Immunological Characterization of the Major Chick Cartilage Proteoglycan and Its Intracellular Localization in Cultured Chondroblasts: A Comparison with Type II Procollagen

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ABSTRACT Polyclonal antibodies were raised in a rabbit against the major proteoglycan of chick sternal cartilage. A total of six antisera was obtained, three after the first booster injection (A1, A2, and A3) and three after the second booster injection (A4, A5, and A6). The A1 antiserum, which was characterized in most detail, immunoprecipitated native as well as chondroitinase ABC-digested or chondroitinase ABC/keratanase-digested cartilage proteoglycan synthesized by cultured chick chondroblasts, but failed to immunoprecipitate the major proteoglycan synthesized by chick skin fibroblasts. This antiserum was also able to immunoprecipitate the cartilage proteoglycan core protein newly synthesized by cultured chondroblasts, but no other major cell protein. However, the late bleed antisera obtained from the same rabbit after a second booster injection reacted with a new chondroblast-specific polypeptide(s) of ~60,000 mol wt in addition to the cartilage proteoglycan. By immunofluorescence procedures, the A1 antiserum stained the extracellular proteoglycan matrix of cultured chondroblasts but not that of skin fibroblasts. Following enzymatic removal of the extracellular matrix and cell membrane permeabilization, this antiserum stained primarily a large, juxtanuclear structure. Additional radioautographic evidence suggests that this structure represents the Golgi complex. Similar immunofluorescent staining with antibodies to the cartilage-characteristic Type II collagen revealed that type II procollagen was localized in numerous cytoplasmic, vacuole-like structures which were scattered throughout most of the chondroblast cytoplasm but were notably scanty in the Golgi complex area. In conclusion, our data suggest the transit of the major cartilage proteoglycan through the Golgi complex of cultured chondroblasts and possible differences in the intracellular distribution of newly synthesized cartilage proteoglycan and Type II procollagen.

The central role of the Golgi complex in effecting posttranslational changes and in packaging proteins for export has been studied in a variety of cell types (for reviews see reference 13). There is evidence that the Golgi complex exerts a similar pivotal role with respect to the structural components of the extracellular matrix in connective tissues (13, 24, 52). Proteoglycans and collagen are two major classes of such matrix components. Different families of these macromolecules have been identified in recent years and often exhibit a tissue-specific distribution. For example, Type II collagen fibers and aggregates of a chondroitin sulfate/keratan sulfate-rich proteoglycan are the major structural components of the cartilage tissue extracellular matrix (26, 46, 51).

In analogy to other secretory glycoproteins, the synthesis of the protein moiety of proteoglycans and collagen is probably carried out on the polysomes of the rough endoplasmic reticulum (ER)[1]. Precursor forms of proteoglycans (12, 38, 72, 73) and collagen (19, 60) have been isolated from microsomal fractions or among in vitro translation products that may still bear the signal peptide (3) necessary for their penetration into the rough ER. Concomitant with synthesis and penetration,

[1] *Abbreviations used in this paper:* BSS, buffered saline solution; ER, endoplasmic reticulum.

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complex type, asparagine-linked oligosaccharides (40) may be added onto the proteoglycan core protein (10, 23, 42). Similarly, hydroxylation of proline and lysine and the initial glycosylations occur while the nascent procollagen molecule is still in the rough ER (4).

There is evidence that both procollagen and proteoglycans move from the rough ER into the Golgi complex for further posttranslational modifications. Isolated smooth microsomal fractions have been shown to synthesize glycosaminoglycans and to contain high levels of sulfotransferases in comparison with rough microsomes (16, 17, 31, 35), suggesting that sulfated glycosaminoglycans are synthesized and assembled onto the proteoglycan core protein in this organelle. These biochemical studies have corroborated microscopic observations demonstrating a concentration of radioactive sulfate (15, 18) and ruthenium red–stained granules (69) in the juxtanuclear Golgi complex of chondroblasts. In a smooth microsomal fraction enriched in Golgi cisternae, Hartwood et al. (21) have detected the presence of disulfide-linked procollagen polypeptides, thus corroborating previous radioautographic localizations (76).

The preparation of specific antisera to different types of proteoglycans and collagens promises to further the understanding of these phenomena. Dessau et al. (11) have shown by immunofluorescence that Type II procollagen is localized in numerous intracellular vesicles in cultured chondroblasts but is absent in the extracellular matrix. The intracellular vesicles do not exhibit any specific distribution and are scattered throughout the chondroblast cytoplasm. Immunoelectron microscopic studies have detected Type I procollagen in the ER of fibroblasts and osteoblasts, and in vesicles associated with the Golgi complex (32, 49, 52). By the use of fluorescent antibodies to Type II collagen and the major cartilage proteoglycan, Vertel and Dorfman (74) have suggested that these two types of macromolecules co-localize in numerous secretory vesicles scattered throughout the cytoplasm of cultured chondroblasts.

We describe here the preparation and specificity of an antiserum to the major native proteoglycan isolated from chicken sternum cartilage. This antiserum has been used to determine the intracellular and extracellular distribution of this proteoglycan in cultured chondroblasts, and to compare its distribution with that of Type II procollagen.

MATERIALS AND METHODS

Isolation of Proteoglycans: The major high density chick cartilage proteoglycan has been described in detail by several groups following its isolation from chicken sternal, tibial, and vertebral cartilage and from cultured cartilage cells (9, 10, 14, 34, 37, 39, 45, 51, 56, 63). Structurally, it closely resembles the major high density proteoglycan isolated from cartilages of other animal species (reviewed by Hascall, 23). Briefly, this macromolecule is composed of a core protein to which numerous keratan sulfate chains, chondroitin sulfate chains, and oligosaccharides are attached covalently. It exhibits significant polydispersity with an average size of $2.5-3.0 \times 10^6$. The protein moiety comprises ~5–10% of its overall weight. Within the cartilage matrix, single proteoglycan monomers bind specifically to hyaluronic acid and form functional multimolecular aggregates.

Accordingly, 8-wk-old chicken sterna were dissected free of adhering tissues, were finely minced, and were extracted for 48 h at 4°C with 10 vol of 4 M guanidium chloride and 0.05 M sodium acetate, pH 5.8, containing 0.1 M 6-aminohexanoic acid, 0.005 M benzamidine, and 0.05 M sodium EDTA as protease inhibitors (50). Extracts were clarified by centrifugation at 30,000 *g* for 20 min and were dialyzed for 48 h against 9 vol of 0.05 M sodium acetate buffer plus protease inhibitors to reduce the guanidium chloride concentration to 0.4 M. 1 g solid cesium chloride was added to each gram of dialyzed extract to produce an initial density of ~1.6 g/ml. Associative density gradients were obtained by centrifugation for 48 h at 86,000 g_{av} at 11°C in a Beckman type 40 fixed angle rotor (Beckman Instruments, Inc., Palo Alto, CA). The bottom two-fifths of each gradient was collected (average density of A1 fraction was 1.67 g/ml; nomenclature of fractions is according to Heinegard, reference 25) and mixed with an equal volume of buffered 7.6 M guanidium chloride containing protease inhibitors. Density was adjusted to 1.55–1.57 by addition of ~0.21 g/ml of solid cesium chloride. Dissociative density gradients were obtained by centrifugation as described above. The bottom two-fifths was collected (A1D1 fraction; average density was 1.61–1.63 g/ml), dialyzed first against 50 mM sodium chloride and then against water, and finally lyophilized. The A1D1 fraction so obtained contained the major cartilage proteoglycan and very small amounts of contaminating low molecular weight proteoglycans of equal buoyant density. To eliminate the latter, lyophilized A1D1 proteoglycans were dissolved in buffered 4 M guanidium chloride containing 1% sucrose and were separated according to molecular size by centrifugation at 95,000 g_{av} on 5–20% linear sucrose gradients in 4 M guanidium chloride for 21 h at 20°C in a Beckman type 27.1 swinging bucket rotor (51). The bottom three-fifths of each gradient (A1D1S1 fraction; S stands for sucrose) was collected, dialyzed, and lyophilized. About 80% of the total proteoglycans was present in this fraction. When tested on Sepharose 2B columns, the A1D1S1 cartilage proteoglycan exhibited the ability to interact with hyaluronic acid (20, 56).

The major high density, high molecular weight proteoglycan synthesized by chick skin fibroblasts has been characterized to a lesser extent than that of chick cartilage (39, 43, 45, 51, 54, 57). Its known structural characteristics are very similar to those of the major high molecular weight proteoglycan isolated by density gradients from human skin fibroblasts (5) and early chick limb bud cells (8, 9, 39, 51, 63). Briefly, this macromolecule is composed of a core protein to which several chondroitin sulfate chains are attached, producing a macromolecule with an estimated molecular size of $\sim 1 \times 10^6$ mol wt. It is unknown whether oligosaccharides are present. Its chondroitin sulfate chains have a significantly higher average size and 6S/4S disaccharide ratio than similar chains of the cartilage proteoglycan, thus representing a distinct metabolic product. This proteoglycan does not bind to hyaluronate under standard experimental conditions and does not appear to contain keratan sulfate. Accordingly, primary day-11 chick embryo skin fibroblasts were established in monolayer cultures as described below (43), and were labeled with 100 μCi/ml of [^{35}S]methionine. After labeling, medium was separated from cell layers and clarified by centrifugation at 12,000 *g* for 10 min and cetylpyridinium chloride was slowly added with gentle stirring to a final concentration of 1%. The precipitated material was collected by centrifugation at 12,000 *g* for 5 min, solubilized in a small volume of 1.25 M magnesium chloride, and precipitated again with 4 vol of ethanol of 4°C overnight (77). The precipitated material was collected again by centrifugation and dissolved in buffered 4 M guanidium chloride containing protease inhibitors. Solid cesium chloride was added to produce an initial density of 1.5 g/ml, and dissociative gradients were produced by centrifugation for 48 h at 86,000 g_{av} at 11°C in a Beckman type 40 rotor. The bottom one-forth of each gradient was collected (D1 fraction; average density of 1.58 g/ml). 4 M guanidium chloride containing protease inhibitors was added to the pooled D1 fractions to lower the density to 1.5 g/ml and density gradients were established again by centrifugation for 48 h. Bottom one-fourths were collected (D1D1 fractions), dialyzed, and lyophilized as described above. The recovered D1D1 fractions were essentially homogeneous and produced a typical peak of chondroitin sulfate-rich fibroblast proteoglycan when analyzed on dissociative linear sucrose gradients (Fig. 2; 43, 51).

Preparation and Terminology of the Antisera: 2 mg of A1D1S1 cartilage proteoglycan was emulsified in complete Freund adjuvant and injected subcutaneously at multiple sites in a New Zealand rabbit. 1 mo later, a booster injection was performed and blood was collected after 6, 8, and 10 d. The respective sera were termed A1, A2, and A3. After an interval of 2 wk, a second booster injection was performed and blood was again collected 6, 8, and 10 d later. The sera obtained after the second booster injection were termed A4, A5, and A6. Unfractionated serum was used for the experiments reported below.

Cell Cultures and Preparation of Radiolabeled Proteoglycans: Monolayer cultures of pure vertebral chondroblasts were established from day-11 chick embryo vertebral cartilage as described (6, 55), and were grown in Dulbecco's high glucose modified Eagle's medium containing 10% fetal calf serum.

Day-11 chick embryo vertebral cartilages were organ-cultured (14) and labeled for 24 h with 25 μCi/ml of [^{35}S]sulfate or [^{35}S]methionine. As shown elsewhere, these cultures synthesize large amounts of the major cartilage proteoglycan (14, 51). Labeled proteoglycans were extracted by 4 M guanidium chloride, and were purified by means of two consecutive dissociative cesium chloride gradients as described above (D1D1 fraction). When tested on linear sucrose gradients, the labeled D1D1 fractions appeared essentially homogeneous and produced the typical peak of the major cartilage proteoglycan (Fig. 1; 51, 56).

Primary day-11 chick embryo skin fibroblasts were established in monolayer cultures as described (43) and were labeled with 100 μCi/ml of [^{35}S]methionine for 24 h. We have reported elsewhere that cultured skin fibroblasts synthesize and secrete copious amounts of both the large, high density, fibroblast-characteristic proteoglycan described above, and low molecular weight proteoglycans (43, 51, 65). After labeling, medium was separated from cell layers, and proteoglycans were isolated as described above.

For radioautography, monolayer chondroblasts were rinsed twice with buffered saline solution (BSS) at 37°C, pulsed for 5 min with 50 μCi/ml of [^{35}S]sulfate in BSS, rinsed three times with cold BSS, fixed with 70% ethanol for 5 min, incubated in 0.01 M sodium sulfate for 10 min, rinsed with 70% ethanol, and finally air-dried. Kodak NTB 2 photographic emulsion was diluted 1:1 with water and applied to the fixed monolayers after immunofluorescent staining (see below). Exposure time ranged between 3 and 10 d.

Radioimmune Assays: For radioimmune assays, antisera were diluted with 0.15 M sodium chloride in 0.02 M sodium phosphate, pH 8.6. 20 μl of diluted sera was mixed with 20 μl of the same buffer containing 0.5–2 μg of D1D1 [^{35}S]sulfate-labeled cartilage or fibroblast proteoglycans. Radioactivity in each sample ranged between 5,000 and 25,000 cpm. Samples were incubated for 16 h at 4°C. 40 μl of 80% ammonium sulfate was added and, 45 min later, samples were centrifuged in an Eppendorf microcentrifuge (47). Supernatants were discarded; pellets were rinsed twice with 50% ammonium sulfate and then solubilized in PBS, and their radioactivity was determined by liquid scintillation counting.

Electrophoretic Analysis of Immunoprecipitated Cellular Proteins: Pure cultures of secondary vertebral chondroblasts were rinsed twice with prewarmed BSS and pulsed with 100–125 μCi/ml of [^{35}S]methionine for 5–25 min in BSS at 37°C. This procedure was performed at each time point with 5–6 × 10^6 cells. After being pulsed, cells were rinsed with, and resuspended in, ice-cold BSS. Each sample was divided into two aliquots containing 10 and 90% of the total cells, which were used for electrophoretic analysis of total newly synthesized proteins and for immunoprecipitation, respectively. Aliquots were centrifuged in a clinical centrifuge for 5 min at 4°C. The cells recovered in the 10% aliquots (~5–6 × 10^5 cells) were resuspended in 200 μl of Laemmli sample buffer (41) containing 5 mM EDTA, 5 mM benzamidine, 5 μg/ml Trasylol and 0.1 mM phenylmethane sulfonyl fluoride as protease inhibitors. Samples were then sonicated for 15 s and boiled for 3 min after addition of 5% (vol/vol) of 2-mercaptoethanol. Following clarification at 12,000 *g* for 3 min, 5–10-μl aliquots containing 1–2 × 10^5 cpm were electrophoresed on 1.2-mm-thick SDS gels (41) of various polyacrylamide concentrations (specified in the Results). Gel dimensions were 170 × 140 mm and electrophoresis was performed under a constant current of 15–35 mA. After electrophoresis, gels were fixed in 50% methanol-10% acetic acid and processed for fluorography with EnHance, and Kodak x-omat XAR5 films were exposed for 1–3 d at −80°C.

The cells recovered in the 90% aliquots (~5 × 10^6 cells) were sonicated for 30 s in 125 μl immunoprecipitation Buffer A (0.15 M sodium chloride, 0.5% Triton X-100, 0.5% SDS, 0.5% sodium deoxycholate, 0.1% BSA, 0.02% sodium azide, 0.5% Trasylol, 0.005 M EDTA, 0.005 M benzamidine in 0.05 M Tris-HCl, pH 7.4). Samples were diluted 10-fold with Buffer B (all the components of Buffer A except SDS) in order to reduce the concentration of SDS to 0.05%. Samples were clarified by centrifugation at 16,000 *g* for 10 min at 4°C. Supernatants were mixed with 100 μl of a 10% suspension of fixed *Staphylococcus aureus* (33) prepared as described below and coated with the appropriate serum or left uncoated. Mixtures were incubated for 15–60 min at room temperature with vigorous shaking, and then bacteria were recovered by centrifugation at 4,000 *g* for 15 min. Pelleted bacteria were resuspended in Buffer C (all the above components except the final concentrations of SDS and BSA were 0.05 and 1%, respectively) and recovered again by centrifugation. This procedure was repeated four times. Finally, pelleted bacteria were suspended in 200–400 μl of Laemmli sample buffer (41) containing protease inhibitors and 5% 2-mercaptoethanol and boiled for 3 min. Samples were clarified by centrifugation at 12,000 *g* for 5 min, and supernatants were used for SDS PAGE. Polyacrylamide concentrations used are indicated in the Results. 20–40-μl aliquots containing 1–4 × 10^4 cpm were used for electrophoresis. Gels were fixed and processed for fluorography as described above.

Immunoprecipitation and Electrophoretic Analysis of Digested Proteoglycans: 10–50 μg of D1D1, [^{35}S]methionine-labeled proteoglycans isolated as described above, was brought to a final volume of 50–100 μl of enriched Tris buffer (78) containing either 0.02 U of chondroitinase ABC, or 0.02 U of chondoitinase ABC and 0.5 U of keratanase. The specific activities of the cartilage and fibroblast proteoglycans were 3 × 10^3 and 2.7 × 10^2 cpm/μg dry wt, respectively. Digestion was performed at 37°C for the indicated periods of time. For electrophoretic analysis, digested samples were quickly mixed with 1 vol of 2 × Laemmli sample buffer (41) containing 10% 2-mercaptoethanol and boiled for 3 min, and 10–40-μl aliquots were analyzed by SDS PAGE followed by fluorography. For immunoprecipitation, digested samples were diluted 10-fold with immunoprecipitation Buffer C and incubated for 90 min at 4°C with coated or uncoated *S. aureus* (see below) with vigorous shaking. Bacteria were recovered by centrifugation and rinsed five times with Buffer C as described above. Final pelleted bacteria were suspended in 200 μl of Laemmli sample buffer containing 5% 2-mercaptoethanol, boiled for 3 min, and clarified by centrifugation at 12,000 *g* for 5 min. 50–100 μl of the supernatants were used for SDS PAGE followed by fluorography.

Preparation of S. aureus: Ten percent suspensions of *S. aureus* (33) were boiled twice in 3% SDS and 10% 2-mercaptoethanol for 30 min. Bacteria were washed five times with Buffer C, coated with appropriate serum by mixing 1 vol of the boiled 10% *S. aureus* suspension with ½ vol of serum, and then shaken at room temperature for 1 h. Unbound serum components were removed by pelleting bacteria by centrifugation and resuspending them in Buffer C. This procedure was repeated five times. Final pellets were reconstituted to 10% with Buffer C.

Immunoblots: Cultured vertebral chondroblasts and skin fibroblasts prepared as described above were harvested by centrifugation in a clinical centrifuge, rinsed twice with cold PBS, and homogenized by sonication for 30–60 s in cold PBS while kept in ice water. Aliquots were removed for protein content determination by standard procedure (44). The remaining sample was quickly mixed with 1 vol of 2 × Laemmli sample buffer containing 2 × protease inhibitors and 10% 2-mercaptoethanol, boiled for 3 min, and clarified by centrifugation. Aliquots containing 150–300 μg of total cellular proteins were separated by one dimensional SDS PAGE in 1.2-mm-thick gels, and separated proteins were transferred electrophoretically to nitrocellulose filters overnight (71) in a Bio-Rad apparatus (Bio-Rad Laboratories, Richmond, CA). Filters were preincubated for 30 min in wash buffer (0.15 M sodium chloride, 0.5% NP-40, 0.02% sodium azide, 5% BSA in 0.05 M Tris-HCl, pH 8.0), incubated with appropriate serum at 1:50 or 1:100 dilution in wash buffer containing 1% BSA for 1 h at room temperature on shaker, and finally rinsed for 50 min with five changes of wash buffer. Bound rabbit antibodies were localized by incubation for 30 min with goat antirabbit IgG at 80 μg/ml in wash buffer, followed by incubation for 30 min with rabbit peroxidase-antiperoxidase complexes (1:200 dilution of manufacturer concentrate) in wash buffer (68). Peroxidase activity was detected by the diaminobenzidine reaction as described (68).

Immunofluorescence: Cell monolayers grown on glass coverslips were rinsed three times with PBS, fixed with 70% ethanol at room temperature for 5 min, and air-dried. When specified, live cultures were pretreated with 4 U/ml of testicular hyaluronidase for 2 or 24 h in complete culture medium and then fixed and air-dried. Fixed cultures were incubated with 1:125 dilution of the A1 antiserum in PBS for 60 min with gentle shaking at room temperature, rinsed for 30 min with three changes of PBS, exposed to 1:250 dilution of rhodamine-conjugated goat-anti-rabbit IgG for 60 min, rinsed again for 30 min, and viewed under epifluorescence using a Zeiss microscope (2).

Similar protocols were followed for immunofluorescent staining of control chondroblasts with rabbit antisera to vimentin (2, 29) and to fibronectin (1). These antisera were used at a dilution similar to that used with the A1 antiserum.

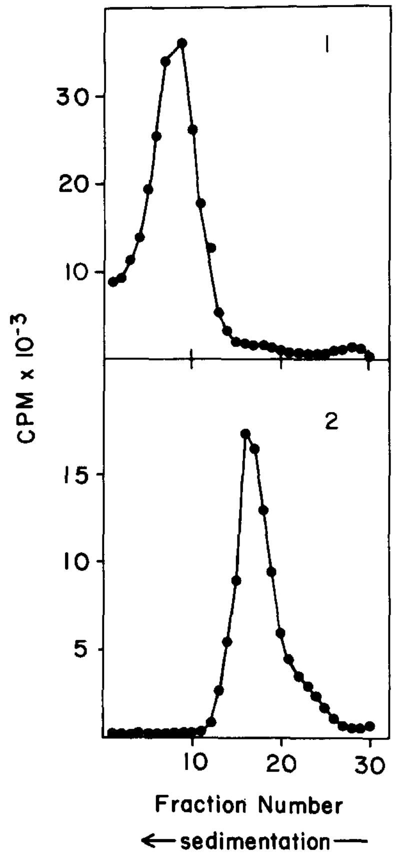
Type II collagen antibodies were a kind gift of Dr. K. von der Mark and were used at 0.07 mg IgG/ml. Their specificity has been described in reference 75.

Materials: [^{35}S]sulfate (1 Ci/mmol) and EnHance were obtained from New England Nuclear (Boston, MA); L-[^{35}S]methionine (850 Ci/mmol) from Amersham Corp., (Arlington Heights, IL); *Proteus vulgaris* chondroitinase ABC and *Pseudomonas* sp. keratanase from Miles Laboratories (Elkhart, IN); testicular hyaluronidase from Calbiochem-Behring Corp., (La Jolla, CA); rhodamine-conjugated goat-anti-rabbit IgG, goat-anti-rabbit IgG, and rabbit antiperoxidase-peroxidase complexes from Cappel Laboratories, Inc., (Cochranville, PA); Trasylol (10,000 U/ml) from FBA Pharmaceuticals (New York, NY); colchicine from Sigma Chemical Co., (St. Louis, MO); and BA85 nitrocellulose filters from Schleicher & Schuell Inc., (Keene, NH). All other chemicals were reagent grade.

RESULTS

Evidence for Specificity of Antisera Against the Major Cartilage Proteoglycan

Figs. 1 and 2 illustrate the homogeneity of the chondroitin sulfate/keratan sulfate-rich cartilage proteoglycan and of the chondroitin sulfate-rich fibroblast proteoglycan on dissociative linear sucrose gradients. Dilutions of the various antisera described in Materials and Methods were absorbed onto *S. aureus*, and radioimmune assays were performed by using the [^{35}S]sulfate-labeled proteoglycans as antigens. As shown in



FIGURES 1 and 2 Proteoglycan analysis on dissociative sucrose gradients. Cultures of vertebral cartilage and skin fibroblasts were labeled with [^{35}S]sulfate and high density, high molecular weight proteoglycans were purified by running two consecutive dissociative cesium chloride gradients (D1D1 fractions). The resulting cartilage and fibroblast proteoglycans appeared homogenous when assayed on dissociative linear sucrose gradients (Figs. 1 and 2, respectively). Identical patterns were obtained with [^{35}S]methionine-labeled proteoglycans.

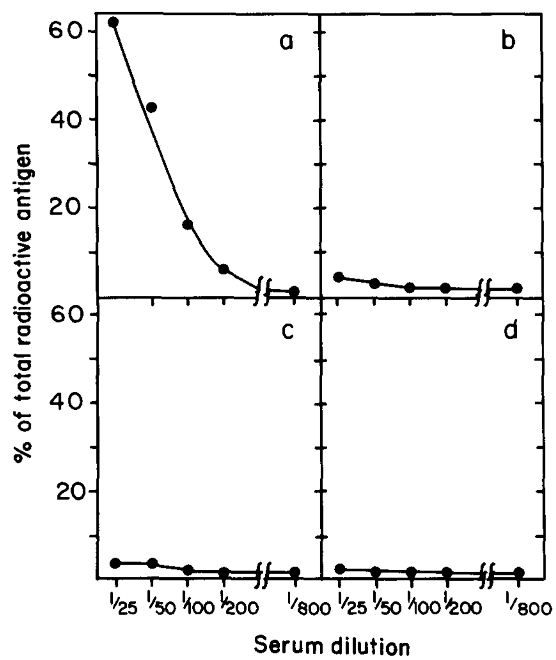


FIGURE 3 Immunoprecipitation of purified proteoglycans with the A1 antiserum. Radiolabeled proteoglycans shown in Figs. 1 and 2 were incubated with serial dilutions of either the A1 antiserum (*a* and *c*) or the preimmune antiserum obtained from the same rabbit (*b* and *d*). Antigen-antibody complexes were recovered by precipitation with 40% ammonium sulfate.

Fig. 3, the A1 antiserum immunoprecipitated the intact cartilage proteoglycan (*a*) but failed to immunoprecipitate the fibroblast proteoglycan (*c*). Preimmune serum from the same rabbit was negative (Fig. 3, *b* and *d*). In typical assays containing 0.5–2 μg of radiolabeled cartilage proteoglycan, ~60–70% of the total radioactivity was immunoprecipitated by 20 μl of a 1:25 dilution of the A1 antiserum. Similar results were obtained with the other antisera (not shown).

To determine the ability of the A1 antiserum to immunoprecipitate proteoglycans from which glycosaminoglycan side chains had been removed, [^{35}S]methionine-labeled proteoglycans were digested with chondroitinase ABC, or with chondroitinase ABC and keratanase. To test the effectiveness of the enzymatic treatments, proteoglycans were digested for various lengths of time, and the resulting products were analyzed by SDS gel electrophoresis followed by fluorography. As shown in Fig. 4, undigested cartilage and fibroblast proteoglycans were too large to enter the separating gel and remained at the bottom of their respective wells (Fig. 4, lanes *a* and *e*). The chondroitinase ABC-digested proteoglycans did enter the separating gel; neither treatments longer than 5 min nor the addition of fresh enzyme appeared to further lower their molecular weights. Note that following chondroitinase ABC digestion, both proteoglycan residues exhibited essentially similar molecular sizes. Large molecular size is exhibited also by the residue of the major chondroitinase ABC-digested proteoglycan synthesized by cultured human skin fibroblasts (5).

Chondroitinase ABC-digested, [^{35}S]methionine-labeled proteoglycans were incubated with (*a*) *S. aureus* coated with the A1 antiserum, (*b*) *S. aureus* coated with preimmune serum, and (*c*) uncoated *S. aureus*. Immunoprecipitated material was then analyzed by gel electrophoresis. As shown in Fig. 5, only chondroitinase ABC-treated cartilage proteoglycan was immunoprecipitated by the A1 antiserum. Note that

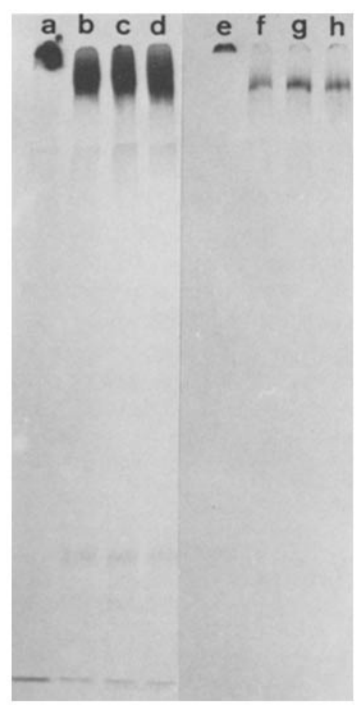


FIGURE 4 Electrophoretic analysis of undigested and chondroitinase ABC-digested proteoglycans. Aliquots of [^{35}S]methionine-labeled cartilage (lanes *a–d*) and fibroblast (lanes *e–h*) proteoglycans shown in Figs. 1 and 2 were either untreated (lanes *a* and *e*) or digested with chondroitinase ABC for 5, 15, or 45 min (lanes *b* and *f*, *c* and *g*, and *d* and *h*, respectively). Samples were then examined on an 8% polyacrylamide gel with a 6% polyacrylamide stacking gel. Radioactivity was detected by fluorography. Note the similar molecular size of proteoglycan residues following chondroitinase ABC digestion. Note also the presence of a polypeptide of lower molecular weight in lanes *b*, *c*, and *d*. It is unlikely that this polypeptide is a degradation product because its relative amount does not increase with time of incubation in chondroitinase ABC. We are testing whether it may correspond to the 60,000-mol-wt polypeptide described below and whether it may be bound to the proteoglycan molecule.

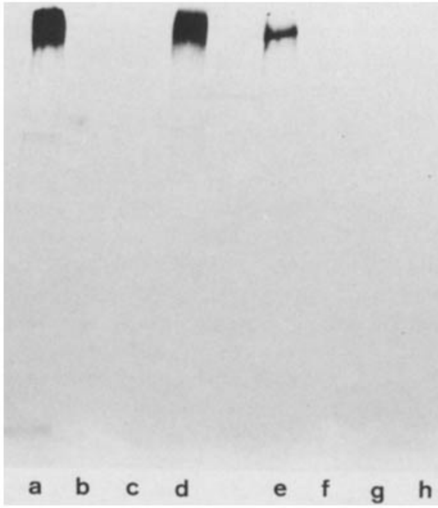


FIGURE 5 Electrophoretic analysis of chondroitinase ABC-digested proteoglycans immunoprecipitated with the A1 antiserum. [^{35}S]-methionine-labeled cartilage and fibroblast proteoglycans were first treated with chondroitinase ABC for 15 min. One aliquot of both the digested cartilage and fibroblast proteoglycans was then removed and immediately processed for electrophoresis (lanes *a* and e, respectively). Similar aliquots were incubated with *S. aureus* left uncoated (lanes *b* and *f*), coated with preimmune serum (lanes c and g), or coated with the A1 antiserum (lanes *d* and *h*), and were processed for immunoprecipitation. Immunoprecipitated material was then analyzed on an 8% polyacrylamide gel with a 6% stacking gel followed by fluorography. Note that the A1 antiserum immunoprecipitates only digested cartilage proteoglycan (lane *d*) but not digested fibroblast proteoglycan (lane *h*). Note also that this antiserum does not immunoprecipitate the lower molecular weight polypeptide visible in lane *a* (see Fig. 4 legend).

FIGURE 6 Electrophoretic analysis of digested proteoglycans. (*a*) Chondroitinase ABC-digested cartilage proteoglycan; (*b*) chondroitinase ABC/keratanase-digested cartilage proteoglycan; (*c*) as in *b* but immunoprecipitated with A1 antiserum; (*d*) chondroitinase ABC-digested fibroblast proteoglycan; (*e*) chondroitinase ABC/keratanase-digested fibroblast proteoglycan; and (*f*) as in e but immunoprecipitated with the A1 antiserum. All samples were analyzed on a 6% polyacrylamide gel followed by fluorography. Note that the A1 antiserum immunoprecipitates only the chondroitinase ABC/keratanase-digested cartilage proteoglycan but not the fibroblast proteoglycan, and that the keratanase treatment further lowers the molecular size of the former but not that of the latter. Some undigested fibroblast proteoglycan is visible in lanes *d* and e.

this antiserum did not precipitate chondroitinase ABC-digested fibroblast proteoglycan. When chondroitinase ABC/keratanase-digested proteoglycans were used as antigens, similar results were obtained (Fig. 6). As shown in Fig. 6, the keratanase treatment further reduced the apparent molecular size of the cartilage proteoglycan, but not that of the fibroblast proteoglycan. This is in agreement with the known presence of keratan sulfate chains in the former and their absence in the latter (10, 51, 57). The results presented here suggest that at least some of the antibodies present in our antiserum are directed against determinants on the cartilage proteoglycan core protein. This has been conclusively demonstrated elsewhere by the A1 antiserum-directed immunoprecipitation of the 340,000-mol-wt proteoglycan core protein produced during cell-free translation of total chick vertebral chondroblast mRNA (54).

To confirm the latter conclusion, cultures of vertebral chondroblasts were pulsed for very short periods of time with [^{35}S]methionine. This brief pulse should label the proteoglycan core protein while still in the rough ER, prior to its transfer to the Golgi complex. Following labeling, cells were homogenized and aliquots were immunoprecipitated with *S. aureus* coated with the A1 antiserum. Fig. 7 shows the fluorogram of the electrophoretic patterns obtained with total cell homogenates (lanes *a–e*) and of those obtained with the A1 antiserum-immunoprecipitated material (lanes *f–j*). Similar results were obtained with the A6 antiserum (not shown). Clearly, the major polypeptide precipitated by the A1 antiserum has a molecular size of ~400,000 mol wt and is likely to represent the cartilage proteoglycan core protein in the rough ER. Following a brief pulse with labeled serine, a similar polypeptide has been observed in cultured chondrosarcoma cells and has been identified as the precursor form of the proteoglycan core protein while still in the rough ER (38). While this large polypeptide was barely detectable in SDS gels of the total cell homogenates, another polypeptide of about 165,000 mol wt was particularly prominent. The latter represents pro-α1(II) procollagen, the single subunit of the cartilage-characteristic Type II procollagen. Its identification under these experimental conditions has been detailed elsewhere (1). Thus, this experiment confirms that the A1 antiserum specifically immunoprecipitates the 400,000-mol-wt protein, likely representing the proteoglycan core protein. It also suggests that the relative rate of synthesis of the core protein is significantly lower than that of Type II procollagen subunits in chondroblasts grown under our conditions[2].

In another experiment, we tested whether the A1 antiserum, as well as the other antisera, would recognize other major chondroblasts proteins. Total homogenates of vertebral chondroblasts and skin fibroblasts that had been separated by one

[2] The core protein and each pro-α1(II) polypeptide appear to have a similar methionine content. The former has been reported to have 7–8 methionine residues per 1,000 amino acids (8, 39). The latter, by our calculations, contains an average of 7.6 methionine residues per 1,000 amino acids. This figure is derived from the following: there are 8 residues in the 1,000 amino acids of the helical region (references in 4), 3 in the 341 amino acids of the carboxy-terminal propeptide (S. Curran and D. J. Prockop, personal communication) and no residues in the 109 amino acids of the amino-terminal propeptide (7), for a total of 11 residues of the 1,450 amino acids of the pro-α1(II) chain.

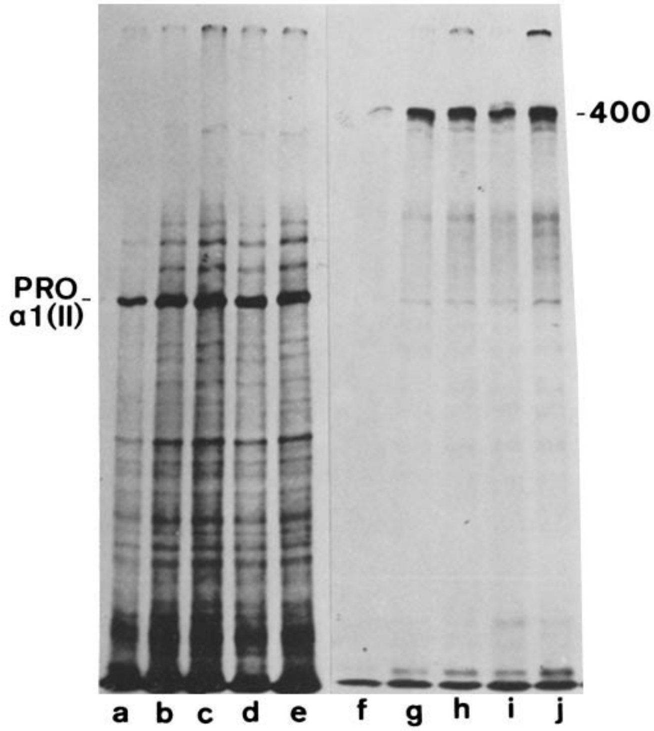


FIGURE 7 Electrophoretic analysis of whole chondroblast homogenates and of the A1 antiserum-immunoprecipitated material. Monolayer cultures of vertebral chondroblasts were pulsed for 5, 10, 15, 20, and 25 min with [^{35}S]methionine (lanes *a–e*, respectively). Aliquots were prepared for electrophoresis while remaining samples were immunoprecipitated with the A1 antiserum (lanes *f–j*). Samples were analyzed on a 6% polyacrylamide gel followed by fluorography. *400*, 400,000-mol-wt putative cartilage core protein; *pro α1(II)*, the single subunit of Type II procollagen. The faint bands visible in lanes *g–j* likely represent nonspecific binding because this material bound also to uncoated or preimmune serum-coated *S. aureus* (not shown).

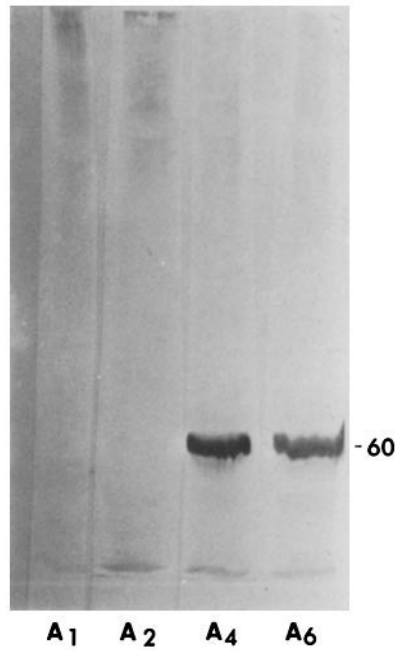


FIGURE 8 Immunoblots of whole chondroblast proteins. Cultured chondroblasts were homogenized, and total cell proteins were first separated by one dimensional electrophoresis and then transferred electrophoretically onto nitrocellulose paper. Single strips were incubated with diluted A1, A2, A4, or A6 antisera which were localized by the immunoperoxidase method. Note that neither A1 nor A2 but both the A4 and A6 antisera bind to the 60,000-mol-wt protein(s).

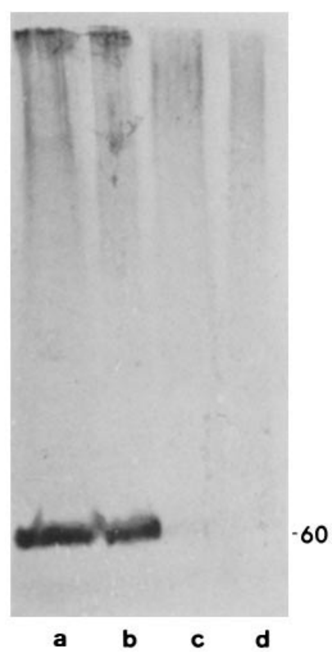


FIGURE 9 Immunoblots of total chondroblast and fibroblast proteins. Chondroblast (lanes *a* and *b*) and fibroblast (lanes *c* and *d*) samples were separated by one dimensional electrophoresis, blotted onto nitrocellulose paper, and exposed to the A6 antiserum. Immunoglobulins were localized by the immunoperoxidase method. Note that only the chondroblast samples display the 60,000-mol-wt protein. The peroxidase activity detected on the top region of the chondroblast lanes may be due to cartilage proteoglycan and their precursors which cannot enter the separating gel. Note the absence of this reaction in the fibroblast lanes.

dimensional electrophoresis were transferred electrophoretically onto nitrocellulose paper. Single paper strips with chondroblast proteins were separately incubated with A1, A2, A4, and A6 antisera, and bound immunoglobulins were localized by the immunoperoxidase method. As shown in Fig. 8, the A1 and A2 antisera did not produce any major detectable band, indicating that (*a*) these antisera do not cross-react with any other major chondroblast protein under these conditions and (*b*) the 400,000-mol-wt protein is present in very low amounts. However, both the A4 and the A6 antisera bound to a major polypeptide(s) of ~60,000 mol wt. This protein appeared to be chondroblast-specific for it was not detected in paper strips containing total skin fibroblast proteins (Fig. 9). We are currently investigating whether it may correspond to a 52,000-mol-wt protein recently isolated from bovine cartilage (61) or to the newly discovered low molecular weight collagen synthesized by chick chondroblasts (67). Because of the above results, all the subsequent experiments were performed with the A1 antiserum.

Localization of the Cartilage Proteoglycan in Cultured Chondroblasts

The experiments reported above led us to conclude that the A1 antiserum is able to distinguish between the major keratan sulfate/chondroitin sulfate-rich cartilage proteoglycan and the major chondroitin sulfate-rich fibroblast proteoglycan. The immunofluorescence experiments using the A1 antiserum reported below confirm and extend this conclusion.

Monolayers of pure vertebral chondroblasts and skin fibroblasts were fixed and incubated with the A1 antiserum, and the bound antibodies were localized by secondary fluorescent antibodies (2, 29). As shown in Fig. 10, the A1 antiserum bound specifically to the extracellular matrix of chondroblasts but not of fibroblasts, revealing a donut-shaped rim of proteoglycan extracellular matrix. This result was confirmed in cocultures of chondroblasts and fibroblasts (Figs. 10, *c* and *f*). A similar donut-shaped matrix has been observed in cartilage in vivo (62). Note that different chondroblasts within the same culture exhibited varying degrees of staining (Fig. 10*b*,

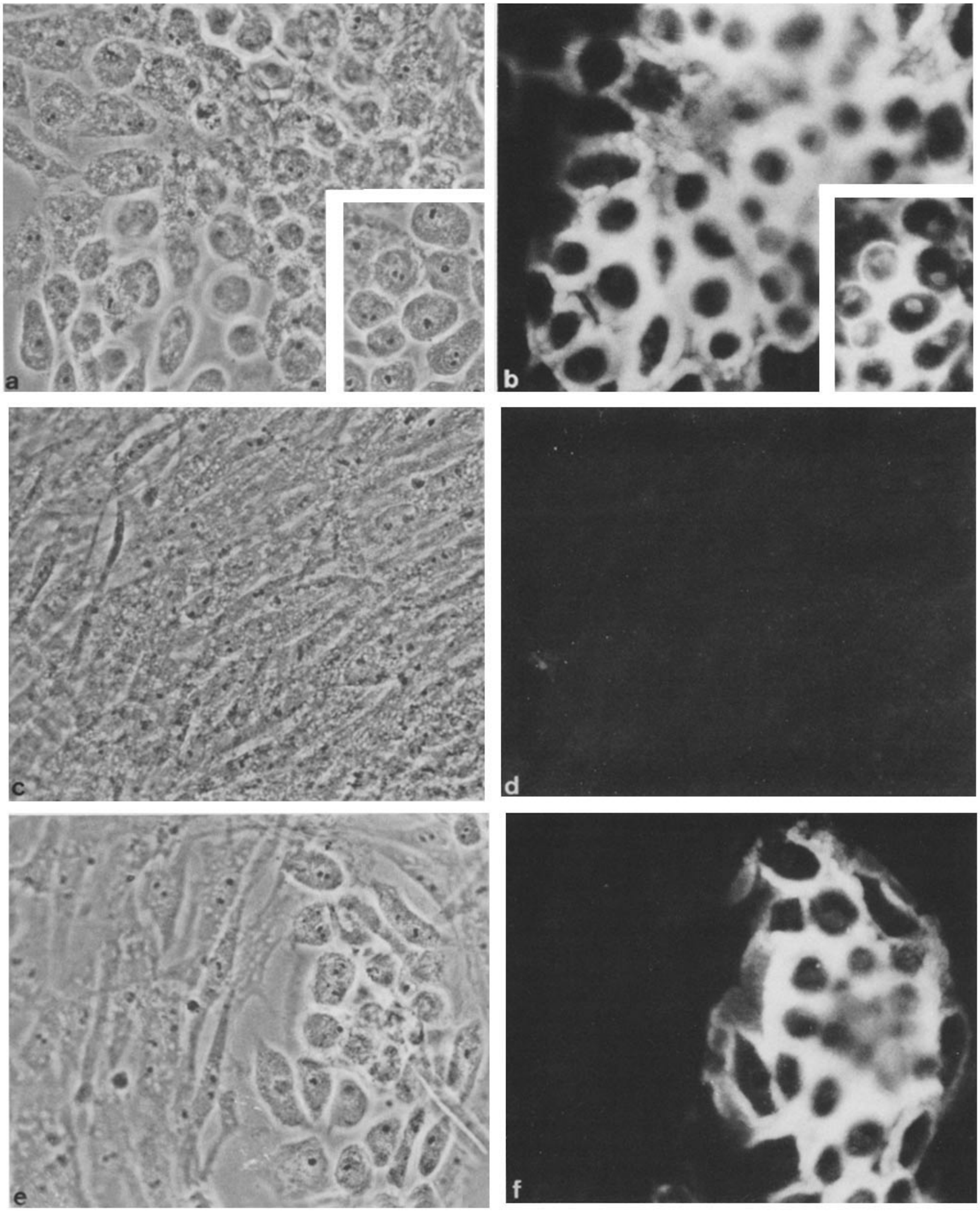


FIGURE 10 Phase-contrast and immunofluorescence micrographs of monolayer chondroblasts and fibroblasts. Cultures of chondroblasts (*a* and *b*), fibroblasts (*c* and *d*), and mixed chondroblasts and fibroblasts (*e* and *f*) were stained with 1:125 dilutions of A1 antiserum localized by rhodamine-conjugated goat antirabbit IgG. Phase-contrast and fluorescent micrographs were taken of the same microscopic field. In *a* and *b*, note that the donut-shaped cartilage proteoglycan matrix is less conspicuous in flatter chondroblasts visible in the upper left corner. *Insets* show that some chondroblasts present in the same culture dish exhibit an intensely positive, juxtanuclear structure that likely represents the Golgi complex (see below). *c* and *d* show the same microscopic field of cultured fibroblasts which did not stain with the A1 antiserum. This was confirmed in co-cultures of chondroblasts and fibroblasts (*e* and *f*). × 500.

upper left corner), suggesting that the amount of extracellular matrix surrounding different chondroblasts varied significantly. These observations confirm earlier reports using a variety of histochemical staining procedures. They also confirm the older observations that the more polygonal and rounded chondroblasts synthesize and/or accumulate greater quantities of matrix than the flatter, "fibroblastic" chondroblasts (6, 28, 30, 56, 66).

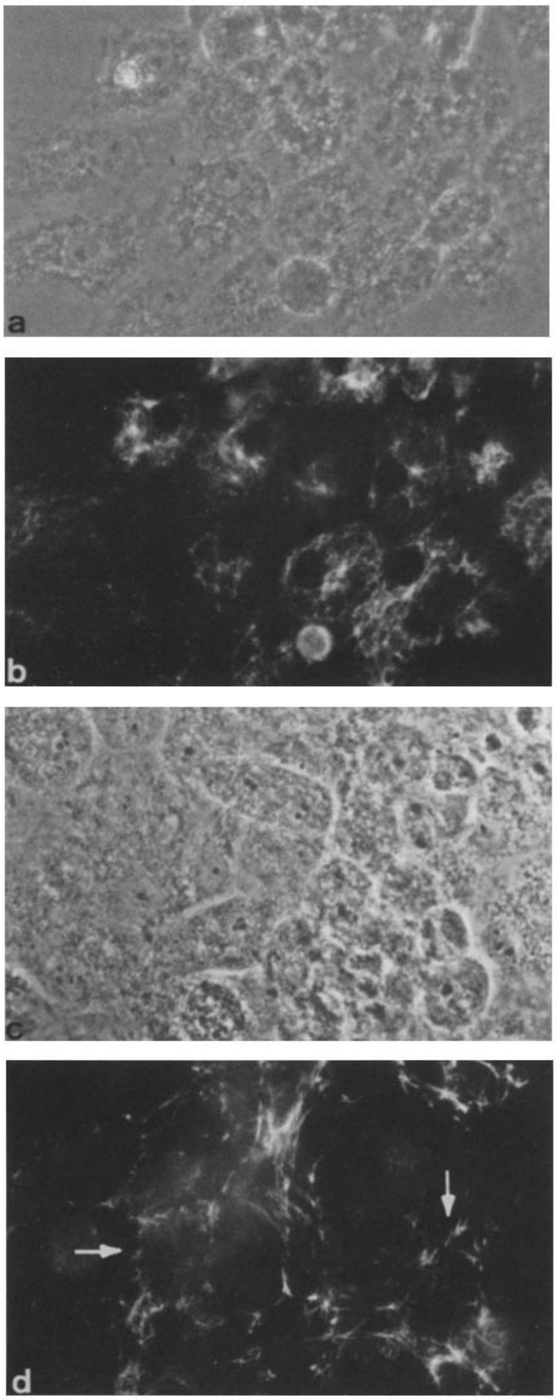


FIGURE 11 Micrographs of monolayer chondroblasts stained with antisera to vimentin and fibronectin. *a* and *b* are phase-contrast and immunofluorescent micrographs of the same microscopic field of a culture stained with antivimentin; *c* and *d* are the same as *a* and *b* but stained with antifibronectin. Arrows in *d* point to the short strands of fibronectin fibrils interconnecting adjacent chondroblasts. × 500.

To confirm the specificity of the A1 antiserum, control chondroblasts were also stained with rabbit antisera to (*a*) vimentin, the fibroblast-characteristic, intermediate-sized filament subunit (2, 29); and (*b*) fibronectin. As shown in Fig. 11, the vimentin antiserum stained the characteristic cytoplasmic network of intermediate filaments (70) while the fibronectin antiserum stained short-stranded, intercellular fibronectin fibrils that interconnect adjacent chondroblasts, as reported by Dessau et al. (11).

In addition to the massive, donut-shaped rim of proteoglycan extracellular matrix, the A1 antiserum revealed in some chondroblasts an intensely stained intracellular structure, generally located to one side of the cell's nonstaining nucleus (Fig. 10*b*, inset). To better visualize this intracellular structure, it was desirable to remove the obscuring extracellular matrix. To this end, the living chondroblasts were first treated with hyaluronidase to remove, at least in part, the matrix, and then fixed and stained with the A1 antiserum. This procedure revealed that numerous chondroblasts displayed this intensely fluorescent intracellular structure, consistently located in a juxtanuclear position, and that the remainder of the chondroblast cytoplasm was only slightly stained (Fig. 12). The

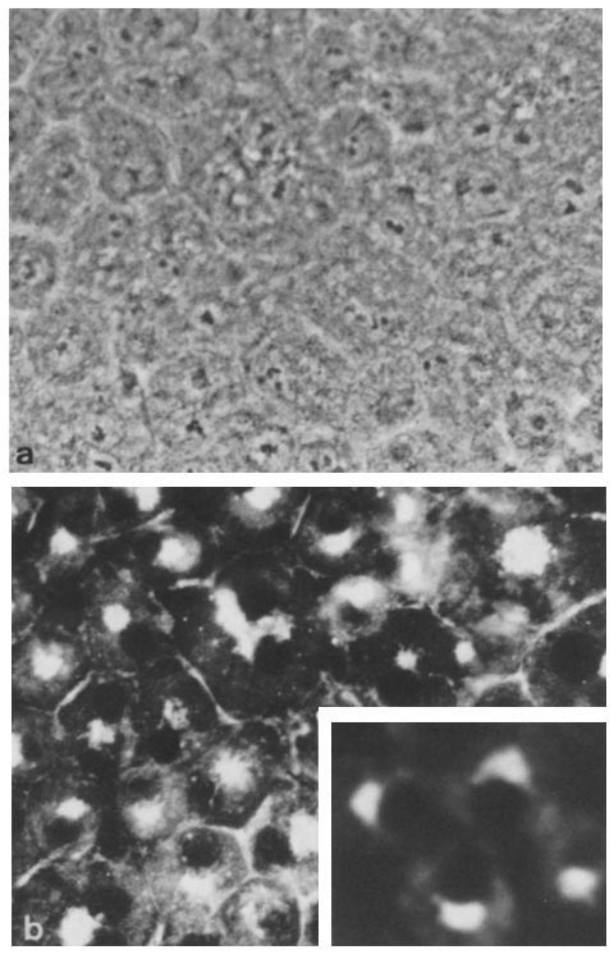
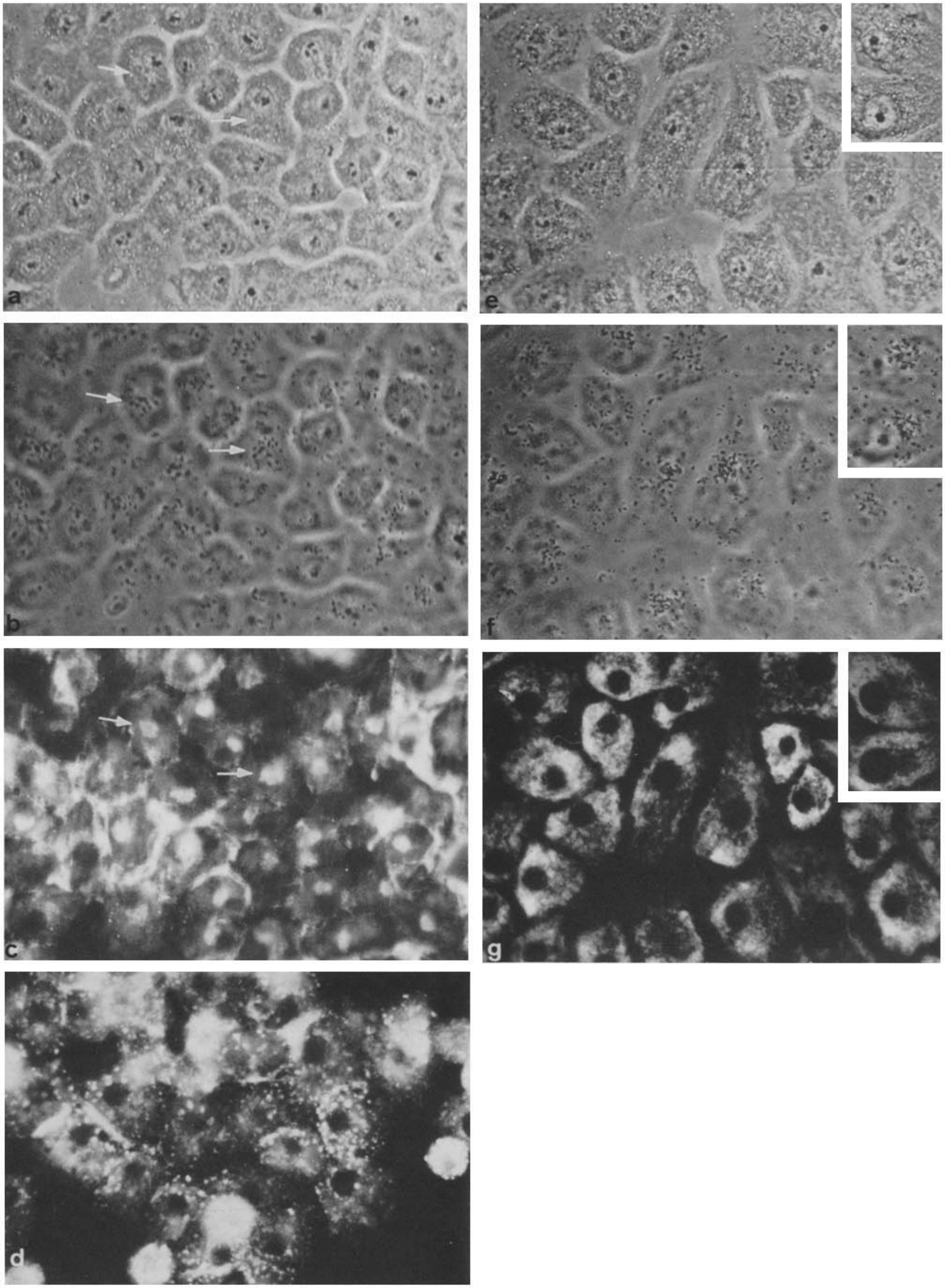


FIGURE 12 Phase-contrast (*a*) and immunofluorescence (*b*) micrographs of chondroblasts stained with the A1 antiserum following a 2-h hyaluronidase treatment. Note that almost every chondroblast displays the highly positive juxtanuclear structure likely representing the Golgi complex. Note also that the remainder of the cytoplasm stains weakly. × 500. *Inset* shows a higher magnification micrograph from the same culture. Note the negative image of the nucleus. × 800.



juxtanuclear position of this structure suggested that it may represent the Golgi complex. Various experiments were performed to strengthen this conclusion. Electron microscopic sections revealed that in monolayer chondroblasts the Golgi complex almost invariably occupies a comparable juxtanuclear position (not shown). In another experiment, the location of the Golgi complex was determined by a brief pulse with [^{35}S]sulfate followed by radioautography. This approach has been widely used to locate the Golgi complex in various cell types including chondroblasts (15, 18). Accordingly, chondroblasts were first pulsed with [^{35}S]sulfate for 5 min, fixed with ethanol, stained with the A1 antiserum, and finally processed for immunofluorescence and radioautography. Micrographs were taken of the same microscopic fields. Fig. 13, *a–c* shows that in numerous chondroblasts the major site of radioactive sulfate uptake was indeed a sizeable, juxtanuclear area (Fig. 13*b*) that coincided with the intracellular area stained intensely by the A1 antiserum (Fig. 13*c*).

It has been established that microtubule-depolymerizing and microtubule-polymerizing agents such as colchicine and taxol, respectively, disperse the Golgi complex into isolated stacks of cisternae (dictyosomes) scattered throughout the cytoplasm (48, 70). Accordingly, monolayer chondroblasts were treated with colchicine for 1 h and then stained with A1 antiserum. The colchicine treatment eliminated the fluorescence staining of the juxtanuclear area and induced the appearance of positive staining vesicles likely to represent scattered dictyosomes (Fig. 13*d*). Comparable findings with taxol at the ultrastructural level will be detailed elsewhere.

In conclusion, these data strongly indicate that the A1 antiserum localizes (*a*) secreted cartilage proteoglycan in the extracellular matrix and (*b*) newly synthesized proteoglycan in the Golgi complex of cultured chondroblasts.

The Localization of Type II Procollagen

An unresolved issue regarding chondrogenesis is how tightly linked are the synthesis and/or secretion of cartilage proteoglycan and Type II procollagen chains (22, 66). More specifically, we asked whether chondroblasts that were stained with antibodies to Type II collagen would also exhibit a conspicuous antibody staining of the juxtanuclear area such as that demonstrated by the A1 antiserum.

Chondroblasts were pulsed with [^{35}S]sulfate before antibody staining. Immunofluorescence observations of untreated and hyaluronidase-treated chondroblast (Fig. 13*g* and inset, respectively) with Type II collagen antibodies revealed extensive cytoplasmic fluorescence, but no extracellular stainable material. The latter finding confirms a previous report (11). The fluorescent material was scattered throughout most of the cytoplasm and in many, but not all, chondroblasts it was greatly reduced in a rounded, juxtanuclear area likely representing the Golgi complex area. Indeed, this poorly staining area exhibited high levels of [^{35}S]sulfate uptake (Fig. 13*f* and inset).

DISCUSSION

The data reported above demonstrate that the A1 rabbit antiserum distinguishes between native, keratan sulfate/chondroitin sulfate-rich cartilage proteoglycan synthesized by vertebral chondroblasts and native chondroitin sulfate-rich proteoglycan synthesized by skin fibroblasts. The antiserum also precipitates cartilage proteoglycan from which both the chondroitin sulfate and keratan sulfate chains have been removed, indicating that it is, at least in part, directed against determinants on the proteoglycan core protein. This is confirmed by its ability to immunoprecipitate (*a*) the newly synthesized 400,000-mol-wt protein which is likely to be the core protein while still in the rough ER and (*b*) a similar polypeptide produced during cell-free translation of total chondroblast mRNA, as shown elsewhere (54). On the other hand, its inability to immunoprecipitate native as well as chondroitinase ABC- or keratanase-digested fibroblast proteoglycan strengthens our original proposal that the core proteins of the major chondroblast and skin fibroblast proteoglycans are products of distinct structural genes (51). However, the roughly similar molecular size of these proteoglycan residues after enzymatic removal of their polysaccharide chains indicates that they are closely related in structure.

These conclusions have been substantiated by immunofluorescence observations showing that the extracellular matrix surrounding definitive chondroblasts, but not skin fibroblasts, is specifically recognized by the A1 antiserum. Elsewhere we have demonstrated that this antiserum binds to the matrix of emerging chondroblasts in cultures prepared from early chick limb buds, but does not bind to the matrix of their precursor cells, the presumptive chondroblasts (64). Moreover, the different, distinct patterns of immunofluorescent staining obtained with the rabbit antisera to vimentin and fibronectin (*a*) confirm that the A1 antiserum recognizes specifically the proteoglycans present in the chondroblast extracellular matrix; (*b*) indicate that the proteoglycan matrix is readily permeable to antibodies directed against either cytoplasmic components such as vimentin filaments or other matrix components such as fibronectin; and (*c*) rule out that the proteoglycan matrix traps or absorbs antibodies nonspecifically.

In addition to the extracellular proteoglycans, the A1 antiserum conspicuously reacts with an intracellular juxtanuclear, rounded area present in virtually every cultured chondroblast. While the definitive identification of this structure will depend upon immunoelectron microscopic studies in progress in our laboratory, several lines of evidence indicate that it may represent the Golgi complex. Firstly, this structure exhibits

FIGURE 13 Micrographs of cultured chondroblasts stained with A1 antiserum and with anti-Type II collagen. *a–c* are, respectively, phase-contrast, radioautographic, and immunofluorescent micrographs of the same microscopic field from a 2-h hyaluronidase-treated culture pulsed with [^{35}S]sulfate, stained with A1 antiserum, and processed for both radioautography and immunofluorescence. Note that the sites of intense, juxtanuclear fluorescence (arrows in *c*) coincide with the sites of high [^{35}S]sulfate uptake (arrows in *b*). *d* is an immunofluorescent micrograph of a culture treated with hyaluronidase for 2 h and with colchicine for 1 h before being stained with A1 antiserum. *e–g* are the same as *a–c*, but are from a control culture stained with anti-Type II collagen. *Insets* are from a sister culture pretreated with hyaluronidase for 2 h before being labeled and stained. Note that in both control (*e–g*) and hyaluronidase-treated (*insets* in *e–g*) chondroblasts the site of low fluorescent, intracellular staining coincides with the site of high [^{35}S]sulfate uptake. Note in *d* the numerous vacuole-like structures scattered throughout the cytoplasm that accompany Golgi complex dispersal by colchicine. × 500.

rapid, high uptake of radioactive sulfate, as shown by the co-distribution of radioautographic grains and A1 antiserum-positive immunofluorescence. The site of localized sulfate uptake has been shown to represent the Golgi complex in various cell types including chondroblasts (15, 18). Secondly, this structure is rapidly dispersed into scattered dictyosomes following a brief treatment with colchicine. The latter effects of microtubule depolymerizing agents such as colchicine have been documented in numerous studies (see references in 48) and in a recent immunofluorescence study (27).

The remainder of the chondroblast cytoplasm (i.e., rough ER and post-Golgi secretory vacuoles) reacts only weakly with the A1 antiserum. Various interpretations of this finding may be considered: (*a*) The juxtanuclear, highly positive structure represents a site of accumulation and concentration of nascent proteoglycans in comparison with the surrounding, weakly reacting rough ER. Proteoglycan precursors in the latter compartment are too diluted to produce a detectable staining. (*b*) The proteoglycan precursors are masked in the rough ER and are not accessible to the antiserum. (*c*) The A1 antiserum contains, in addition to antibodies to the proteoglycan core protein, other antibodies directed against determinants that are assembled onto the nascent proteoglycan in the juxtanuclear, highly positive structure. If the latter is indeed the Golgi complex, then examples of possible new antigenic determinants assembled onto the core protein in this organelle are O-linked oligosaccharides and keratan and chondroitin sulfate chains (23, 24). (*d*) The completed proteoglycans in post-Golgi secretory vacuoles are transported and secreted very rapidly. (*c*) is suggested by some decrease in the amount of cartilage proteoglycan immunoprecipitated after treatment with either chondroitinase ABC or keratanase (not shown). (*d*) is indicated by the successful isolation of completed radiolabeled proteoglycans from the culture medium of rat chondrosarcoma chondrocytes following a 1–2 min pulse with radioactive sulfate (36).

There are striking differences in the pattern of intracellular fluorescence in chondroblasts stained with Type II collagen antibodies when compared with those stained with the A1 antiserum. The anticollagen antibodies stain numerous structures scattered throughout the chondroblast cytoplasm that often have a distinct, vacuole-like appearance. In numerous chondroblasts, fluorescence is greatly reduced in a rounded, juxtanuclear area that most likely represents the Golgi complex. This is strongly indicated by its high, rapid uptake of radioactive sulfate. We do not have definitive explanations for the apparently different, intracellular distribution of Type II procollagen and proteoglycan in cultured chondroblasts. It is unclear whether the numerous, intracellular positive structures depict Type II procollagen in the rough ER before its transit through the Golgi complex, and/or its presence in post-Golgi secretory vacuoles. It is also unclear whether the apparent reduction in Type II procollagen staining in the juxtanuclear area is due to lack of antibody penetration or masking of antigenic determinants. In either case, our data suggest that the bulk of Type II procollagen in cultured chondroblasts resides outside of the Golgi complex. A similar localization for Type I collagen has been also observed in corneal and tendon fibroblasts (49). Obviously this type of cytological data regarding the different intracellular distribution of the Type II procollagen and cartilage proteoglycan does not allow us to distinguish between (*a*) the very different rates of synthesis of these two macromolecules as suggested by our pulse-labeling experiments, or (*b*) the longer period proteoglycans might spend in the Golgi complex undergoing more numerous posttranslational modifications. Our findings may also indicate that the concentration of proteoglycans per unit of volume is higher in the Golgi complex than in the rough ER, and that the fraction of the total intracellular proteoglycan present in the Golgi complex is higher than that of Type II procollagen.

Vacuole-like structures were observed by Dessau et al. (11) and Vertel and Dorfman (74) in their studies localizing Type II procollagen in cultured chondroblasts. Neither of these groups, however, reported the significant reduction of binding of these antibodies in the juxtanuclear area that likely contains the Golgi complex. It is noteworthy that their experiments and ours have made use of the Type II collagen antibodies prepared by von der Mark and associates. This is of importance because our observations do not confirm the proposal of Vertel and Dorfman (74) that Type II procollagen and the cartilage proteoglycan often co-localize in vacuole-like intracellular structures. It is unclear at this time whether differences in cell culture conditions or differences in the properties of the antisera account for these differing observations. Work to be reported elsewhere using monoclonal antibodies to the major cartilage proteoglycans also demonstrates a preferential staining of the Golgi complex in cultured chondroblasts (Sasse, Pacifici, and Holtzer, manuscript in preparation).

The A1 antiserum immunoprecipitates a newly synthesized polypeptide of about 400,000 mol wt. Its apparent size does not vary significantly with increasing labeling time. If indeed this polypeptide represents the newly synthesized proteoglycan core protein, then one would expect to observe a gradual increase in size as new carbohydrate side chains are added. Because this was not observed in our experiments, the following is a likely explanation. The bulk of the 400,000-mol-wt polypeptides represents newly synthesized core proteins bearing a complete set of *N*-linked oligosaccharides while in the rough ER. Once they are transferred to the Golgi complex, assembly of O-linked oligosaccharides and glycosaminoglycans occurs. The latter is a very efficient biosynthetic step and the increase in overall molecular weight is rapid. The resulting proteoglycan intermediate, as well as complete proteoglycan monomers, is indeed immunoprecipitated by the A1 antiserum but is too large to be separated in our electrophoretic system. In addition, our finding of a sizeable pool of 400,000-mol-wt polypeptides of essentially identical molecular size also supports the proposal for the presence of a large intracellular precursor pool of proteoglycan core protein in rat chondrosarcoma cells, likely restricted to the rough ER (36). Our observations also suggest that the rate of synthesis of pro-α1(II), the single subunit of Type II procollagen, is significantly higher than that of the cartilage proteoglycan core protein in cultured chondroblasts.

One of the criteria used here to establish the location of the Golgi complex in chondroblasts has been its dispersal by colchicine. The rapidity of this effect, within 1 h, suggests that attempts to establish a role for microtubules in secretion, through the common strategy of using microtubule-depolymerizing agents as well as microtubule-stabilizing agents such as taxol, must also take into consideration the rapid dispersal of the Golgi complex by these drugs. It will be interesting to learn whether microtubules indeed play such a role and how the dispersal of the Golgi complex can in itself lead to the rapid inhibition of secretion (58, 59).

In the present study, we have also reported the detection of a new chondroblast-specific protein(s) of ~60,000 mol wt, which is readily recognized by the A4 and A6 antisera. Currently, we know nothing of its location and function in hyaline cartilage. Antibodies to this protein may have arisen as a result of (*a*) common immunogenic determinants with the cartilage proteoglycan, or (*b*) contamination by this protein in the A1D1S1 proteoglycan fraction used as an immunogen. (*b*) appears to be a likely explanation in view of the possible formation of disulfide bridges among different proteins during extraction by 4 M guanidine. By the use of the A6 antiserum, we have recently demonstrated (1) that the synthesis of the 60,000-mol-wt protein is coordinately inhibited, along with other chondroblast specific proteins, in definitive chondroblasts transformed by Rous sarcoma virus. Work is in progress to determine whether this protein corresponds to a protein of similar size isolated from bovine cartilage (61) or to a low molecular weight collagen synthesized by chick chondroblasts (67).

We are currently preparing monoclonal antibodies directed against the different major structural components of the cartilage proteoglycan. These antibodies will enable us to study the transit of these macromolecules through the various cellular compartments involved in their biosynthesis.

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M. Pacifici is on a leave of absence from the University of Rome, School of Medicine.

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