Kinetics of Intracellular Processing of Chondroitin Sulfate Proteoglycan Core Protein and Other Matrix Components

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Abstract. Pulse-chase labeling techniques are used in conjunction with subcellular fractionation and quantitative immunoprecipitation to define the kinetics of intracellular translocation and secretion of proteoglycan core protein, along with link protein and type II collagen. In embryonic chick chondrocytes the core protein is processed very rapidly, exhibiting a \( t_{1/2} \) in both the rough endoplasmic reticulum and golgi region of less than 10 min. Link protein appears to be processed as rapidly as the core protein, but the kinetics of type II collagen secretion is 3-4 times slower. These results are consistent with possible segregation and coordinate intracellular processing of link protein and core protein, macromolecules which are known to associate extracellularly. In contrast, rat chondrosarcoma chondrocytes translocated and secreted the core protein much more slowly \( (t_{1/2} = 40 \text{ min}) \) than the chick cells, perhaps due to the significantly reduced levels of galactosyltransferase I observed in the transformed chondrocytes.

Cartilage chondroitin sulfate proteoglycan (CSPG) consists of a core protein to which are attached various types of carbohydrate chains, including N- and O-linked oligosaccharides in addition to the characteristic chondroitin sulfate and keratan sulfate glycosaminoglycan chains (Rodén, 1980). Obviously, the core protein must undergo extensive posttranslational modifications leading to the assembly of completed CSPG. Our previous work (Geetha-Habib et al., 1984) using pulse-chase labeling of cultured chick sternal chondrocytes followed by subcellular fractionation and immunoprecipitation indicates that the core protein is synthesized in the RER where N-linked oligosaccharides and probably O-linked xylose residues are added (Hoffman et al., 1984). A protein of apparent \( M_r \sim 370,000 \) has been identified as the putative newly synthesized, N-glycosylated form of core protein (Geetha-Habib et al., 1984). Evidence that the \( M_r \sim 370,000 \) protein is indeed the precursor comes from both functional and immunological studies. Based on labeling pattern, sensitivity of intracellular precursors to chondroitinase, keratanase and endoglycosidase H, and subcellular distribution of chondroitin sulfate glycosyltransferases, it has been concluded that after translocation to the Golgi region, the N-linked oligosaccharides are modified to the complex form and the glycosaminoglycan chains are synthesized yielding a proteoglycan of final \( M_r \) of \( 1-4 \times 10^6 \).

Most other studies of CSPG biosynthesis have used the rat chondrosarcoma as a source for chondrocytes (Kimura et al., 1981b, 1984). Results from these experiments are analogous to what has been observed in the chick sternal chondrocyte system, except there is evidence that xylosylation may not occur to a large extent in the rough endoplasmic reticulum (RER) in this system (Kimura et al., 1984, Nuwayhid et al., 1986). The temporal characteristics of CSPG assembly have also been established in rat chondrosarcoma chondrocytes (Fellini et al., 1984; Mitchell and Hardingham, 1981; Kimura et al., 1984a). In these cells the core protein appears to reside in the RER for a rather long time \( (t_{1/2} = 60-90 \text{ min}) \) with very little modification, and then extensive processing occurs in the Golgi region very rapidly \( (t_{1/2} = 10 \text{ min}) \).

While our earlier work using chick sternal chondrocytes was able to define some of the topological characteristics of CSPG assembly, the temporal parameters of this process were not reported. Our early attempts to delineate the kinetics of core protein processing using chick chondrocytes yielded results that were very different from those obtained in the rat chondrosarcoma system, suggesting significant basic differences in the two cell types or that variations in culture conditions or metabolic state of the cells might be important factors. Consequently, pulse-chase experiments have been designed to more accurately define the temporal characteristics of CSPG assembly in chick sternal and rat chondrosarcoma cultures, and to allow direct comparisons to be made between the two systems. As well we examine the kinetics of intracellular processing of the other prominent extracellular matrix molecules found in cartilage, i.e., link protein, fibronectin, and type II collagen, and find different rates of processing for the various extracellular matrix proteins.
However, the kinetics of link protein and core protein processing are similar, suggesting possible coordinate transport.

Materials and Methods

1-[^35]S[Methionine (800 Ci/mmol) was purchased from Amersham. UDP-
[^3]C[Galactose (337 mCi/mmol) and [^35]S[U]late (carrier-free) were from New England Nuclear (Boston, MA). UDP-Galactose and cycloheximide were from Sigma Chemical Co. (St. Louis, MO). Normal rat serum, normal rabbit serum, goat anti-rabbit serum, and goat anti-rabbit serum were all from Miles Scientific (Naperville, IL). Monoclonal mouse anti-link protein
(12C2) was a generous gift of Dr. B. Cardin (University of West Vir-
ginia, Morgantown, WV). Polyclonal rabbit anti-human plasma fibronectin antisera was obtained from Bethesda Research Laboratories, (Bethesda, MD). Polyclonal rabbit anti-chick type II collagen antisera (cross absorbed from type I collagen) was a generous gift of Dr. T. F. Lindemannery (Tufts University, School of Medicine, Boston, MA). PD-10 columns con-
taining Sephadex G-25 were from Pharmacia Fine Chemicals, (Ficsatow, NJ). Aqueous counting scintillant was from Amersham. Collagenase was from Worthington while trypsin, F, media, gentamycin, penicillin, and streptomycin were all from Gibco, (Grand Island, NY). 6-Aminocaproic acid, benzamidine HCl, phenylmethylsulfonyl fluoride (PMSF), pepstatin, and N-ethyl maleimide were all purchased from Sigma Chemical Co. Pro-
teoglycan monomer from chick epiphysis was prepared in the presence of protease inhibitors as described (Hascall and Kimura, 1982). A Toyoda Soda TSK-4000 SW sizing column (7.5 cm × 60 cm) was purchased from Anspec (Ann Arbor, MI) and used in conjunction with a Varian 5000 Liquid Chromatography instrument.

Cell culture

Cultures of chick sternal chondrocytes were established from 14 d embryos according to previously described procedures (Geetha-Habib et al., 1984; Cahn et al., 1967) with a few important modifications. Briefly, cells were plated at an initial density of 2.5 × 10^6/60 mm Falcon Primaria culture dish in F12 medium supplemented with 10% FCS and 50 μg/ml gentamycin. The cells were allowed to attach to the dish and subsequent growth was maintained by a complete change of the F12 media containing FCS and gentamycin on the third day. On day 4 a rubber policeman was used to scrape the tissue was dispersed by gently forcing it through a 20 cm 3 syringe without tissue fibronectin. The yield and purity of the various fractions from unlabeled cells were determined by ultracentrifugation in a Beckman L2-65 ultracentrifuge (type 65 rotor) to pellet the rough microsomes. At each substep, aliquots of the pellet and supernatant were saved for further characterization.

Subcellular Fractionation

Subcellular fractionation of chondrocytes was performed as previously de-
scribed (Geetha-Habib et al., 1984) with a few modifications which helped to improve the yield of the membrane preparations. Cells were allowed to swell in 10 mM Hepes-NaOH buffer, pH 7.2, containing 0.25 M sucrose, 1 mM DTT, and a mixture of protease inhibitors (0.1 M 6-aminocaproic acid, 5 mM benzamidine HCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mM NaEDTA). Cells were then homogenized with 100 strokes in a tight-fitting glass dounce homogenizer, frozen, thawed and homogenized with 50 more strokes. Homogenates were then centrifuged at 300 g for 10 min to remove nuclei, unbroken cells, and large membrane fragments. The supernatant was centrifuged again at 10,000 g for 10 min and the resultant supernatant was spun at 100,000 g for 1 h in a Beckman L2-65 ultracentrifuge (type 65 rotor) to pellet the rough microsomes. At each substep, aliquots of the pellet and supernatant were saved for further character-
ization.

Discontinuous sucrose gradients were prepared according to Wibo et al. (1981) with modifications to optimize results in this system. The 10,000 g pellet was suspended in 54% (w/vol) sucrose containing 10 mM Hepes-
NaOH, pH 7.2, 1 mM DTT, and the mixture of protease inhibitors. This solution was homogenized in a dounce (25 strokes) to disperse all clumps. A discontinuous gradient was then made with 1 ml of 80% sucrose on the bottom of a cellulose nitrate tube (5.0 ml) overlaid by the sample in 54% sucrose (w/vol) followed by 1.5 ml 40% sucrose (w/vol) and 1.0 ml 21.6% sucrose (w/vol) solutions. All sucrose solutions contained the Hepes, DTT, and protease inhibitors as described. The 100,000 g pellet was resuspended in the same manner (except that it was brought up in the 80% sucrose [w/vol]), and was placed on the bottom of the gradient tube and overlaid by the 54, 40, and 21.6% (w/vol) sucrose solutions. The gradients were centrifuged in a SW50.1 rotor at 40,000 rpm (4°C) for 2 h, and the inter-
faces were drawn off and diluted with the 0.25 M sucrose buffer mentioned above. Membranes were then collected by centrifugation in a type 65 rotor at 40,000 rpm for 1 h at 4°C, and pellets were resuspended in an appropriate buffer for further analysis. Our previous work using this protocol (Geetha-Habib et al., 1984) demonstrated that the 10-1 fraction (top interface from the gradient containing 10,000 g pellet material) was highly enriched in golgi-derived membranes, while the 100-3 fraction (bottom interface from the gradient containing 100,000 g pellet material) was enriched in RER-
derived membranes.

Characterization of Subcellular Fractions

The yield and purity of the various fractions from unlabeled cells was monitored by the following assays.

RNA content was used as a marker for RER and was determined by the modification of the procedure of Munro and Fleck, as described (Munro and Fleck, 1966; Hutchinson and Munro, 1961; Munro et al., 1975). UDP-galactose ovalbumin galoactosyltransferase (UDP-galactose β-D-N-
acetylglucosamine-glycopeptide β 1→4 transferase; EC 2.4.1.22), was used as a marker for the smooth endoplasmic reticulum and the golgi apparatus (Brew et al., 1975). The procedures for both of these assays were described in detail previously (Geetha-Habib et al., 1984).

Protein concentrations were determined by the method of Bradford (Brad-
ford, 1976) using Bio-Rad protein dye reagent.

Macromolecular incorporation of isotope into various fractions was de-
termined by TCA precipitation. 50 μg of BSA was added to an aliquot of labeled material, and this was diluted by an equal volume of 20% TCA, 1 mM unlabeled methionine or leucine and put on ice. Precipitated protein was recovered by centrifugation, washed twice with 5% TCA, and dissolved in 0.1 N NaOH. The dissolved samples were transferred to a 5 ml plas-
tic vial, neutralized with HCI, and then 4.5 ml of Aqueous Counting Scintil-
ant (Amersham) was added, and the samples were counted in a Packard Tri-Carb 460 C&D liquid scintillation system.

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Immunoprecipitation

The labeled membrane preparations from the gradients were resuspended in 10 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl, 0.5% (wt/vol) sodium deoxycholate, 1% (wt/vol) Nonidet P-40, 0.1 M 6-aminocaproic acid, 5 mM benzamidine HCl, 1 mM PMSF, 0.3 mM pepstatin, and 1 mM EDTA (lysis buffer).

To immunoprecipitate CSPG core protein, an aliquot of labeled material was diluted to 200 μl with lysis buffer, and 20 μl of the monoclonal antibody SI03L, which has been shown to precipitate chick core protein specifically was added (Upholt et al., 1981). After 2 h of incubation at 25°C, 5 μl of normal rat serum and 200 μl of rabbit anti-rat IgG (Miles Scientific) were added and incubated 2 more hours at 4°C. The immunoprecipitates were centrifuged (5 min) in a Beckman microfuge and washed once in lysis buffer, a second time in lysis buffer containing 0.1% SDS, and a third time in 10 mM Tris-HCl, pH 7.4, containing 0.1% Nonidet P-40 (Brassil and Lodish, 1981). The immunoprecipitates were solubilized in sample buffer and used for gel electrophoresis as described below.

The procedure just outlined was also used to immunoprecipitate the samples for fibronectin and type II collagen. Titration experiments indicated that the following concentrations were optimal. 5 μl of polyclonal rabbit anti-human plasma fibronectin antiserum (Bethesda Research Laboratories) was used as the primary antibody to immunoprecipitate fibronectin from the fractions. 2 μl of normal rabbit serum (Miles Scientific) was used as carrier and 100 μl of goat anti-rabbit serum (Miles Scientific) was used as the secondary antibody. 10 μl of polyclonal rabbit anti-chick type II collagen antiserum was used as the primary antibody to immunoprecipitate type II collagen. This antiserum was a generous gift of Dr. T. F. Linsenmeyer (Tufts University School of Medicine). The carrier and the secondary antibody were the same as for the fibronectin immunoprecipitation.

The mouse monoclonal antibody 8A4 (a generous gift of Dr. B. Caterson, West Virginia University) was used to immunoprecipitate link protein from the subcellular fractions. 8A4 apparently recognizes unaggregated link protein most effectively (Caterson et al., 1985); therefore, the samples were boiled in 1% SDS for 2 min in preparation for immunoprecipitation. 8A4 was added to the samples after dilution down to 0.1% SDS, and Sialyltransferase Pansorbin (Calbiochem) was used to bring down the Ag-Ab complexes. Preabsorption of the samples to the Pansorbin followed by centrifugation was also routinely carried out, since it successfully removed non-specific precipitable material. Immunoprecipitates were washed and handled as described above. Appropriate controls omitting primary or secondary antibodies were routinely done in each case.

Gel Electrophoresis

SDS-polyacrylamide slab gel electrophoresis was carried out by the method of Laemmli (Laemmli, 1970), using 3.75 or 10% resolving gels and 3% stacking gels. Smears were heated at 100°C for 5 min in sample buffer, 0.0625 M Tris-HCl, pH 6.8, containing 2% SDS (wt/vol), 10% glycerol (vol/vol), and 1% DTT (wt/vol). Gels for autoradiography were fixed in 25% methanol (vol/vol) and 10% acetic acid (vol/vol) for 0.5 h, infiltrated with Amplify (Amersham) for 0.5 h, and then dried for 2 h at 60°C in a Bio-Rad gel drier. Dried gels were exposed to Kodak X-Omat XAR-5 film at -70°C. In some cases the autoradiographs were scanned using a LKB 2202 scanner with unidirectional flow between compartments. Cycloheximide and excess unlabelled methionine were used to prevent further production of labeled core protein in the RER. Assuming first order loss of label from each compartment (Fellini et al., 1984), and that the flow was ordered $A \rightarrow B \rightarrow C$, the distribution of label can be described by the following equations:

$$\begin{align*}
\frac{dA}{dt} &= -k_1A(t) \\
\frac{dB}{dt} &= k_1A(t) - k_2B(t) \\
\frac{dC}{dt} &= -k_2B(t)
\end{align*}$$

Where $A =$ CPM in the RER, $B =$ CPM in the Golgi region, $C =$ CPM in the media, and $K_1$ and $K_2$ are the rate constants for intercompartmental flow. These equations were integrated, taking into account that even after long chase times the RER appears to contain some residual core protein ($K$), and that some core protein has already reached the Golgi region by the end of the 10-min pulse time ($K$). Integration then yielded the following:

$$A = Ae^{-k_1t} + \left(\frac{Ak_1}{k_2 - k_1}\right) (e^{k_2t} - e^{k_1t}) + Be^{-k_2t}$$

The data were fit to these equations using a Hewlett Packard computer with a program by Yamaoka et al., (1981), and modified by Dr. Ferenc Kezdy (University of Chicago, Chicago, IL).

Incorporation of [35S]Sulfate into CSPG After Treatment with Cycloheximide

Labeling of chick sternal or rat chondrosarcoma chondrocytes with [35S]SO$_4$ at various times after cycloheximide treatment to estimate and compare the rates of core protein processing was done essentially as described by Kimura et al. (1984), with a few modifications. Chick sternal or rat chondrosarcoma chondrocytes prepared as described were suspended at 37°C in F12 media at a concentration of 5 × 10$^5$ cells/ml and aliquoted into several tubes. 100 μg/ml cycloheximide was added (except to the control samples) and the cells were incubated at 37°C. At various times after initiation of cycloheximide treatment, [35S]sulfate (10 μCi/ml) was added. Labeling was continued to 37°C for 10 min and then incorporation of label was stopped by placing the sample on ice and adding an equal volume of 8 M GuHCl at 4°C containing 100 mM 6-aminocaproic acid, 10 mM Na$_2$EDTA, 10 mM benzamidine HCl, 2 mM PMSF, 5 mM N-ethyl maleimide, and 100 mM Tris-HCl at pH 7.4 (8 M extraction buffer). Each sample was vortexed vigorously and spun in a SA600 rotor in a Sorvall centrifuge at 9,000 rpm (10,000 g) at 4°C for 15 min to pellet the unextracted material. The supernatant, which contains media and cell extract, was saved and a fraction was run on a PD-10 column in 4 M GuHCl buffer with 100 mM Tris-HCl (pH 7.4), containing 1 M Na$_2$SO$_4$, 0.15 M NaCl, and the same mixture of proteases as in the 8 M GuHCl buffer described above. The void volume was counted to estimate macromolecular incorporation of sulfate.

Estimation of Chondroitin Sulfate Chain Length

[35S]Sulfate labeled samples prepared as described above were dialyzed vs. H$_2$O, lyophilized, and then dissolved in 0.5 M NaOH, 1 M Na borohydride and left at 25°C for 24 h (Fellini et al., 1981; Carlson, 1968). The samples were neutralized with concentrated acetic acid and run on a 75 mm × 60 cm TSK 4,000 column (Varian 5000 Liquid Chromatography Instrument) eluted with 0.15 m NH$_4$OAc, 0.1 M Na$_2$SO$_4$ (pH 7.0), which had been calibrated with glycosaminoglycan standards of M, 15,000, 25,000, and 45,000, provided by Dr. M. Mathews (University of Chicago). Aliquots were collected and counted.

[35S]Methionine-labeling Experiments

Chick sternal and rat chondrosarcoma chondrocyte cultures were labeled with [35S]methionine as described, yielding cell and media samples for each chase time point. The media was combined with a 4 M GuHCl extract of the cell fraction for each time point and the samples were prepared for...
dissociative CsCl centrifugation to isolate proteoglycan monomer (Hascall and Kimura, 1982). 5-mg chick epiphyseal proteoglycan monomer was used as carrier and each sample was made 4 M in guanidine HCl and brought to a density of 1.50 g/ml with CsCl. The samples were then centrifuged at 35,000 rpm for 48 h at 5°C in a 50.2 Ti rotor. Proteoglycan monomers (D1) were removed from the bottom fourth of the gradients, dialyzed vs. H2O, and counted. Samples were also run on 3.75% gels which were put up for autoradiography. The autoradiographs were scanned with an LKB densitometer to confirm that the [35S]methionine label isolated was incorporated into proteoglycan.

Comparison of Glycosyltransferase Activities

Particulate and soluble fractions were obtained from chick epiphyses or rat chondrosarcoma and assayed for xylosyltransferase and galactosyltransferase I activity as previously described (Schwartz et al., 1976).

Results

Intracellular Processing and Secretion of CSPG Core Protein

We have previously reported an effective procedure for the subcellular fractionation of chick sternal chondrocytes (Geetha-Habib et al., 1984), using differential centrifugation of cell homogenates followed by discontinuous sucrose gradients. This procedure yielded highly enriched membrane preparations, as characterized by electron microscopy, [3H]-puromycin incorporation, RNA content, and several enzyme assays (phosphodiesterase, acid phosphatase, and UDP-galactosyltransferase). The results indicated that the 10-1 fraction (lightest interface from the gradient containing the 10,000 g pelleted material) was highly enriched in smooth endoplasmic reticulum (SER) or golgi membranes, while the 100-3 fraction (densest interface from the gradient containing the 100,000 g pelleted material) was relatively enriched in RER membranes. Such a fractionation scheme is requisite for the examination and localization of intracellular precursors found during the process of CSPG assembly. In the present studies, modifications have been made in cell culture and harvesting procedures to increase incorporation of radioactive precursors, and in the fractionation procedures in order to increase yields (See Materials and Methods). However, as shown in Table I, the fractionation scheme is still successful as the 10-1 fraction remains highly enriched in Golgi or SER membranes with very little cross-contamination, while the 100-3 fraction remains enriched in RER membranes.

The modified subcellular fractionation procedure was then used in conjunction with an in vitro pulse-chase labeling protocol to study the kinetics of intracellular translocation and secretion of the core protein. Chick sternal chondrocytes were pulse labeled for 10 min followed by chase incubations for various times in the presence of cycloheximide. The cells were fractionated and the membrane preparations obtained were immunoprecipitated with a monoclonal antibody (SI03L) which is specific for the core protein of chick cartilage proteoglycan (Upholt et al., 1981). Immunoprecipitates were then visualized by autoradiography as shown in Figs. 1 and 2. Controls omitting primary or secondary antibodies have shown that material of apparent Mr < 240,000 represents nonspecific immunoprecipitate (Geetha-Habib et al., 1984).

Examination of the autoradiographs in Fig. 1 shows that after the 10-min labeling time (0-min chase sample), the chondrocytes contain a high concentration of the immunospecific ~370,000-Mr core protein, but this protein rapidly decreases in concentration upon reincubation at 37°C in the presence of cycloheximide. It is also evident that during the pulse-labeling period (10 min) some of the core protein has already been translocated to the Golgi region (10-1 frac.

Figure 1. Autoradiographs of core protein immunoprecipitated from the various subcellular fractions and analyzed by SDS–PAGE. [35S]Methionine pulse-chase labeled chondrocytes were fractionated and immunoprecipitated with SI03L. The immunoprecipitates were then analyzed on 3.7% SDS–polyacrylamide gels. (Lanes A–E) Whole cell homogenates from cells chased for 0, 2, 5, 10, and 20 min, respectively. (Lanes F–J) Golgi-enriched fractions from the same chase times. (Lanes K–N) Media from cells chased for 0, 2, 5, 10, and 20 min, respectively. Molecular weight standards indicated in the margin are: (1) Myosin (200,000); (2) β-galactosidase (116,000); (3) phosphorylase B (92,000).
tion), and that most of this material has been partially glycosylated, as suggested by the predominance of slower migrating forms (Fig. 1 F). The concentration of proteoglycan intermediates then increases in the Golgi region until it peaks at the 5-min chase time (Fig. 1 H). At this point the Golgi region contains a fairly continuous array of core protein forms of increasing relative molecular weight, starting at Mr $\approx 370,000$. These observations confirm the precursor nature of the Mr $\approx 370,000$ protein and suggest that the extensive glycosylation of the core protein occurs in the Golgi region in a rapid and continuous manner under the present experimental conditions. After 5 min under chase conditions, proteoglycan intermediates decrease in concentration in the Golgi, as more extensively glycosylated forms predominate (Fig. 1, H-J). The media (Fig. 1, K-N) show an increase in secreted proteoglycan with advancing chase time as expected. Some precursor Mr $\approx 370,000$ core protein is present in the media, and remains constant with time. Most likely a small amount of cell lysis occurs due to the addition of warm (37°C) buffer at the start of the chase to cells that have been washed at 4°C, thus leakage some precursor to the media fraction artifactually. Nevertheless, an insignificant percentage of the cells are being lysed, since the amount of precursor is <1% of the immunospecific material in the media at later chase times.

Fig. 2 represents an autoradiograph of immunoprecipitates from the RER-enriched subcellular fraction (100-3) from a similar experiment, showing that the core protein precursor of Mr $\approx 370,000$ is highest in concentration in the RER immediately after the 10-min pulse, and that it decreases in concentration very rapidly. In fact, little core protein is detectable in the RER at later times. These results are qualitatively consistent with synthesis of the core protein, followed by rapid translocation of this precursor out of the RER.

Quantitation of CSPG Intermediates in Subcellular Compartments

Immunoprecipitates were also run on gels which were sliced and counted to provide quantitation of the radioactivity in the Mr $\approx 370,000$ precursor and other proteoglycan intermediates in the subcellular fractions (See Materials and Methods). The radioactivity incorporated into core protein forms in the 10-1 fraction have been adjusted since the yield of Golgi membranes in this fraction, although highly reproducible (4.6, 4.4, and 4.4% in 3 different experiments), was considerably less than 100%. Hence, the amount of core protein present in the Golgi region was estimated by multiplying the CPM present in the 10-1 fraction by the term (100/yield of Golgi membranes) (See Materials and Methods). The estimates obtained are dependent upon the 10-1 fraction being a representative sample of the Golgi membranes, a point that is inherently difficult to establish with certainty. We show that our estimates of the amount of core protein in the Golgi region are internally consistent with the amounts of precursor protein present in the RER and of completed proteoglycan in the media at the different time points, i.e., that the estimates obtained fit the three compartment model for intracellular processing fairly well (Fig. 3). Therefore, our estimates of the amount of proteoglycan intermediates in the Golgi apparatus, obtained by correcting for the incomplete yield of enriched Golgi membranes present in the 10-1 fraction, appear to be reasonably accurate.

Similar attempts to determine the radioactivity of the Mr $\approx 370,000$-precursor in the RER using data derived from the 100-3 fraction have been less successful, even when correction was made for the incomplete yield of RER membranes in this subcellular fraction. The CPM of precursor in the RER calculated from the 100-3 fraction were uniformly less than 40% of what was recovered from the whole cell homogenates. Difficulty in recovering core protein from rough membrane preparations (yield = 30% of expected) was also observed by Fellini et al. (1984) in rat chondrosarcoma chondrocytes using different fractionation methods. In contrast, recovery of proteoglycan intermediates from Golgi preparations was not problematic in either study. Apparently, the core protein more easily escapes from the RER during subcellular fractionation. Perhaps the core protein associates more tightly with the Golgi apparatus due to interaction with the extensive glycosylation machinery present in this organelle. Due to these difficulties the 100-3 fraction was not routinely used for quantitative purposes. Instead, the amount of pre-Golgi region located precursor has been estimated by subtracting the CPM of the Mr $\approx 370,000$ core protein in the Golgi region from the CPM of this precursor in the whole cell homogenate. That this is a reasonable approximation is supported by data showing that the 10-1 fraction contains almost no cross-contamination with RER-derived membranes (Table I). Also, calculations show that the Golgi region never has more than 5% of the total Mr $\approx 370,000$-precursor in the cell.

Fig. 3 shows the data for the CPM of proteoglycan intermediates in the three compartments as a function of chase time. To fit theoretical curves to these data, unidirectional flow of material was assumed, as was first order kinetics for the loss of material from each compartment (Fellini et al., 1984). The process can then be represented by the equation $A^{\delta}B^{\beta}C$, where $A = CPM$ of the Mr $\approx 370,000$ precursor in the pre-Golgi compartment (RER), $B = CPM$ of all core
Comparison of Core Protein Processing by Chick Sternal and Rat Chondrosarcoma Chondrocytes

The rapid kinetics of core protein processing observed in chick sternal chondrocytes contrasts with what has previously been found using rat chondrosarcoma chondrocytes. In other studies it has been shown that the core protein has a very long intracellular dwell time ($t_{90} = 60-90$ min) in the rat cells (Fellini et al., 1984; Mitchell and Hardingham, 1981; Kimura et al., 1981a). Consequently, experiments were designed to directly compare the kinetics of core protein translocation in these two cell types.

To this end, a modification of the previously reported procedures (Mitchell and Hardingham, 1981; Kimura et al., 1981a) of $^{35}$S sulfate pulse labeling of chondrocytes at various times after cycloheximide treatment has been developed as described in Materials and Methods. Theoretically, the [$^{35}$S] sulfate incorporation into glycosaminoglycan after inhibition of protein synthesis should be proportional to the amount of unprocessed core protein left in the cell, which in turn should be a function of the rate of core protein processing. Therefore, cultured rat chondrosarcoma or chick sternal chondrocytes were harvested, incubated at 37°C in the presence of 100 $\mu$g/ml cycloheximide, and at various times after this, 100 $\mu$Ci/ml of $^{35}$S sulfate was added and labeling was continued for 10 min. Subsequent dilution by an equal volume of 8 M GuHC1 buffer at 4°C served both to terminate labeling and to begin extraction of the cells. After 30 min on ice with vigorous vortexing, the samples were centrifuged and the incorporation of $^{35}$S sulfate into macromolecules into the su-

### Table I. Characterization of Subcellular Fractions*

| Fraction  | UDP-galactosyltransferase (Relative specific activity) | RNA content (Relative A$_{260}$/mg) |
|-----------|--------------------------------------------------------|-----------------------------------|
| Homogenate| 1.0                                                    | 1.0                               |
| 10-1      | 9.55                                                   | .063                              |
| 100-3     | 1.61                                                    | 2.43                              |

* Microsomal fractions obtained from subcellular fractionation of chick sternal chondrocytes were assayed for UDP-galactosyltransferase activity and RNA content as described in Materials and Methods. The 10-1 fraction represents membrane vesicles from the lightest interface of a gradient containing 10,000 g pellet material, while the 100-3 fraction was derived from the heaviest interface from a gradient containing 100,000 g pellet material.
permatant fraction (media plus 4 M GuHCl cell extract) was determined by gel filtration on PD-10 columns (Fig. 4 A). SDS-PAGE followed by autoradiography of the material from the void volume of the PD-10 columns indicated that >97% of the macromolecular [35S]sulfate had been incorporated into proteoglycan (not shown). Controls in which the cells were incubated without cycloheximide have also been included.

The amount of [35S]sulfate incorporated by the cells decreases with apparent first-order kinetics exhibiting $t_{1/2}$ values for the chick and rat chondrocytes of ~10 and 40 min, respectively (Fig. 4 A). However, before any correlation could be made between [35S]sulfate incorporation and the amount of unprocessed core protein remaining in the cell, the data had to be corrected for changes that occur in the average amount of sulfated carbohydrate associated with the proteoglycan. The carbohydrate entities of CSPG are known to be nearly uniformly sulfated (~90-95% of the GalNAc residues); however, it has been observed that the carbohydrate content per core protein varies inversely with the amount of proteoglycan being synthesized, and that this change in carbohydrate content is due primarily to variations in the average length of the chondroitin sulfate chains (Lohmander et al., 1979; Kato et al., 1978). In contrast, the number of such chains per core protein has been shown to be a constant feature of proteoglycan structure (Fellini et al., 1981a).

To estimate average chondroitin sulfate chain lengths, the samples were digested with alkaline borohydride and then released [35S]sulfate labeled carbohydrate chains were run on a TSK4000 sizing column calibrated with carbohydrate standards of known relative molecular weight (Figs. 5 and 6). As expected, the average chain size increases with time after cycloheximide treatment for both the chick and the rat samples, but there is an important qualitative difference between the two systems. The average chain length of the chick samples increases rapidly with time after cycloheximide treatment, and then levels off (Fig. 5 I), while the rat samples exhibit a much more gradual change in this parameter (Fig. 6 I). These observations suggest that the chick chondrocytes are more rapidly cleared of core protein than the rat chondrocytes, in agreement with the previous experiments.

The CPM of [35S]sulfate incorporated at each time point was then adjusted for the observed changes in relative chain lengths (See Fig. 4 B). After correction for this factor, the incorporation of label should be proportional to the amount of unprocessed core protein remaining in the cell, as discussed earlier. Computerized fit of the data shows that the amount of core protein precursor left in the cell decreases with first-order kinetics for both cell types, but that the chick cells are still processing the core protein much more rapidly than the transformed rat cells ($t_{1/2}$ = 7.9 and 36 min, respectively).

**[35S]Methionine-labeling Experiments**

Rat chondrosarcoma and chick sternal chondrocytes were also pulse labeled for 10 min with [35S]methionine to follow core protein processing and secretion directly. After various chase times in the presence of cycloheximide, proteoglycan monomer was purified from combined media plus 4 M guanidine-HCl cell extract by dissociative CsCl gradient centrifugation. Labeled monomer samples (DI) were run on SDS-polycrylamide gels, detected by autoradiography, and radioactivity incorporated into secreted proteoglycan at each time point (Fig. 7) was quantitated by scanning with an LKB densitometer. As before, the chick chondrocytes processed the core protein much more rapidly than the rat chondrocytes.
Comparison of the kinetics of secretion of proteoglycan by chick sternal or rat chondrosarcoma chondrocytes. Proteoglycan monomer was isolated from the media of chondrocytes pulse-chase labeled with \([35S]\)methionine by dissociative CsCl centrifugation. A 6-h time point was also included to provide an estimate of the total CPM of proteoglycan secreted. (○) Chick sternal chondrocytes. (○) Rat chondrosarcoma chondrocytes. The pulse labeling time period was 10 min as in the earlier experiments.

\((t_{1/2} \text{ values } \sim 15 \text{ and } 60 \text{ min, respectively})\), again demonstrating a significant difference in this aspect of proteoglycan synthesis by the two cell types.

Comparison of the Activities of Xylosyltransferase and Galactosyltransferase I in Embryonic Chick and Rat Chondrosarcoma Chondrocytes

In an attempt to explain the apparent difference in intracellular transit rates for proteoglycan core protein in the two chondrocyte systems, the chain initiating glycosyltransferases, which might be rate-limiting in the critically important processing steps, were investigated. Accordingly, particulate and soluble fractions were obtained from chick and rat chondrocytes and assayed for the two enzymes that initiate chondroitin sulfate glycosaminoglycan synthesis, xylosyltransferase and galactosyltransferase I (Table II). The most notable finding was that the rat chondrocytes exhibited markedly (24-fold reduced activities of galactosyltransferase I in both the soluble and particulate fractions. In contrast, the xylosyltransferase levels were comparable in the two cell types. The limited availability of galactosyltransferase I in the rat chondrocytes may at least partially account for the slower rate of core protein processing observed in this cell type (See Discussion). Furthermore, the reduced levels of galactosyltransferase I, an integral Golgi membrane protein, may also account for the increased tendency of xylosyltransferase to segregate into the soluble fraction in the rat cells (Table II). Xylosyltransferase, which is relatively soluble (50%), is known to associate specifically with galactosyltransferase I (Schwartz and Rodén, 1975; Schwartz, 1975), and this interaction may influence the compartmentalization of xylosyltransferase.

Comparison of the Kinetics of Processing of Core Protein, Link Protein, Fibronectin, and Type IICollagen

Because of the significant differences observed in intracellular transit and secretion rates of CSPG, it was of interest to investigate the kinetics of processing of the other predominant extracellular matrix components, some of which are known to associate noncovalently with CSPG extracellularly. To this end, subcellular fractions of chick sternal chondrocytes pulse-chase labeled with \([35S]\)methionine or \([\text{H}]\)leu-
cine were immunoprecipitated with antibodies specific for core protein, link protein, fibronectin and type II collagen as described in Materials and Methods. Immunoprecipitates were analyzed by SDS–PAGE followed by fluorography, and the resulting autoradiographs were scanned with an LKB densitometer to quantitate the relative amounts of each of these proteins in the subcellular fractions as a function of chase time (Fig. 8). Fibronectin appears to be processed by the chick chondrocytes almost as rapidly as the core protein, since both of these proteins reach peak concentrations in the Golgi between 5 and 10 min (Fig. 8 B) and are cleared from the cells with a half-life of ~15 min (Fig. 8, A and C). On the other hand, type II collagen seems to be processed much more slowly. It does not reach maximal concentration in the Golgi region until 1 h (Fig. 8 B), and is cleared from the cells with a much longer half-life than core protein or fibronectin (Fig. 8, A and C). These observations suggest that the synthesis of two of the most prevalent molecules of the extracellular matrix of cartilage, chondroitin sulfate proteoglycan and type II collagen, is not tightly coupled intracellularly.

The situation for link protein is somewhat more complex (Figs. 9 and 10). Link protein in the Golgi region and the media fractions from chick chondrocytes (Fig. 9) exists in a more heterogeneous and slightly higher relative molecular weight form than the precursor form present in the cell homogenates. These changes evidently reflect the processing of N-linked oligosaccharides which is known to occur in the Golgi region. All of the link protein that was secreted was processed as rapidly as the core protein; however, as much as 50% of the newly synthesized link protein remained in an unprocessed form within the cell, suggesting excess synthesis. The stoichiometry of link protein to core protein secreted by chick chondrocytes was estimated by comparing the relative incorporation of [3H]leucine into these proteins. After specific immunoprecipitation followed by SDS–PAGE and slicing and counting the appropriate regions of the gels, the incorporation of the isotope into secreted core protein was determined to be 5.41 times greater than the incorporation into secreted link protein. Taking into account the relative molecular weights (340,000 for the core protein [Upholt et al., 1981] and 40,000 for the link protein [Neame et al., 1986; also See Fig. 9]) and leucine concentrations (60 residues/I,000 for the core protein [DeLuca et al., 1978] and 85 residues/I,000 for the link protein [Neame et al., 1986]), this
would correspond to a stoichiometry of 1.10 molecules of link protein secreted per molecule of core protein. Hence, it appears that the chick chondrocytes synthesize excess link protein but secrete link protein in a 1:1 ratio with proteoglycan core protein. From other studies link protein appears to be associated with core protein immediately after secretion, since exogenous monomer is incapable of dissociating newly synthesized endogenous PG monomer and link protein (Kimura et al., 1980), and it is therefore possible that link protein and core protein may even begin to interact intracellularly. The results of these experiments are consistent with obligatory intracellular interaction or some other manner of coordinated posttranslational processing of these two proteins (see Discussion). Alternatively, the similar kinetics of processing of the core and link proteins may be merely coincidental.

**Discussion**

Our previous work on assembly of chondroitin sulfate proteoglycan in chick sternal chondrocytes demonstrated the initial synthesis of a precursor containing N-linked oligosaccharides, which is translocated in an energy-dependent manner to the Golgi region where processing of the N-linked oligosaccharides and extensive addition of keratan sulfate and chondroitin sulfate chains occurs. Although the temporal parameters of this process were not reported, the processing of core protein in embryonic chick sternal chondrocytes was found to occur extremely rapidly, in striking contrast to the long intracellular dwell time reported for proteoglycan core protein in rat chondrosarcoma chondrocytes (Fellini et al., 1984). To better understand this difference in one important parameter of the synthetic process of very similar molecules, experiments were designed to directly compare the kinetics of translocation and secretion of core protein in these two cell types.

Direct comparison of the kinetics of core protein processing by the two cell types (See Figs. 4–7) appears to verify that the chick chondrocytes are capable of processing the core protein 3–4 times more rapidly than the chondrosarcoma cells. It should be mentioned that at least one other proteoglycan, proteodermaetan sulfate is synthesized, processed and secreted by human skin fibroblasts (Glossl et al., 1984) fairly rapidly, with kinetics similar to that of the chick chondrocyte rather than the rat chondrosarcoma system. These results would suggest that proteoglycans, although requiring extensive posttranslational modification, are processed efficiently and in a timely fashion in most cell types, and that the rat chondrosarcoma system may be aberrant in this respect. It is also interesting to note that the difference in rate of transport of the core protein out of the RER can account for the disparity in the kinetics of the secretion process by the two cell types. This is not unusual in light of intracellular transport rates reported for several other secretory proteins (see below). However, most impressive is the ability of both types of chondrocytes to carry out the series of processing and modification reactions (estimated to be greater than 25,000 individual reactions per molecule) in an efficient and rapid manner in the Golgi region. This feature of the biosynthetic process is similar for both cell types, each exhibiting $t_0$ for the core protein in the Golgi region of $\sim 10$ min.

Since our previous results in chick chondrocytes, as well as those in rat chondrosarcoma, suggest that very little processing of the core protein occurs while residing in the RER, the apparent long dwell time in RER in rat chondrosarcoma is puzzling. Perhaps the significantly decreased specific activity of galactosyltransferase I previously observed in the rat chondrocytes (Stoolmiller et al., 1975) and in the present study (Table II) is an important factor. Galactosyltransferase I is the first particulate glycosyltransferase encountered by the core protein upon entering the Golgi apparatus (Schwartz and Rodén, 1975), and it may play an essential role in directing or routing the core protein for further processing in a membranous subcompartment of the Golgi region containing the remainder of the glycosylating enzymes. Such an organization of glycosyltransferases and substrate has been proposed to explain the high efficiency and rapidity of the glycosylation process (Stoolmiller et al., 1972; Horwitz and Dorfman, 1968; Schwartz et al., 1974). Furthermore, it has been demonstrated that xylosyltransferase associates specifically with galactosyltransferase I (Schwarz and Roden, 1975; Schwartz, 1975), and it has therefore been hypothesized that this interaction may help “dock” the core protein into the proper region of the Golgi for further glycosylation to take place. Such “docking” of a xylosyltransferase-core protein complex with directed release of the core protein may also be necessary for recycling of xylosyltransferase and thus for continued core protein translocation. Limited availability of galactosyltransferase I in rat chondrosarcoma chondrocytes may then be responsible for the enhanced intracellular pool of unprocessed core protein, the decreased rate of translocation and secretion of core protein, and the large amounts of soluble xylosyltransferase observed in this cell type. The severe deficiency of galactosyltransferase I activity in rat chondrosarcoma may also be partly responsible for the lag in elongation of chondroitin sulfate chains by rat chondrosarcoma chondrocytes after cycloheximide treatment (See Figs. 5 I and 6 I). Apparently arguing against a rate limiting or regulatory role of galactosyltransferase I is the fact that B-xylosides, which act as substrate analogues for this enzyme, are known to stimulate chondroitin sulfate GAG synthesis by chondrocytes (Schwartz, 1977; Galligani et al., 1975). However, the B-xylosides have been shown to be effective only at high concentration (>1.0 mM), and at such nonphysiologic concentrations of substrate it is difficult to draw any definite conclusions about the role of the enzyme in question.

Decreased expression of galactosyltransferase I may represent one of the altered characteristics of the transformed phenotype of the chondrosarcoma chondrocytes. For instance, transformation may increase the sensitivity of the chondrocytes to certain regulatory hormones, e.g., cortisol. The levels of this hormone have been shown to be inversely proportional to the specific activity of galactosyltransferase I in untransformed guinea pig chondrocytes. In contrast, the activities of the other linkage region and chain elongation glycosyltransferases appear to be relatively insensitive to cortisol levels (Bird et al., 1986). Of course, there are other significant differences between the rat chondrosarcoma and embryonic chick systems which may account for the disparity in the rates of core protein processing. For example, the lack of keratan sulfate glycosaminoglycan chains on the rat proteoglycan may affect the rate of processing, particularly if the absence of these chains is primarily due to a selec-
tive deficiency of the enzymes required for keratan sulfate synthesis.

As mentioned previously, the rapid processing of proteoglycan core protein was examined in relation to the processing and secretion of the other prominent matrix components to determine whether the deposition of the extracellular matrix molecules of cartilage is tightly coupled. Some of these molecules are known to associate after secretion, in particular core protein and link protein, and it is possible that they may begin to interact intracellularly. Our results suggest that this is not the case for core protein and type II collagen, since the latter protein is processed 3–4 times slower than the former, thus indicating that the processing of these two of the prominent components of the extracellular matrix is not tightly coupled intracellularly.

On the other hand, the data are consistent with possible intracellular interaction or coordinate intracellular processing of link protein and core protein. Indeed these proteins exhibited very similar rates of intracellular processing and translocation and were secreted in near one-to-one stoichiometry. It is also interesting to compare the results of our studies with those obtained by Vertel et al., (1985) using double immunofluorescence to localize core protein, link protein, and type II collagen within chondrocytes. They observed co-localization of core protein and link protein in one type of pre-Golgi vesicle while type II collagen appeared to be present in another class of RER-derived vesicle. Taken together, these studies suggest that the cell may segregate core protein and link protein at a very early stage of intracellular processing. Such a system may be necessary to insure secretion of these two proteins in proper stoichiometry for maximal association with hyaluronic acid.

However, our results suggest that if intracellular association and concomitant translocation of link protein and core protein does occur, all of the synthesized precursors do not follow this pathway. In both cell types, significant amounts of both proteins remain intracellular (mostly in the RER) for very long chase times. For instance, in chick chondrocytes ~10–20% of the core protein and 50% of the link protein remains intracellular even after 2 h of chase time. Furthermore, the results of additional experiments with rat chondrosarcoma cells suggest that an even greater proportion (70–80%) of the newly synthesized link protein remains in an unprocessed intracellular form (our unpublished results). In these preliminary experiments, the fraction of link protein (20–30%) which was processed and secreted exhibited kinetics similar to the link protein in chick chondrocytes, i.e., t1/2 values in both the RER and the Golgi region of less than 10 min, suggesting that at least a small proportion of the link protein is not processed coordinately with the core protein in the rat chondrosarcoma. However, the large proportion that remains in the cell and is processed more slowly, may be influenced by association with the long-lived core protein precursor. It is difficult to speculate on the significance of the apparent excess synthesis of link protein compared with core protein by both cell types, except that it may promote more efficient secretion of the proteoglycan. As mentioned above, even in the more efficient chick chondrocyte system, ~15% of the precursor core protein is not processed as rapidly as the rest. It is possible that this fraction of the newly synthesized core protein has missed some processing step in the RER which attaches a signal critical for routing. The fate of the more slowly processed core protein or link protein has not been further investigated.

Similar attempts to follow the kinetics of the processing and secretion of several proteins from one cell type (usually hepatocytes or pancreatic acinar cells) have been reported (Lodish et al., 1983; Scheele and Tartakoff, 1985; Fries et al., 1984). Typically, the rates of processing of the different proteins vary considerably, with exit from the RER being by far the greatest cause of variation. It has been hypothesized that individual receptors are present in the RER for each protein, and that the recognition and uptake of a protein by its receptor is the rate-limiting step in the secretion pathway. Alternatively, the variation in the rates of exit from the RER may be due to selective retention of certain proteins.

Whatever the mechanism of translocation, variations in the rate of processing and secretion have been reported. For instance, the reported half lives for the secretion of albumin from hepatoma cells or normal hepatocytes range between 23 and 45 min, depending on the cell type used and the incubation temperature (Lodish et al., 1983; Fries et al., 1984; Strous and Lodish, 1980). In general, faster rates of processing were demonstrated at higher temperatures and in untransformed cells, which correlates well with what we have observed in chondrocytes. Chondroitin sulfate proteoglycan appears to be processed in chick sternal chondrocytes as rapidly (t1/2 for secretion ~15–20 min) as albumin, which is particularly notable when one considers the extensive post-translational processing that the core protein undergoes on the way to becoming a proteoglycan.

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