Mammalian Eyes and Associated Tissues Contain Molecules That Are Immunologically Related to Cartilage Proteoglycan and Link Protein

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ABSTRACT   Monospecific antibodies to bovine nasal cartilage proteoglycan monomer and link protein were used to demonstrate that immunologically related molecules are present in the bovine eye and associated tissues. With immunofluorescence microscopy, reactions for both proteoglycan and link protein were observed in the sclera, the anterior uveal tract, and the endoneurium of the optic nerve of the central nervous system. Antibody to bovine nasal cartilage proteoglycan also reacted with some connective tissue sheaths of rectus muscle and the perineurium of the optic nerve of the central nervous system. Antibody to proteoglycan purified from rat brain cross-reacted with bovine nasal cartilage proteoglycan, indicating structural similarities between these proteoglycans. ELISA studies and crossed immunoelectrophoresis demonstrated that purified dermatan sulphate proteoglycans isolated from bovine sclera did not react with these antibodies but that the antibody to cartilage proteoglycan reacted with other molecules extracted from sclera. Two molecular species resembling bovine nasal link protein in size and reactivity with antibody were also demonstrated in scleral extracts: the larger molecule was more common. Antibody to link protein reacted with the media of arterial vessels demonstrating the localization of arterial link protein described earlier. Tissues that were unstained for either molecule included the connective tissue stroma of the iris, retina, vitreous body, cornea, and the remainder of the uveal tract. These observations clearly demonstrate that tissues other than cartilage contain molecules that are immunologically related to cartilage-derived proteoglycans and link proteins.

The matrix of hyaline cartilage is composed of type II collagen and proteoglycan monomer. Cartilage proteoglycan is of high buoyant density and composed of a core protein to which are attached side chains of chondroitin sulfate and keratan sulfate (1) as well as O-linked and N-linked oligosaccharides (2, 3). The average molecular weight of the largest proteoglycans that sediment in density gradient fraction AIDI (according to the nomenclature of Heinegård [4]) has been estimated to be of the order of 2.5 × 10⁶ (5). Cartilage proteoglycan can bind specifically to hyaluronic acid (6-9) to form macromolecular aggregates (10). This interaction is stabilized by link protein (9, 11-14). The hyaluronic acid–binding region of cartilage proteoglycan has been isolated from link-stabilized aggregates by digestion with trypsin (9, 15) or clostripain (16).

Monospecific antibodies to cartilage proteoglycan (17-19) and link protein (17-19) have been recently described. These antibodies to native proteoglycans react with different domains associated with the proteoglycan protein core including the hyaluronic acid–binding region (17-19). It has been reported that unsaturated uronic acid residues linked to N-acetylgalactosamine 4-sulfate left on the protein core after digestion with chondroitinase ABC can also react with antibodies prepared against chondroitinase ABC–digested proteoglycan monomer (20). We have confirmed these observations with monoclonal antibodies (A. R. Poole and C. Webber, manuscript in preparation). Moreover, hyaluronidase-treated chick cartilage pro-

teoglycan can elicit monoclonal antibodies among which are those that react with chondroitin 6-sulfate oligosaccharides attached to core protein (21). Recently, monospecific antibodies were used to localize proteoglycan (22–25) and link protein (22, 24, 25) in hyaline cartilages with immunofluorescence and with immunoelectron microscopy (26, 27).

Proteoglycans have now been identified biochemically in many tissues such as aorta (28–30), ovarian follicular fluid (31), gingiva (32), lung (33), basement membrane (34), and brain (35, 36). These proteoglycans exhibit fundamental differences in their glycosaminoglycan and protein compositions, their molecular sizes, and their ability to aggregate with hyaluronic acid.

The eye is a complex organ made up of many different tissues performing specialized functions. It has been shown to contain cartilage type II collagens (37–40) in the sclera (but not in mammals), retina, and vitreous. The cornea (41–43) and sclera (44) contain proteoglycans that are structurally and functionally different from cartilage proteoglycans. In this study we demonstrate, using antibodies to native cartilage proteoglycan and link protein, that the sclera, but not the cornea, also contains molecules immunologically related to cartilage proteoglycans and link proteins and confirm that arterial vessels contain molecules immunologically related to cartilage link protein: these are present in the media. In addition, we have detected molecules with immunological relationships to cartilage proteoglycan and link protein coexisting in the optic nerve of the central nervous system and the anterior uveal tract of the eye. Purified proteoglycan from rat brain was also shown to be immunologically related to cartilage proteoglycan.

MATERIALS AND METHODS

Tissue Preparation

Bovine eyes from ~1-y-old animals were removed immediately after death. For immunohistochemistry they were cut into strips along the median axis (from corneal optic center to optic nerve). As described earlier (22), tissue was frozen in gelatin with liquid nitrogen, sectioned along the same axis, fixed with formaldehyde for 5 min at room temperature, and treated with chondroitinase ABC, where indicated. Sclera were also dissected free of other tissue and extracted with guanidine hydrochloride as described below.

Preparations of Proteoglycans and Link Protein

The isolation of bovine nasal cartilage proteoglycans and link protein has already been described in detail (12). Purified and extensively characterized proteoglycan from rat brain (35, 36) was generously supplied by Dr. R. K. Margolis and D. Aquino, Department of Pharmacology, New York University School of Medicine. Proteoglycans I and II containing dermatan sulfate from bovine sclera were purified and characterized as reported earlier (44). Bovine sclera were dissected from freshly enucleated eyes and extracted in 5 vol (vol/wt) of 4 M guanidine hydrochloride with inhibitors as described (43). The extract was adjusted to contain 0.5 g of CsCl/g, centrifuged in a Beckman 50Ti rotor (Beckman Instruments, Inc., Palo Alto, CA) for 68 h, and the tubes were cut into D1 to D4 fractions (43). The D4 fraction, which contained the bulk of the protein was dialyzed overnight against 0.125 M Tris-HCl, pH 7.0, and concentrated ~1.5-fold with a B-15 cell (Amicon Corp., Lexington, MA).

Antibodies to Proteoglycans and Link Protein

The preparation and characterization of antisera (R7, R90, R131, and S27) and F(ab')₂ antibody subunits to native bovine cartilage proteoglycan and link proteins have all been described (12, 19, 22). A rabbit antiserum (R130) was prepared against bovine nasal cartilage link protein and low buoyant density proteoglycan present in fractions AID5 and AID6. The preparations and reactivities are as described for antiserum R7 (12, 19). Monospecific IgG antibodies, which react with purified high buoyant density bovine nasal proteoglycans (AIDIDI) were prepared from serum R130 by affinity chromatography and

shown to be specific (19). In addition, a monospecific sheep antiserum (S27) to bovine articular proteoglycan (AIDI) was used (19). Both sets of antibodies (R130 and S27) react with and precipitate native high buoyant density proteoglycan (AIDI) and native low buoyant density proteoglycans. They also react with purified hyaluronic acid–binding region isolated from bovine nasal cartilage (19). F(ab')₂ antibody subunits (R131 and R90 [19, 22]) derived from rabbit antisera to bovine nasal cartilage link protein were rendered monospecific by absorption with bovine nasal cartilage proteoglycan (AIDI), as previously reported (19): this removes trace amounts of antibody to cartilage proteoglycan. A rabbit antiserum to purified rat brain proteoglycan (35, 36) was generously supplied by Dr. R. Margolis and D. Aquino, Department of Pharmacology, New York University School of Medicine.

A rabbit antiserum to dermatan sulfate–rich proteoglycan (47) isolated from fetal bovine epiphyseal cartilage[1] was prepared as follows: 1 mg of proteoglycan[1] was injected intramuscularly in an emulsion of 0.5 ml Freund's complete adjuvant (Difco Laboratories, Detroit, MI) and 0.5 ml of phosphate-buffered saline (21) on days 0, 28, and 49. The rabbit was bled out by cardiac puncture under anesthesia on day 58 and serum was separated as described (12). Immunoglobulins and antibodies were concentrated by precipitation with 50% ammonium sulfate (12, 19).

Rabbit IgG was purified from a nonimmunized rabbit by DEAE cellulose chromatography (22). F(ab')₂ subunits of IgG were also prepared from the sera of nonimmunized sheep and rabbits as well as from sera S27, R90, and R131 as described (19, 22). The preparation of unlabeled and fluorescein-labeled pig F(ab')₂ antibody subunits to sheep F(ab')₂ and to rabbit F(ab')₂ have also been described (22).

SDS Polyacrylamide Gel and Transfer Electrophoresis (Electroblotting) of Sclera D4 Fraction

1.5-mm-thick nonreducing SDS 10% polyacrylamide gels (45) were prepared, using a 3% stacking gel. Samples were first dialyzed against 0.125 M Tris-HCl, pH 6.8, containing 0.1% SDS. They were then boiled for 3 min and centrifuged at 10,000 g for 3 min. Sample volumes of 40 μl were applied and gels were run at 6 mA overnight. The gel was cut between the electrophoretic tracks into longitudinal strips and these were soaked three times, each for 10 min, in a transfer buffer of 24 mM Tris, 192 mM glycine (46), and 20% methanol, pH 8.3. The separated proteins were then transferred for 2 h at 800 mA to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH; ex. Bio-Rad #162-0113) using electroblot transfer equipment (E. C. Apparatus Corp., St. Petersburg, FL). Nitrocellulose strips were removed and soaked with 3% bovine serum albumin to block residual nonspecific binding sites.

After 1 h, strips were immersed in solutions of appropriate unabsorbed rabbit anti–link protein serum (R90) or nonimmune rabbit serum (control), concentrated by ammonium sulfate precipitation (12) to a final protein concentration of 2.5 mg/ml. These solutions also contained 3% bovine serum albumin and 10% normal rabbit serum. After 30 min at room temperature, the strips were washed in five rinses of phosphate-buffered saline (19) for 30 min. A peroxidase-labeled pig anti-rabbit F(ab')₂ conjugate (as prepared for enzyme-linked immunoabsorbent assay (ELISA), described below) was then added at appropriate dilution in 3% bovine serum albumin and 0.1% Tween 20 for 30 min. The strips were again washed as before and immersed in a fresh solution of 2,5-diaminobenzidine (carcinogenic!) in 10-ml of phosphate-buffered saline containing 0.005% hydrogen peroxide at room temperature. The reaction was stopped after 5–10 min by the addition of a few drops of 10% sodium azide. Papers were then washed in water and air-dried. Antibody reactivity was revealed by the presence of brown, insoluble reaction product. This method was also used to examine these D4 fractions with antibodies to cartilage proteoglycan, namely R130 and S27. Control sheep serum and peroxidase labeled pig anti-rabbit or sheep F(ab')₂ were used as described above.

Crossed Immunoelectrophoresis of Scleral Extracts

Plates were prepared and stained as described previously (12, 19), with the following exceptions. Agarose gels of 0.6% were used. 12-μl volumes of unconcentrated D4 fractions in 4 M guanidine hydrochloride were used for each plate. The fractions were first electrophoresed at 5 V/cm for 2 h and then at right angles at 3 V/cm for 16 h into gels containing concentrated sheep antibody (S27) to articular cartilage proteoglycan (1.25 mg/ml), or concentrated rabbit antibody

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[1] H. Choi, L. Rosenberg, A. Reiner, and A. R. Poole. Manuscript in preparation.

(R90) to bovine link protein (0.31 mg/ml) or nonimmune concentrated rabbit or sheep immunoglobulins at 0.31 and 1.25 mg/ml, respectively, as controls.

Tissue Staining

Frozen tissue sections were prepared, fixed, and stained by the indirect method as reported previously (22). Purified antibody IgG to proteoglycan from antiserum R130 was used as before at a concentration of 0.05 mg/ml. F(ab')₂ derived from antiserum R90 to link protein was used as described for F(ab')₂ derived from antiserum R131. These steps were controlled by nonimmune IgG or F(ab')₂ of the same species and by antibody preparations that had been preabsorbed with bovine nasal proteoglycan (for R130 affinity-purified antibody) or bovine articular proteoglycan (for S27), or with bovine nasal link protein (for R131 and R90) as reported earlier (19, 22). All the photographs published here were taken with green-only fluorescence emission. The preparation of sections and microscopy has already been discussed (21).

ELISAs of Proteoglycans

These assays were performed using Immulon flat bottom multiwell plates (Dynatech, Alexandria, VA; no. 1-223-29). Proteoglycans dissolved in 0.1 M Tris-acetate buffer, pH 7.2, were diluted 300- to 500-fold in 0.1 M carbonate buffer, pH 9.0. 50-µl volumes were added to each well to give the following total amounts of proteoglycan per well: chondroitinase ABC–treated bovine nasal proteoglycan (5 µg); rat brain proteoglycan (1 µg); bovine scleral proteoglycan (0.5 µg). After 18 h at 4 °C, unbound proteoglycan was removed by washing with a solution of phosphate-buffered saline containing 0.1% polyoxyethylene sorbitan monolaurate (Tween 20; Sigma Chemical Co., St. Louis, MO). The assay was then continued as described previously (48) using sheep and rabbit antibodies followed by peroxidase-labeled pig anti-sheep or -rabbit F(ab')₂.

RESULTS

Treatment of Tissue Sections with Antibodies to Proteoglycan and Link Protein

Tissue sections initially treated with either nonimmune rabbit IgG or with nonimmune rabbit or sheep Fab' exhibited a complete lack of green fluorescein fluorescence (Figs. 1 d and 4 b) with the exception of those cases described below where some weak green fluorescence was observed. Fluorescent staining achieved with antisera was blocked by prior absorption with corresponding antigen as reported earlier (22) demonstrating specificity.

Antibodies to Cartilage Proteoglycan

Sections treated first with anti–cartilage proteoglycan antibody (either S27 Fab' or R130 purified antibody IgG) both exhibited strong fluorescence in extracellular sites throughout the sclera. This was sometimes patchy, particularly in the anterior part of the sclera (Fig. 1 a), or more evenly distributed in other sites (Fig. 1 c). Staining was also seen in the anterior uveal tract at the base of the ciliary body, between the ciliary processes and ciliary muscle (Fig. 1 e). The remainder of the uveal tract (Fig. 1 c), the retina, and the cornea were unstained (Fig. 1 b). However, the junction between the stained sclera and the unstained cornea was strikingly marked by an abrupt transition between scleral cells surrounded by stained matrix and corneal cells without any associated staining (Fig. 1 b). These differences were emphasized by sudden changes in the fibrillar collagenous organization of these two tissues as one passed from the "wavy" fibrillar organization of the sclera into the more homogeneous fibrillar composition of the cornea. A very weak green autofluorescence was observed in controls in the anterior uveal tract but was too weak to detect photographically (Fig. 1 f). Results were identical for antibody preparations S27 and R130 (only R130 results are shown) and were unchanged after sections had been pretreated with chondroiti-

nase ABC to remove chondroitin sulfate and dermatan sulfate (results not shown).

The connective tissue sheaths of rectus muscles frequently, but not always, stained with antibodies to cartilage proteoglycan (Fig. 2). The reasons for this limited staining are not yet clear. Examination of the optic nerve revealed that antibody to cartilage proteoglycan reacted weakly with the tissue surrounding the myelin sheaths (endoneurium) and with the epineurium (Fig. 3 c and e). The perineurium was not specifically stained since in control sections of optic nerve treated with nonimmune sheep Fab' or rabbit IgG there was nonspecific staining of the perineurium (Fig. 3 a). Arterial vessels did not stain with antibodies to cartilage proteoglycan (R130 and S27) as shown in Fig. 5 c.

Antibodies to Cartilage Link Protein

The use of antibody Fab' to link protein revealed similar staining in only some tissues. Thus the sclera (Fig. 4 a and c) and the anterior uveal tract at the base of the ciliary body (Fig. 4 e) both stained. As observed for proteoglycan, the anterior sclera stained less evenly and showed more clearly defined wavy staining than was observed elsewhere in the sclera. The cornea, retina, and remaining uveal tract were unstained. Some very weak autofluorescence was observed in control sections in the sclera and anterior uveal tract (Fig. 4 d and f). No staining was observed in the stromal connective tissue of the iris (Fig. 5 a and b). The iris was also completely unstained by antibodies to cartilage proteoglycan and this included all arterial (Fig. 5 c) and venous vessels both here and in the rest of the eye and its associated tissues. The endoneurium surrounding the myelin sheaths in the optic nerve also stained selectively (Fig. 3 d and f) and nonspecific binding of nonimmune Fab' to the perineurium was again observed (Fig. 3 b). The epineurium did not stain for link protein (Fig. 3 d and f). The connective tissue sheaths of striated rectus muscle never stained. The media of arterial vessels present in the iris and the uveal tract and on the outer side of the sclera selectively stained with R131 antibodies to link protein (Fig. 5 a and b): only the autofluorescent internal elastic lamina and adventitia were observed in control sections. Surprisingly, antibody R90 did not react with arterial media although its immunoreactivity was otherwise very similar to antibody R131. Again, similar results were obtained without (results shown) or with pretreatment of fixed tissue sections with chondroitinase ABC.

Antibodies to cartilage proteoglycan and link protein never reacted with fibrous connective tissues such as those constituting the stroma of the iris and the connective tissues immediately outside and surrounding the sclera. There was no convincing evidence of any specific staining of the vitreous using either antibodies to proteoglycan or to link protein.

Reactions of Antisera with Isolated Proteoglycans and Link Protein
Scleral Proteoglycans

ELISAs were used to determine immunological relationships of proteoglycans purified from tissues that stained with antibody (R130) to bovine nasal proteoglycan. Since it was technically not possible to study all tissues and to achieve a detailed characterization of other proteoglycans in the framework of this study, it was decided to examine previously well-characterized proteoglycans isolated from these or related tissues. Proteoglycans (I and II) have been isolated from bovine sclera
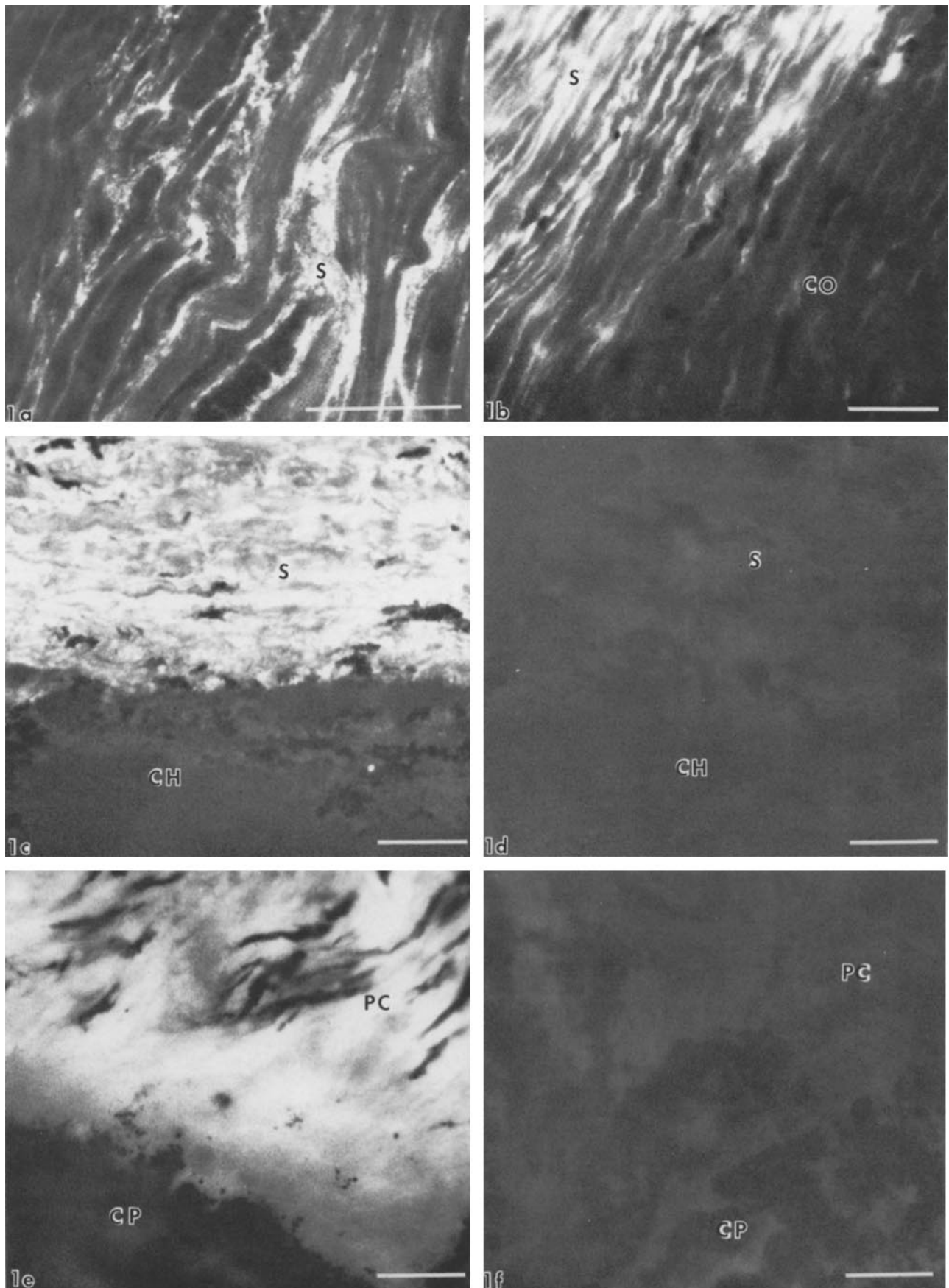
FIGURES 1–5 Localizations of link protein and proteoglycan in fixed sections untreated with chondroitinase ABC. Sections were initially treated with IgG antibodies or their Fab′ subunits followed by fluorescein-labeled antibody Fab′ to the first antibody used. All methods are described fully in the text. Photomicrographs reveal only green fluorescence. All bars, 50 μm.

FIGURE 1 Longitudinal sections showing intense staining with antibody (R̄130) to cartilage proteoglycan in the anterior sclera (a and b), middle sclera (c), and anterior uveal tract under the ciliary processes (e) around the pigmented cells (PC). Staining was absent from the remaining uveal tract (choroid, CH), ciliary processes (CP) and cornea (CO). Control sections of sclera and uvea (d) and ciliary body (f) were initially treated with nonimmune IgG and were unstained. s, sclera.
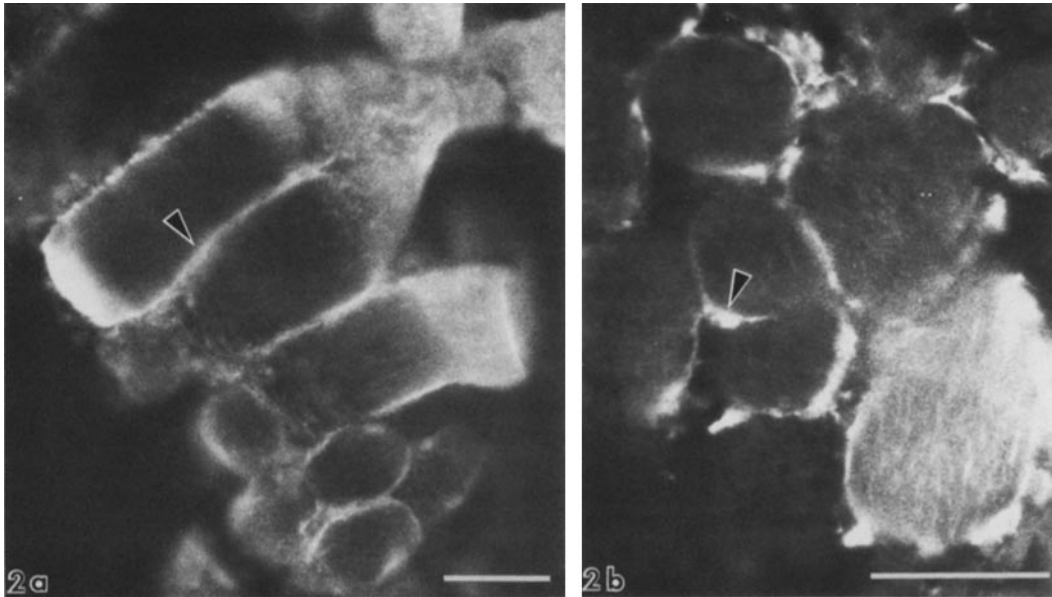
FIGURE 2 Longitudinal (a) and transverse (b) sections of rectus muscle stained with rabbit IgG antibody (R130) to cartilage proteoglycan. Staining was observed in the connective tissue sheaths (arrowheads) surrounding individual muscle fibers.

and shown to contain dermatan sulfate (44). Antibody to dermatan sulfate–containing proteoglycans isolated from bovine epiphysis reacted with both purified scleral proteoglycans I and II (Fig. 6). Antibodies to bovine nasal (R130) and articular (S27) proteoglycans showed no reaction with these proteoglycans indicating that the reaction of R130 and S27 antiproteoglycan antibodies with the sclera must be with molecular species other than these dermatan sulfate proteoglycans.

Sclera were extracted with guanidine hydrochloride. Whole extracts and D1 to D4 fractions thereof were examined with SDS PAGE and electroblot transfer for molecules reactive with antibodies to bovine nasal cartilage (R130) or articular cartilage (S27) proteoglycan. There was no convincing evidence that molecules (other than link protein) which reacted with these antibodies had penetrated the gels. High buoyant density proteoglycans from nasal cartilages were not detected within these gels even after treatment with chondroitinase ABC. Thus it was concluded that the reactivities of these antibodies with bovine sclera must be with molecular species that were too large to penetrate these acrylamide gels or that could not be transferred from gels to nitrocellulose. When these extracts and D4 fractions were subjected to crossed immunoelectrophoresis, sheep antibody to bovine articular cartilage proteoglycan (S27) produced two moderately strong and one weak precipitin reaction when fraction D4 was tested (Fig. 7). No reactivity was seen with other fractions and extracts nor with gels containing nonimmune sheep immunoglobulin. Thus, extractable molecules immunologically related to cartilage proteoglycan are present in sclera.

Scleral Link Protein

Analysis of scleral extracts and D4 fractions by use of crossed immunoelectrophoresis and the rabbit antibody (R90) to bovine link protein also demonstrated the presence of immunoreactive material which gave a small rocket (Fig. 7), revealing the presence of an extractable species related to link protein. By reference to purified bovine nasal link protein examined under the same experimental conditions, SDS PAGE and electrophoretic transfer demonstrated that this reactive mate-

rial resided in scleral D4 fractions. The material was weakly stained but the finding was reproducible. Two species were observed which reacted with the antibody to link protein. Their molecular sizes corresponded to those of the link proteins of bovine nasal cartilage (Fig. 8). There was significantly more of the larger species present. The weakness of the reactions was probably due to the limited amount of link protein present in this fraction. No such reactions were observed when electroblots were initially treated with nonimmune rabbit serum instead of the antiserum.

Proteoglycan of the Central Nervous System

Since the optic nerve is also part of the central nervous system, it was important to determine whether reactivity of R130 anti–bovine nasal proteoglycan antibodies with the optic nerve could be accounted for by the presence of proteoglycan which has been purified from the central nervous system (34, 35). Although this proteoglycan was isolated from rat brain, ELISA analyses revealed that an antiserum to this proteoglycan reacted, like antiserum R130, with bovine nasal proteoglycan (Fig. 9). Antisera R130 and S27 did not, however, cross-react with rat brain proteoglycan. Thus, the single cross-reaction demonstrates an immunological and hence structural relationship between proteoglycan of the central nervous system and that isolated from hyaline cartilage, as suggested by the immunofluorescence studies.

DISCUSSION

Until the advent of specific immunological methods for the identification and localization of proteoglycans and related molecules we had no precise way to identify definitively these molecules in tissues using histochemical methods. Metachromatic dyes have been commonly used (see discussion in reference 22). Although they bind to the glycosaminoglycans of proteoglycans, their reactivity is not always specific. Moreover, they provide us with no clear, unequivocal information regarding either the presence of different glycosaminoglycans (characteristic of different proteoglycans) or the similarity (or dissim-
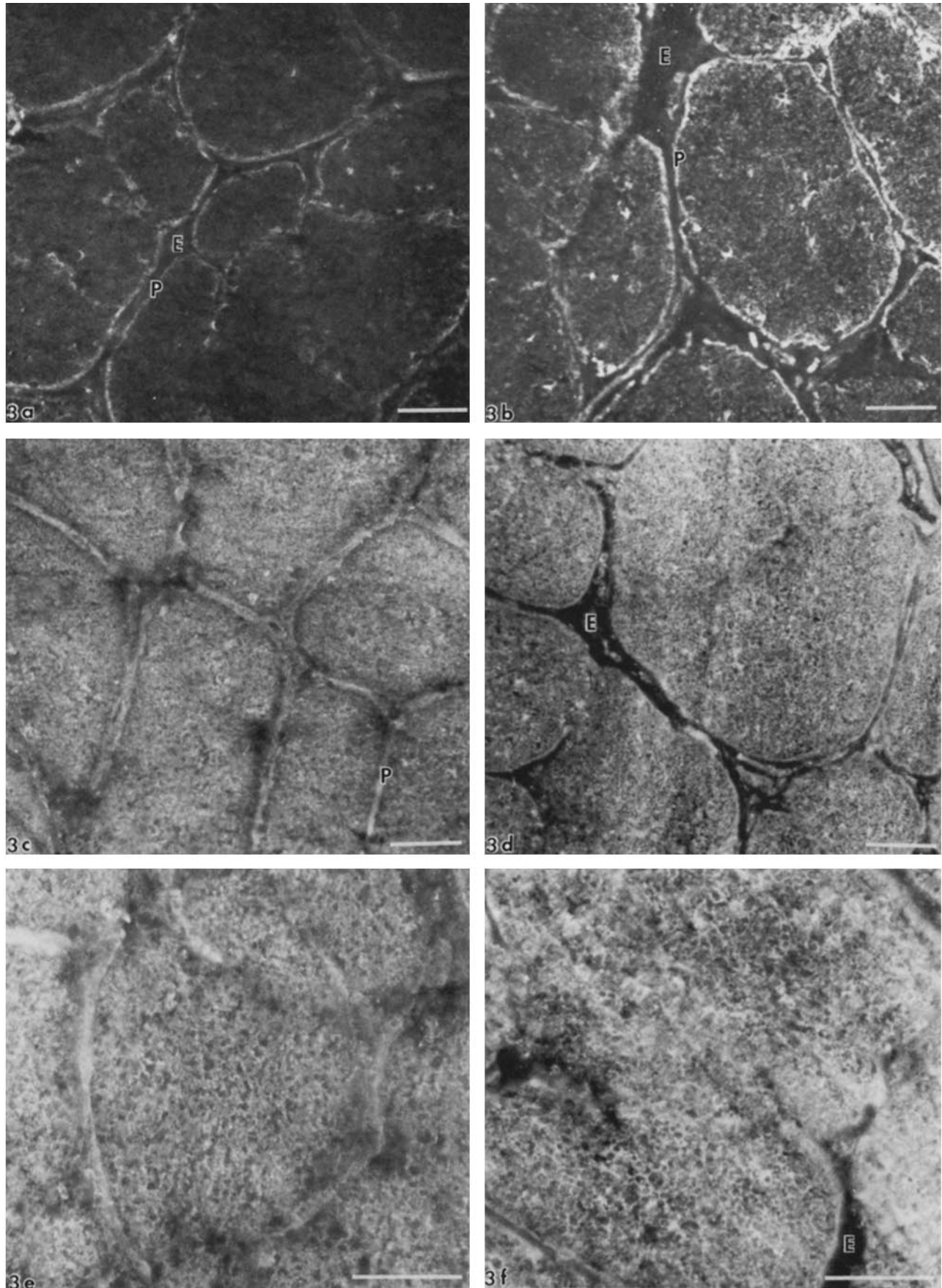
FIGURE 3 Transverse sections of the optic nerve to show fine latticework of staining for proteoglycan with IgG R130 anti-cartilage proteoglycan antibody (c and e) and link protein with antibody R131 (d and f) in tissue surrounding unstained myelin sheaths. The epineurium (E) was unstained for link protein (d and f) but stained for proteoglycan (c and e). Control sections were initially treated either with nonimmune rabbit IgG (a, for proteoglycan) or Fab' (b, for link): nonspecific binding of IgG was observed in the perineuria (P) and sometimes within the nerve.
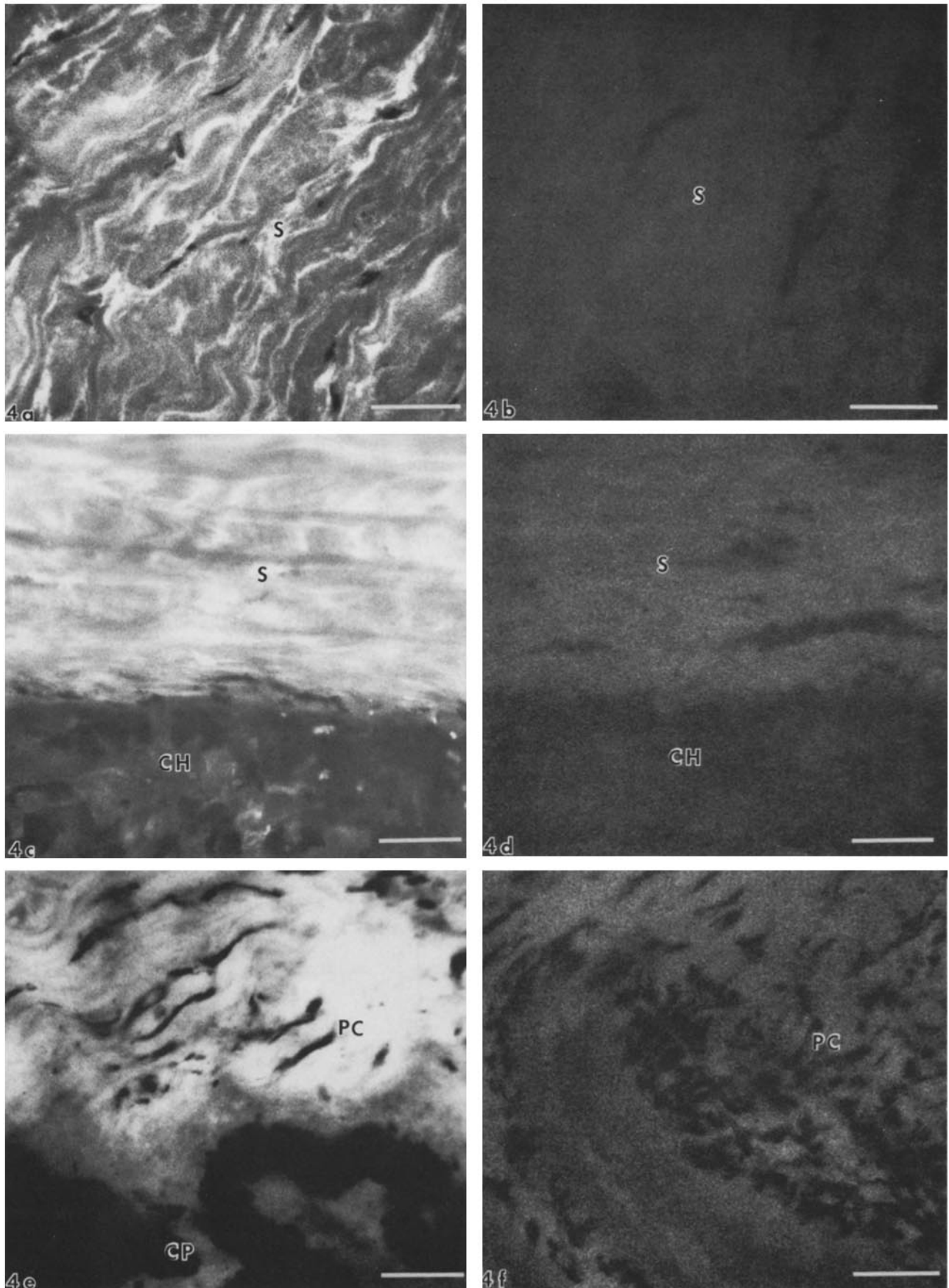
FIGURE 4 Longitudinal sections showing intense staining with antibody to cartilage link protein (R131) in the sclera (*S* in *a* and *c*) and in the ciliary body (*e*). Staining was absent from the remaining uveal tract (*CH*) and the ciliary processes (*CP*). Control sections of sclera, anterior uvea, and ciliary body (*b, d,* and *f*) were initially treated with nonimmune rabbit Fab': they did not stain. Some weak autofluorescence was observed in the sclera (*d*) and ciliary body (*f*). The pigmented cells (*PC*) can be seen in *e* and *f*.
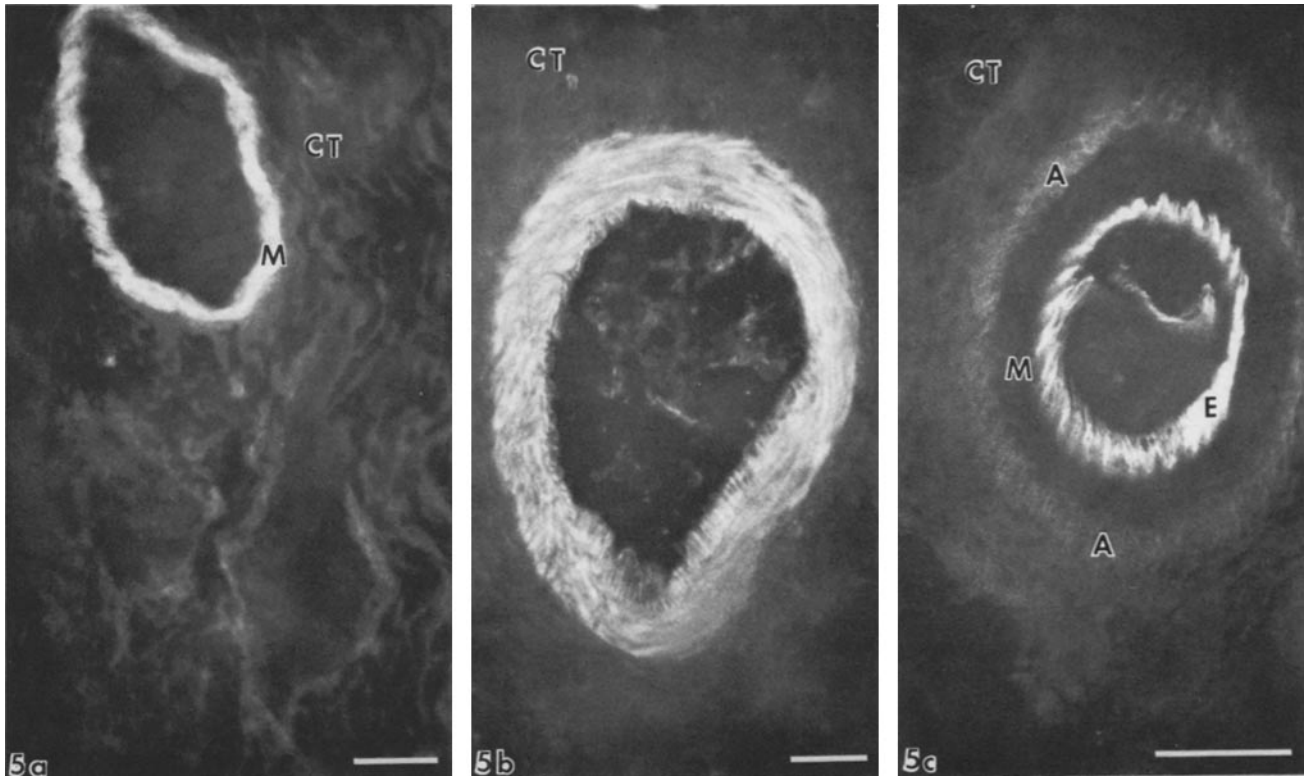
FIGURE 5   Longitudinal sections of the iris to show transverse sections of arterioles (*a* and *b*) and venules (*a*) staining with rabbit antibody (R131) to link protein. The media of arterial vessels were intensely stained but the connective tissue stroma of the iris (*CT*) and venules were unstained. In *c* can be seen a cross section of an arteriole vessel unstained after treatment with rabbit antibody (R130) to cartilage proteoglycan. Only the autofluorescent internal elastic lamina (*E*) and the autofluorescent adventitia (*A*) can be seen. The media (*M*) and connective tissue stroma (*CT*) did not stain.
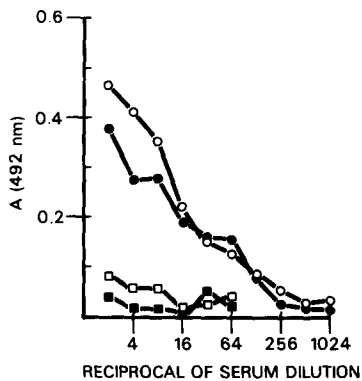

FIGURE 6   ELISA assays of bovine scleral proteoglycans I (O, □) and II (●, ■) using R107 (O, ●), an antiserum to bovine epiphyseal dermatan sulfate proteoglycan, and R130 (□, ■), an antiserum to bovine nasal proteoglycan.
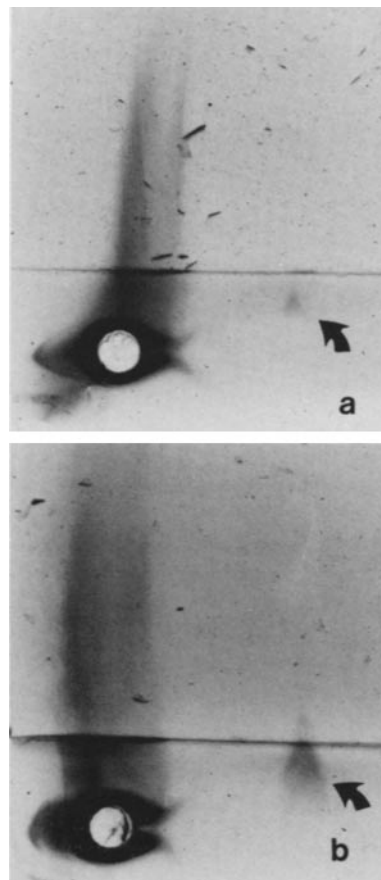

FIGURE 7   Detection of proteoglycan- and link protein-related molecules in a scleral D4 fraction using crossed immunoelectrophoresis. Upper gels contained (*a*) rabbit antibody (R90) to bovine link protein, and (*b*) sheep antibody to bovine articular proteoglycan. Smears of scleral protein can be seen around and over the origin wells. The identifiable specific immunoprecipitates are indicated by arrows.

ilarity) of the core proteins of proteoglycans. Hence, specific comparisons of proteoglycans are not normally possible.

The unique specificities of antibodies raised against native proteoglycans and functionally related molecules such as link protein now permit us to analyze and localize these molecules and their immunologically related counterparts in cartilage and other tissues. All the evidence at our disposal indicates that antibodies like ours, which were prepared against proteoglycans not treated with glycosidases, react with antigenic determinants associated with the core proteins and not with oligosaccharides of degraded glycosaminoglycans. Reactions with glycosaminoglycans, namely chondroitin sulfate, have only been observed when antibodies were raised against and reacted with molecules treated with chondroitinase ABC (20, 21; A. R. Poole, unpublished results). Our previous work described the relative organization of proteoglycans and link proteins in
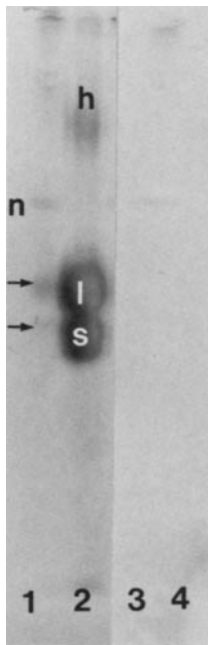
FIGURE 8 Detection of link protein related molecules in a scleral D4 fraction using SDS gel electrophoresis followed by electrophoretic transfer of scleral fraction D4 (tracks 1 and 3) and purified bovine nasal link protein (tracks 2 and 4) to nitrocellulose, then stained with unabsorbed antiserum to link protein (tracks 1 and 2) and nonimmune rabbit serum (tracks 3 and 4). The large (l) and small (s) link proteins are indicated in track 2 together with the weakly stained corresponding molecules found in bovine sclera track 1 (arrows). These species do not stain in tracks 3 and 4. The reaction of the unabsorbed antiserum with contaminant hyaluronic acid binding region is indicated (h);[2] nonspecific reaction with D4 can be seen in tracks 1 and 3 (n).
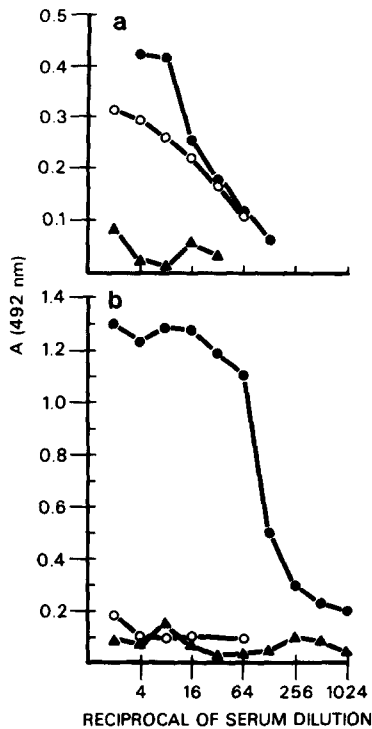

FIGURE 9 ELISA assays of (a) bovine nasal proteoglycan and (b) rat brain proteoglycan. AS PG (●) is an antiserum to rat brain proteoglycan and NRS (▲) is nonimmune rabbit serum. R130 (○) is an antiserum to bovine nasal proteoglycan.

articular cartilage (22) and mineralizing cartilages (24, 25). Our present study extends this work to tissues other than cartilage, namely those present in or associated with the mammalian eye. These new immunofluorescent studies have revealed the presence of molecules immunologically related to cartilage components in various tissues. The reactivities of these antibodies to cartilage proteoglycans and link protein will be discussed in the light of our own and other recent biochemical observations on such molecules extracted and isolated from these and other related tissues.

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[2] Demonstrated in experiments to be reported elsewhere: A. R. Poole, A. Reiner, J. S. Mort, J. Kimura, V. Hascall, L. Rosenberg. Manuscript in preparation.

Neuroglial cells of the central nervous system, isolated from the brain, can incorporate radiolabeled sulfate into a large, chondroitin sulfate–rich aggregatable proteoglycan resembling that found in cartilage (49). Margolis and co-workers (35, 36) have also described a chondroitin sulfate proteoglycan isolated from rat brain. We have now shown that an antiserum to this proteoglycan from rat brain cross-reacts with bovine nasal cartilage proteoglycan and that antibodies to this bovine proteoglycan also react with the bovine optic nerve, a part of the central nervous system. This would imply that proteoglycans of the central nervous system have structural similarities (associated with the core protein) to those found in cartilage and that a proteoglycan related to that observed by Margolis et al. is present in the optic nerve. Proteoglycan of the central nervous system may provide important supportive roles in protecting nerve fibers and maintaining the integrity of these tissues. These molecules are probably present in intracellular sites in brain as revealed by immunoelectron microscopy (R. U. Margolis, personal communication).

The reactivity of antibody to cartilage link protein with the optic nerve indicated the presence of related molecules in this tissue. The observations that some of the proteoglycans of the central nervous system can aggregate with hyaluronic acid (36) implies that these aggregates may, as in cartilage, be stabilized by a link protein. Very recently a protein was isolated from human brain with a molecular weight of ~68,000 (50). Since this protein, called hyalurononectin, can bind to hyaluronic acid it may be related to link protein and be functionally related to the proteoglycan-related material detected by our antibodies in the optic nerve.

A reaction of antibody to link protein with arterial vessels confirms a recent observation (51) in which bovine aorta was shown to contain a molecule corresponding in molecular size to the larger species (link protein 1) found in bovine nasal cartilage and reactive with an antibody to cartilage link proteins. Our observations demonstrate that this molecule is present in the media of these vessels. Moreover, since only one of our link antisera was reactive with arterial media there are clearly some differences between link proteins in bovine cartilage and in arterial vessels. These may be related to the presence of only the large link species in vessel walls or to accessibility of link protein to antibody in situ.

Proteoglycans resembling cartilage proteoglycans have also been isolated from aorta (28, 29) and they can aggregate with hyaluronic acid. Again, link protein may play a role in these interactions. Work by others (51) has indicated that these high buoyant density proteoglycans are immunologically related to those found in cartilage. Our observations using other antisera failed to confirm this. Although we and others (51) both employed antibodies reactive with the hyaluronic acid–binding region, isolated molecules were examined before (51), whereas we examined molecules in situ. In this latter case, the immunoreactive part of the molecule may not have been accessible to antibodies in situ. Thus, negative results with immunohistochemistry must be confirmed by work on isolated molecules.

Corneal proteoglycans are structurally very different from and smaller than cartilage proteoglycans (34, 41, 42) and have different protein compositions and far fewer glycosaminoglycan chains. These were not detected in situ by our antibodies to proteoglycan. Reactivity was, however, observed with sclera. Recent studies of scleral proteoglycans (44) have revealed that they are also very different from those found in cartilage. Molecules with protein cores with molecular weights of

>100,000 (proteoglycan I) and 46,000 (proteoglycan II) have been described, to which are bound dermatan sulfate. Whereas these molecules reacted well with an antibody to proteoglycan containing dermatan sulfate isolated from bovine epiphyseal cartilage, they showed no reactions with the antibodies to cartilage proteoglycans that stained sclera. Antibody to cartilage proteoglycan did, however, react with isolated molecules in scleral D4 fractions, indicating that molecules related to cartilage proteoglycan are present and extractable.

Our attempts to detect these other immunoreactive species by electrophoresis of extracted material in polyacrylamide gels followed by transfer proved inconclusive. Since 10% acrylamide gels were employed, we would imagine that these molecules are of a size, like that of cartilage proteoglycans, too great to permit it to enter our gels. This immunoreactive molecule remains to be characterized.

Studies of link protein–related species using similar technology clearly showed that bovine sclera, like bovine aorta (51), contains molecules very similar in molecular weight and immunoreactivity to those found in bovine nasal cartilage. Interestingly, as with aorta, the larger link species predominated in sclera whereas similar amounts of a large and a small species of link are found in bovine cartilage (12). The functional significance of these molecular species remains to be established. Since there was no evidence that the recently characterized scleral dermatan sulfate proteoglycans react with hyaluronic acid, this scleral link protein may interact with the immunologically identified but as yet unisolated proteoglycan-related species detected by us in sclera.

The reaction of anti–cartilage proteoglycan antibodies with some muscle sheaths suggests the presence of structurally related muscle proteoglycans of which we know little. Fibroblastic mesenchymal cells derived from fetal rat triceps muscle can differentiate into chondrocytes and secrete a cartilaginous matrix (52). The immunological relationship with cartilage may not be all that surprising since proteoglycan synthesis in the sheath may be determined by these mesenchymal fibroblasts.

A good example of changing phenotypic expression giving rise to different connective tissue macromolecules is shown by chondrocytes which, when cultured, can initially synthesize type II collagen and then start to manufacture other collagen types (53, 54). Since antigenic determinants are shared by these collagens, it would appear that, although the proteoglycans synthesized by cells derived from the same stem cells may change when these cells experience different environmental conditions, they may, like the collagens, still retain certain structural similarities on their core proteins.

Earlier work on the collagens present in ocular tissues revealed that cartilage type II collagen is synthesized by cultured neural retina cells (37), which are involved in the synthesis of the type II collagen of the vitreous body (38, 39). We could not detect any clear-cut reactivities of our antibodies with these tissues. Interestingly, the presence in sclera of birds (39, 40) and elasmobranch fishes (55) of hyaline cartilage containing type II collagen may explain why link protein and molecules related to cartilage proteoglycans are found in this tissue, although in mammals the cartilage plate is lost during evolution and there is no evidence for the presence of type II collagen in mammalian sclera (56). Thus, the structures of proteoglycans in different tissues would appear to vary independent of collagen although there is an evolutionary basis for scleral molecules related to those found in cartilage. Moreover, proteogly-

cans may have protein cores that are structurally and immunologically more tissue specific than the different collagen types, which share protein structure and antigenicity in part. This may be because the core proteins of proteoglycans and their associated oligosaccharides, which together probably determine the major antigenic domains of native molecules, are structurally very variable; also these probably vary independent of the glycosaminoglycans of these molecules, which in a native state would not ordinarily determine their immunological character.

Finally, the presence of molecules immunologically related to cartilage proteoglycans in other tissues is of interest in the field of autoimmunity to these molecules in rheumatic diseases. Immunity to purified cartilage proteoglycan has been demonstrated in patients with relapsing polychondritis (57, 58) and rheumatoid arthritis (59, 60). Patients with relapsing polychondritis (61) often suffer from anterior uveitis. Patients with polychondritis commonly display scleritis (61, 62), and scleritis is also observed in rheumatoid arthritis (63). Thus, a primary immune response to cartilage proteoglycan could lead to a cross-reaction with immunologically related proteoglycans in other tissues of the kind described here. The pathology of the extra-articular manifestations of these diseases, such as anterior uveitis, scleritis, neuropathies, and muscular rheumatism, may be related to autoimmunity to cartilage proteoglycans.

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