferases I and II for UDP-GlcA were similar (approx. $50\ \mu\text{M}$).

Q-Sepharose ion-exchange chromatography of the solubilized enzymes is shown in Figure 3. All the GlcA transferase I activity was found together with approximately one-third of the GlcA transferase II activity in fractions 65–78, requiring 0.1–0.3 M NaCl for elution. Approx. 20% of the total protein (measured as A_{280}) was found in these fractions, whereas approx. 30% required higher concentrations of NaCl for elution, and approx. 50% did not adhere to the column (results not shown). The remaining two-thirds of the GlcA transferase II activity, with less than 10% of the total protein, was found in later fractions, requiring even higher NaCl concentrations for elution. The enzyme from this later peak has been purified and identified by SDS/PAGE and photoaffinity labelling as an 80 kDa protein [20]. Fractions 65–78 were pooled and chromatographed sequentially on a heparin-Sepharose column and then a WGA-agarose column (results not shown) as described in the Materials and methods section. Both GlcA transferases I and II bound quantitatively to the heparin-Sepharose column and were found in similar fractions on addition of 1.0 M NaCl to the eluting buffer. After dialysis of the active fractions and resuspension in buffer with 0.15 M NaCl, both enzymes bound quantitatively to the WGA-agarose column and were eluted by the addition of 0.5 M GlcNAc. After these purifications both enzymes remained stable at -20°C for many months. Neither enzyme bound to columns of chondroitin-Sepharose or chondroitin sulphate-Sepharose.

The requirement of high salt concentrations for elution from the Q-Sepharose demonstrated that the enzymes were either highly anionic or were tightly associated with highly anionic substances, suggesting the presence of endogenous nascent proteochondroitin sulphate. To examine this, aliquots of pooled fractions 65–78 from the Q-Sepharose column were treated with

Figure 4 Lability of solubilized GlcA transferases after chondroitin ABC lyase treatment

Pooled fractions 65–78 from the Q-Sepharose column (Figure 3) were treated with chondroitin ABC lyase and reappplied to the same Q-Sepharose column as described in the Materials and methods section: (A) GlcA transferase I; (B) GlcA transferase II. Aliquots from the unbound fractions of the Q-Sepharose column were stored at -20°C and assayed at varying times for comparison with untreated enzymes. Columns in each group, from left to right: untreated, treated, treated with chondroitin added, and treated with chondroitin pentasaccharide added.

chondroitin ABC lyase, which specifically degrades chondroitin sulphate, and then rechromatographed on the same column. After this treatment no GlcA transferase I or GlcA transferase II activities were retained, but they were recovered quantitatively before elution with NaCl was initiated. Similarly, chondroitin ABC lyase-treated enzymes did not bind to either the heparin-Sepharose or WGA-agarose columns. Control incubations with heat-inactivated chondroitin ABC lyase had no effect, and retention of GlcA transferase I or GlcA transferase II on Q-Sepharose, heparin-Sepharose or WGA-agarose could not be restored by the addition of chondroitin, chondroitin sulphate or chondroitin hexasaccharide.

Chondroitin ABC lyase-treated GlcA transferases with or without added chondroitin ($100\text{ }\mu\text{g/ml}$) or chondroitin pentasaccharide ($100\text{ }\mu\text{g/ml}$) were stored at -20°C . In sharp contrast with the stability of enzymes that had not been subjected to chondroitin ABC lyase treatment, after such treatment both enzymes lost most activity in 1 day at -20°C , and essentially all activity in 3 days (Figure 4). The addition of chondroitin or chondroitin pentasaccharide did not stabilize the treated enzymes. This suggested that, even after solubilization, the GlcA transferases were firmly associated with the nascent proteo-chondroitin substrate at the site of synthesis, and this association greatly stabilized the enzyme activities.

Table 2 Chondroitin polymerization on endogenous proteo-chondroitin associated with partly purified enzyme

Nucleotides added	^{14}C incorporated (c.p.m.)	^3H incorporated (c.p.m.)
UDP- ^{14}C GlcA	160	—
UDP- ^{14}C GlcA + UDP-GalNAc	7200	—
UDP- ^3H GalNAc	—	450
UDP- ^3H GalNAc + UDP-GlcA	—	18600

To confirm this presence of nascent proteo-chondroitin, we incubated pooled enzyme fractions from the WGA-agarose step with appropriate sugar nucleotides (Table 2) in the absence of exogenous acceptors such as chondroitin oligosaccharides. When UDP- ^{14}C GlcA or UDP- ^3H GalNAc was used alone, a limited incorporation was observed into endogenous acceptors. The addition of unlabelled UDP-GalNAc or UDP-GlcA respectively to the incubations resulted in more than 40-fold increased incorporation. Because endogenous nascent proteo-chondroitin is required for chondroitin polymerization [2,14], this demonstrates its presence in the solubilized enzyme preparation. This also demonstrates the presence of GalNAc transferase II in the partly purified enzyme preparation.

DISCUSSION

The previously reported [6,7] presence of two separate GlcA transferases has now been confirmed by our examination of the effects of the oligosaccharides on the incorporation of ^{14}C GlcA into endogenous nascent proteo-chondroitin acceptors (Table 1). We found that the chondroitin pentasaccharide was an effective inhibitor, whereas methyl-Gal-Gal was not inhibitory at any concentration used. Thus one GlcA transferase only adds GlcA to non-reducing Gal, whereas the other GlcA transferase adds GlcA only to non-reducing GalNAc. (We have previously shown that GlcA transferase II can add GlcA to 6-sulphated but not to 4-sulphated GalNAc [21]. However, it is not known whether GlcA transferase I can add GlcA to either 6-sulphated or 4-sulphated Gal.)

We expected to find that the density-gradient distribution of GlcA transferase I would be similar to that of the linkage-region Gal transferases, with all activity in earlier *cis*/medial fractions. Thus it was surprising to find the GlcA transferase I activity in the same dual distribution as the GlcA transferase II activity in medial Golgi and *trans*-Golgi/*trans*-Golgi network fractions (Figure 2). Therefore, in contrast with the formation of linkage-region Gal-Gal, which does not occur in later *trans*-Golgi fractions, the incorporation of GlcA on the linkage-region oligosaccharide can take place in all the Golgi fractions where polymerization takes place. Furthermore, after solubilization, some of the GlcA transferase II was found to accompany GlcA transferase I on chromatography with Q-Sepharose (Figure 3), heparin-Sepharose and WGA-agarose (results not shown).

The solubilized GlcA transferase I and GlcA transferase II bound to Q-Sepharose (Figure 3) and to heparin-Sepharose as though these enzymes were highly charged, and both bound to the WGA-agarose lectin affinity column as though there were glycoprotein-like substituents. However, our previous work [4,14,21] has indicated that cartilage microsomes contain endogenous proteo-chondroitin sulphate at the site of synthesis, so that we considered it more likely that both enzymes were tightly associated with this highly charged glycosaminoglycan-containing substance, which had not been separated by detergent and

1 M NaCl during the solubilization procedure. This was indicated by treatment with chondroitin ABC lyase, which eliminated all binding of the solubilized enzymes to Q-Sepharose, heparin-Sepharose and WGA-agarose. Furthermore the strong association of the enzymes with nascent substrate would explain the lack of binding of these enzymes to chondroitin-Sepharose and chondroitin sulphate-Sepharose columns. In addition, the presence of this intact nascent proteochondroitin was found to stabilize the enzymes on solubilization, because enzyme activity was lost rapidly after the chondroitin ABC lyase treatment (Figure 4).

It is of interest that only one-third of the solubilized GlcA transferase II, but all of the GlcA transferase I, could be eluted from Q-Sepharose (Figure 3) with 0.1–0.3 M NaCl, whereas much higher NaCl concentrations were needed for elution of the other two-thirds of the GlcA transferase II. This suggests that all the GlcA transferase I and some of the GlcA transferase II was binding to nascent proteochondroitin sulphate that was at an earlier stage of synthesis, whereas the large portion of the GlcA transferase II requiring the higher concentrations of NaCl for elution was binding to nascent proteochondroitin sulphate that already contained substantial amounts of highly anionic chondroitin sulphate at a later stage of synthesis. This is consistent with a juxtaposition of all the GlcA transferase I and some of the GlcA transferase II together in the Golgi with nascent proteochondroitin containing a substantial amount of unsubstituted linkage Gal-Gal-Xyl as well as short, growing, chondroitin sulphate chains. Furthermore the elution of some GlcA transferase II, but no GlcA transferase I, at higher concentrations of NaCl is consistent with juxtaposition with nascent proteochondroitin sulphate that already contained longer chondroitin sulphate chains and no unsubstituted linkage oligosaccharide.

It is not clear how complexing of the solubilized GlcA transferases with proteochondroitin sulphate takes place. However, our results indicate that membrane-attached nascent proteochondroitin is in juxtaposition with membrane-attached enzymes, thus promoting a high degree of efficiency in positioning for synthesis of the oligosaccharide linkage region and the growing chondroitin chains on proteochondroitin acceptor. Our results indicate that the solubilized enzymes remain complexed with nascent proteochondroitin sulphate directly, and/or with small pieces of membrane into which both the enzymes and the

proteochondroitin are inserted. The latter possibility is more likely as this can be supported by our previous findings that proteochondroitin sulphate synthesized with microsomal preparations remains highly resistant to solubilization, and apparently remains attached to some membranous fragments even after it is no longer precipitable by high-speed centrifugation [14]. Moreover, the attached nascent proteochondroitin is not readily displaced by the addition of chondroitin, chondroitin sulphate or chondroitin oligosaccharides, nor does the addition of these substances stabilize the enzymes after the nascent proteochondroitin is degraded by chondroitin ABC lyase.

This research was supported by the Medical Research Service of the Department of Veterans Affairs and by Research Grant AR-41649 (to G.S.) from the National Institutes of Health.

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