We have studied binding to collagen of the 59-kDa protein present in most connective tissues. Collagen fibril formation, measured as increasing turbidity, was markedly retarded and reduced by the presence of small amounts of this protein. This was true for both collagen I and collagen II. The effect was also observed when pepsin-treated collagens were used, indicating that interaction with the telopeptides is not involved. The proportion of collagen precipitated in the assay was not or only marginally reduced. Thus, the altered optical properties indicate that structurally different fibrils are formed in the presence of the 59-kDa protein.

The 59-kDa protein bound to collagen I or collagen II that had been insolubilized on polystyrene 96-well microtiter plates, as measured by enzyme-linked immunosorbent assay. Analogously, binding to the collagens was demonstrated for the PG-S2 low Mr proteoglycan, previously shown only to inhibit collagen fibrillogenesis. The two matrix components showed similar strength of binding, i.e. $K_d$ 35 nM for the 59-kDa protein and 16 nM for PG-S2 at 20°C.

The results do not reveal if the collagen interaction site of the 59-kDa protein is different from that of PG-S2. Our observations do, however, suggest that the 59-kDa protein, as well as PG-S2, have functions related to the regulation of collagen organization in tissues.

Connective tissues have supportive and protective roles in the body. They offer specific mechanical properties which are determined by their extracellular matrix composition and structure. Major constituents are a network of insoluble fibrils and water-soluble polymers. The fibers provide tensile strength and consist of collagen polypeptides forming elongated triple helical molecules. Several collagen molecules aggregate in an ordered fashion to form fibrils and fibril bundles (Pierz, 1984). In tissues like cartilage, the major class of soluble polymers is the proteoglycans. These are negatively charged molecules that assume extended conformations. One function is to resist compressive forces and organize water molecules and ions. The major proteoglycans consist of a central protein core with a large number of attached glycosaminoglycan chains (for references see Heinegård and Sommarin, 1987).

Collagens and proteoglycans seem structurally similar from one tissue to another, while the functional adaptation of the tissues is related to the organization of the matrix components at the supramolecular level. The different requirements between tissues are most likely met by the fine and different tuning of the assembly of these macromolecules into structures of higher order, e.g. proteoglycan aggregates and collagen fibrils. At present, the mechanism and the regulation of these processes are largely unknown. It is therefore important to study factors that may be involved in the assembly of matrix components. The formation of collagen fibrils and their assembly into bundles in the extracellular matrix is one such key process.

It was proposed earlier that glycosaminoglycans and proteoglycans affect the interactions between collagen molecules and thus the formation of fibrils. Several studies have brought support for this hypothesis. Various glycosaminoglycan preparations have been mixed with collagen and effects on collagen precipitation and fibrillogenesis in vitro have been demonstrated (Wood, 1969; Öbrink, 1973b; Oegema et al., 1975; Snowden and Swann 1980). Weak electrostatic interactions between glycosaminoglycans and collagen have been characterized (Öbrink, 1973a).

More recently, as techniques for the purification and characterization of proteoglycans have improved, it has become possible to study the interactions of intact, distinct proteoglycans. A pronounced capacity of a small dermatan sulfate proteoglycan isolated from bovine tendon to inhibit development of collagen fibrils in vitro has been demonstrated in our laboratory (Vogel et al., 1984). This interaction depends on the core protein, while the isolated DS$^1$ side chains showed no effect. Proteoglycans of similar size and shape as the small proteoglycan from tendon have been isolated from several other connective tissues. Two different groups have been identified, referred to PG-S1 and PG-S2 (Heinegård et al., 1985). The core proteins of these proteoglycans are of similar size ($M_r \approx 45,000$), but they are structurally distinct within each group, according to peptide patterns. The small proteoglycans from nasal cartilage (chondroitin sulfate side chains) and aortic intima (DS side chains), both of the PG-S1 type, do not show any specific effects on fibril formation in vitro of collagen I or II. The small proteoglycans from tendon (DS side chains), sclera (DS side chains), and articular cartilage (DS side chains), representing the PG-S2 species, are all capable of interacting with collagen I and II. Support for the physiological relevance of the interaction is the localization of dermatan sulfate proteoglycans at the "d" and "e" bands of collagen fibrils in nonmineralized connective tissues, e.g. tendon, skin, cornea, and sclera, as revealed by electron microscopy (Scott and Orford, 1981; Scott and Haigh, 1985; Young, 1985).

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Recent studies also draw attention to minor collagens. The existence of mixed fibrils has been demonstrated containing both collagen I and III (Henkel and Sharp, 1988), and collagen II and IX (Eyre et al., 1985). Other molecules of interest are the noncollagenous matrix proteins. Several of these have been isolated during recent years, while knowledge of their function is still very limited. The 59-kDa protein discussed in the present paper was originally purified from articular cartilage (Heinegård et al., 1986). The 59-kDa protein discussed in the present paper was originally purified from articular cartilage (Heinegård et al., 1986), but it is present in all cartilages where it typically represents 0.1-0.3% of tissue wet weight. The protein is also present in variable amounts in many other connective tissues.

**Experimental Procedures**

**Collagen Preparation**—The procedure for preparation and analysis of collagens was essentially the same as described previously (Vogel et al., 1984). In order to efficiently remove proteoglycans and the anionic 59-kDa protein, a DEAE-cellulose chromatography step was included as follows. Precipitated material was redissolved in 1 M NaCl, 50 mM Tris/HCl, pH 7.5, and transferred to 0.2 M NaCl, 50 mM Tris/HCl, pH 7.5, by extensive dialysis. This solution was passed through a column of DE32 (Whatman) equilibrated with the same buffer. Nonbinding material was precipitated by dialysis against 20 mM sodium phosphate, pH 7.2, and further treated as originally described.

In order to verify purity of preparations, reduced samples were electrophoresed on sodium dodecyl sulfate-polyacrylamide gradient-gels (3–12%) in the buffer system of Laemmli (1970). The gels were stained with Kwencid Blue R (BDH Chemicals). Furthermore, to detect and remove any trace amounts of proteoglycans and 59-kDa protein, both being anionic, samples containing 2 μg of collagen were passed through a DEAE-Sepharose Fast Flow (Pharmacia LKB Biotechnology Inc.) column (1.0 x 4.4 cm), eluted at 42 °C with 2 mM urea, 20 mM Tris/HCl, pH 7.0. While collagen does not bind to the anion-exchanger, any proteoglycans and 59-kDa protein would bind and be eluted with increased salt concentrations.

**Fibrillogenesis Assay**—Freeze-dried collagen was redissolved in 0.5 M acetic acid. Acid-extracted collagen I, at 4 mg/ml was dialyzed against 0.1 M acetic acid and stored in aliquots at −20 °C. One of these samples was thawed and diluted 10-fold with water before each experiment. Neutral salt-extracted collagen II at 0.8 mg/ml and pepsin-extracted collagen I and collagen II at 1.2 mg/ml and 2 mg/ml, respectively, were dialyzed against 10 mM acetic acid in the cold and used within a few days. The fibrillogenesis buffers contained 0.14 M NaCl, 30 mM sodium phosphate, pH 7.3, as final concentrations. In some experiments the buffer also contained 30 mM TES. To monitor fibril formation, a Cary 210 spectrophotometer with a water-jacketed five-place cuvette chamber was used. Glass-cuvettes of 1 ml were placed in the photometer and warmed to the temperature desired for the particular fibrillogenesis assay. All stock solutions were deaerated and equilibrated to the appropriate temperature before being combined. Fibril formation was induced by mixing 0.5 ml of double-concentration buffer with 0.25 ml of water containing the components to be tested, and finally with 0.25 ml of collagen solution in a cuvette.

**Collagen Assay**—After 20 h of fibrillogenesis, at plateau level of turbidity development, contents of cuvettes were centrifuged at 10,000 x g for 5 min in a table-top centrifuge (Spinco) at 37 °C. An aliquot (0.80 ml) of the supernatant was retrieved, hydrolyzed in 6 M HCl at 110 °C for 24 h, and the hydroxyproline was determined colorimetrically (Steigemann and Sadow, 1967). The measured hydroxyproline contents of supernatants were compared with control samples of equal composition but not warmed to induce fibril formation. For these control solutions, contents of collagen were diluted with water and compared with equal volumes of double-concentration buffer. Various buffers were tested initially, but it was found that coating the saline/phosphate buffer also used for the fibrillogenesis assay was preferable. Microtiter plates of polystyrene (Nunc-Immuno Plate IF, art no. 439454, A/S Nunc) were coated with solutions of collagen at 10 μg/ml in the saline/phosphate buffer (200 μl/well). After incubation overnight at room temperature in a humidified chamber, the plates were extensively rinsed with 0.15 M NaCl, 5 mM sodium phosphate, pH 7.4. To prevent further binding of matrix components to the hydrophobic surface, the remaining wells were incubated for 6 h with 200 μl of bovine serum albumin (Serva Feinbiochemica) at 10 mg/ml in 0.15 M NaCl, 5 mM sodium phosphate, pH 7.4. In subsequent steps the buffer contained 0.14 M NaCl, 30 mM sodium phosphate, 0.05% (v/v) Tween 20, pH 7.3, and the temperature was 18-22 °C. After extensive rinsing, samples of 200 μl containing the matrix components to be tested, were added. The plates were left overnight to permit binding of components to the cost. Next the plates were rinsed, incubated for 2 h with the first antibody in appropriate dilution (200 μl), rinsed again, and incubated for another 30 min with the second antibody, i.e. swine anti-(rabbit IgG) antibody conjugated to alkaline phosphatase (Orion Chemical) at a dilution of 1:200. Finally, after the plates had been rinsed, bound antibodies were detected by adding substrate solution (200 μl), i.e. p-nitrophenyl phosphate (Sigma) at 1 mg/ml in 1 M diethanolamine, 1 mM MgCl2, pH 10. The absorbance at 405 nm was measured with a Multiscan photometer (Flow Industries) immediately after substrate addition and again after 1-4 h of incubation at room temperature. The increase in absorbance due to formation of colored product was taken as the value of enzyme activity.

All analyses were done in triplicate. Every test of proteoglycan or protein binding was supplemented with controls, where either the collagen-coat or the tested component had been omitted.

Data from titration experiments were analyzed according to the theory established for fluid-phase ligand-macromolecule binding. Hypothesizing that the collagen coats represent one single class of homogenous binding sites, Scatchard-type plots were drawn. A modified form of the equation originally described by Scatchard (1949) was used:

\[
\frac{\Delta A}{C} = \frac{\Delta A_{\text{max}}}{K_d} - \frac{\Delta A}{K_d}
\]
where $K_d$ is the dissociation constant, $\Delta A$ is the measured value of absorbance, $\Delta A_{\text{max}}$ is the maximum absorbance at saturation, and $C$ is the concentration of free ligand in solution. Since the proportion of matrix components binding to the coat was low in all cases, the total concentration of the matrix component was taken as C. The molecular mass of PG-S2 used for the calculations was 74,600 Da (Franzén and Heinegård, 1984).

**RESULTS**

Samples of collagen I from tendon and collagen II from cartilage appeared pure when electrophoresed on sodium dodecyl sulphate-polyacrylamide gels, and also when gels were overloaded (results not shown).

Each of the collagen preparations formed fibrils at physiological pH and elevated temperature, in accordance with previously reported data (Vogel et al., 1984). To obtain optimal fibrillogenesis curves, it was necessary to have the initial concentrations and temperature individually set for each kind of collagen preparation. Therefore, the kinetics of turbidity development cannot be compared between the three different preparations of collagens tested.

**Purity of Collagens**

Ion-exchange chromatography in 2 M urea was used to determine whether any anionic proteins and proteoglycans, remaining in the collagen preparations, would be released under more dissociative conditions. Samples of pepsin-extracted collagens that were passed through DEAE-Sepharose contained no detectable amounts of material binding to the column. The preparation of acid-extracted collagen I contained small amounts (less than 0.1% as judged from measurements of absorbance at 206 nm) of material binding to the anion-exchanger. This minute material was not further analyzed. None of the collagens recovered after DEAE-cellulose chromatography could be induced to undergo fibrillogenesis. The same applied to control samples of collagen that had been dissolved at 42°C in the 2 M urea solution but never passed through the ion-exchanger. Apparently even the mildly denaturing conditions used irreversibly eliminated the capability of forming fibrils. Collagen chromatographed on DEAE-Sepharose could, however, be used in the solid-phase assay system discussed below. The amounts of 59-kDa protein or PG-S2 bound to coats of collagen I and II, thus purified, were equal to the amounts bound to urea-treated collagens where the ion-exchange step had been omitted (data not shown). In controls where collagen coats were incubated with buffer containing no matrix components, the nonchromatographed collagen showed the same low binding of antibodies as did the collagen recovered from DEAE-cellulose chromatography (data not shown). These results support the view that all collagen preparations had the same purity with regard to the 59-kDa protein and PG-S2. Coats of collagen previously exposed to the urea solution showed somewhat (10–25%) lower capacity for matrix protein binding compared with renatured collagen (data not shown). It is notable that the 59-kDa protein interacts well with denatured collagen. This fact demonstrates that dissociative conditions irreversibly alter the ability of collagen molecules to interact with each other but not necessarily their ability to interact with other molecules.

**Fibrillogenesis Assay**

*Acid-extracted Collagen I*—This fibrillogenesis system corresponds to those used in most studies of fibril formation *in vitro* (Gross and Kirk, 1958; Williams et al., 1978; Gelman et al., 1979a; Holmes et al., 1986). A typical turbidity curve is shown in Fig. 1 (No additive). It is characterized by three different phases, i.e. a lag period where there is no detectable change in turbidity, a growth phase representing a rapid turbidity change, and a plateau where again there is a constant level of turbidity. When examined by electron microscopy, the generated fibrils of collagen I have the same appearance as native fibrils (Wood, 1984), particularly distinct when positioned in a buffer containing TES (Williams et al., 1978). Pepsin-digested collagens do not readily form fibrils in the presence of TES. Therefore saline/phosphate buffer without TES was used for fibrillogenesis of the pepsin-extracted collagens. For comparison also fibrillogenesis of acid-extracted collagen I in this buffer was included in the study.

Presence of the 59-kDa protein remarkably inhibited the development of turbidity in saline/phosphate buffer containing TBS (Fig. 1). Small amounts of this protein, representing less than 5% (w/w) of the collagen, were sufficient to markedly delay the fibrillogenesis. At concentrations of the 59-kDa protein exceeding 20 μg/ml (molar ratio [matrix protein]/[collagen] exceeding 1) no turbidity increase occurred (data not shown). When samples with no added inhibitor had reached the plateau level of turbidity, usually 5–10% of the total collagen remained in solution when determined after centrifugation to remove precipitated collagen. When fibrils had been formed in the presence of 5 μg of 59-kDa protein, 15–18% of the total collagen remained in the supernatants.

In saline/phosphate buffer without TES, the 59-kDa protein was an efficient inhibitor of turbidity development (Fig. 1).

**Fig. 1.** Effect of the 59-kDa protein on fibrillogenesis of acid-extracted collagen I in saline/phosphate buffer containing 30 mM TES. A 100-μg portion of collagen and various amounts of the 59-kDa protein were combined in 1 ml of saline/phosphate buffer containing TES, equilibrated to 30°C. After mixing, absorbance at 400 nm was monitored at 3-min intervals.
the phosphate buffer showed a typical precipitation curve also inhibit fibrillogenesis of collagen I, two preparations developed (Fig. 3, top). The proportion of the collagen precipitated was 40%. The 59-kDa protein when added to the collagen gave very efficient inhibition of collagen fibrillogenesis, with only 13% of the collagen precipitated. This shows that the protein also binds to native collagen II and inhibits its fibrillogenesis.

To demonstrate that the protein binds to collagen II not containing telopeptides, and also inhibits fibrillogenesis of this preparation, collagen isolated after pepsin digestion of cartilage guanidinium chloride extraction residues was used (Fig. 3, bottom). This preparation, at 500 μg/ml in the saline/phosphate buffer, formed fibrils at 37 °C as shown by the increased turbidity. Only 10–15% of the collagen was incorporated in the precipitate. The 59-kDa protein strikingly decreased the rate of turbidity development. The amounts of precipitated collagen in the presence of 59-kDa protein, were 7–11% of the total.

The reason for the more restricted tendency of this collagen preparation to form fibrils is probably that preextraction of the cartilage with 4 M guanidinium chloride prior to collagen extraction causes denaturation of collagen molecules to various extents. As discussed above, even mildly denaturing conditions irreversibly alter the fibril-forming properties of collagen.

The result, however, shows that pepsin-extracted collagen II also binds the protein. We chose to use this preparation for subsequent experiments with collagen II, since the amount of neutral salt-extracted collagen II was very limited.

Combination of 59-kDa Protein and PG-S2—A series of fibrillogenesis experiments were designed to compare quant-

**FIG. 2. Effects of the 59-kDa protein on fibrillogenesis of collagen I, with and without telopeptides, in saline/phosphate buffer without TES.** Top, a 100-μg portion of acid-extracted collagen, i.e., containing telopeptides, was allowed to fibrillate in the presence and absence of 5 μg of the 59-kDa protein in 1 ml of saline/phosphate buffer at 30 °C. Absorbance at 400 nm was monitored at 3-min intervals. Bottom, a 300-μg portion of pepsin-extracted collagen was allowed to fibrillate in the presence and absence of 15 μg of the 59-kDa protein, using the buffers described above.

2, top). After fibrillogenesis in this buffer, less than 5% of the total collagen remained in solution. This was true also for samples containing up to 5 μg of 59-kDa protein.

Lowering the temperature to 4 °C in these systems after completion of fibril formation produced in all cases a loss of absorbance of less than 10%. Fibrils formed in the presence of 59-kDa protein appeared as stable when the temperature was lowered as those formed with no additives present. This implies that cross-linking through lysine-derived aldehydes is not inhibited (Gelman et al., 1979a).

**Pepsin-extracted Collagen I**—Treatment of collagen with pepsin removes the nonhelical telopeptide ends, while the triple helical region is left intact. The nonhelical parts of the collagen molecule seem to play a role in fibril formation, since their removal affects both the kinetics of assembly and the morphology of the product (Gelman et al., 1979b). However, at three times higher concentrations of pepsin-extracted collagen I in saline/phosphate buffer without TES, appropriate fibrillogenesis curves were generated at 30 °C.

Addition of 15 μg of 59-kDa protein to 300 μg of collagen I (5% w/w in this system efficiently inhibited turbidity development (Fig. 2, bottom). The amounts of collagen remaining in the supernatants after precipitation of fibrils was 9–11% of the total. These values were not affected by the presence of the 59-kDa protein.

**Collagen II**—To demonstrate the capacity of the protein to also inhibit fibrillogenesis of collagen II, two preparations were used, one with and one without telopeptides.

Collagen II molecules containing telopeptides were extracted from bovine fetal epiphyseal cartilage with sodium chloride and purified as described previously (Vogel et al., 1984). Fibrillogenesis assay with 200 μg/ml of the collagen in the phosphate buffer showed a typical precipitation curve (Fig. 3, top). The proportion of the collagen precipitated was 40%. The 59-kDa protein when added to the collagen gave very efficient inhibition of collagen fibrillogenesis, with only 13% of the collagen precipitated. This shows that the protein also binds to native collagen II and inhibits its fibrillogenesis.

To demonstrate that the protein binds to collagen II not containing telopeptides, and also inhibits fibrillogenesis of this preparation, collagen isolated after pepsin digestion of cartilage guanidinium chloride extraction residues was used (Fig. 3, bottom). This preparation, at 500 μg/ml in the saline/phosphate buffer, formed fibrils at 37 °C as shown by the increased turbidity. Only 10–15% of the collagen was incorporated in the precipitate. The 59-kDa protein strikingly decreased the rate of turbidity development. The amounts of precipitated collagen in the presence of 59-kDa protein, were 7–11% of the total.

The reason for the more restricted tendency of this collagen preparation to form fibrils is probably that preextraction of the cartilage with 4 M guanidinium chloride prior to collagen extraction causes denaturation of collagen molecules to various extents. As discussed above, even mildly denaturing conditions irreversibly alter the fibril-forming properties of collagen.

The result, however, shows that pepsin-extracted collagen II also binds the protein. We chose to use this preparation for subsequent experiments with collagen II, since the amount of neutral salt-extracted collagen II was very limited.

Combination of 59-kDa Protein and PG-S2—A series of fibrillogenesis experiments were designed to compare quan-

**FIG. 3. Effect of the 59-kDa protein on fibrillogenesis of collagen II, with and without telopeptides.** Top, a 200-μg portion of neutral salt-extracted collagen II, i.e., containing telopeptides, was allowed to fibrillate in the presence and absence of 10 μg of the 59-kDa protein in 1 ml of saline/phosphate buffer equilibrated to 37 °C. After mixing, absorbance at 400 nm was monitored at 3-min intervals. Bottom, a 500-μg portion of pepsin-extracted collagen II, i.e., without telopeptides, was allowed to fibrillate in the presence and absence of 25 μg of the 59-kDa protein, using the buffer described above.
titatively the effects of the 59-kDa protein, PG-S2, and mixtures of the two components. At suitably low concentrations of added components (molar ratio [total added components]/[collagen] ~1:7) in the assay system with acid-extracted collagen I, a mixture of about equal proportions of 59-kDa protein and PG-S2 inhibited the turbidity increase more efficiently than did either component alone at corresponding molar ratios of inhibitor (Fig. 4). Similar observations were made for fibrillogensis of collagen II (Fig. 5).

Solid-phase Assay

The assay based on passive binding of collagen to plastic surfaces was developed for simple, quantitative measurements of protein/proteoglycan binding. The system is less complex than that of fibrillogensis, where the interactions studied also include those between collagen molecules. On the other hand the properties of coated molecules may differ from those in physiological conditions and in solution. The coating efficiency and the properties of the coated molecules may vary with coating conditions, particularly if the molecules are able to self-interact. Therefore, when comparing binding capacities between different components, it is preferable to use a single type of cost and vary the composition of the ligand solution.

It was found advantageous to use neutral rather than weakly acidic solutions of collagen when coating the plates. Generally binding of components was more pronounced after coating at physiological pH. This was probably due to a larger degree of collagen binding to the plastics. In addition assembly of collagen molecules could have a role, even if the collagen concentrations are low (10 μg/ml). Both the configuration and the number of sites on collagen available for interaction could thus depend on the coating conditions.

When collagen coats had been incubated with the 59-kDa protein (1 μg/ml), binding could be easily observed (Fig. 6). This was true for each of the preparations of collagen used. Controls representing nonspecific binding, omitting the 59-kDa protein or the collagen, showed low values of absorbance. Similarly, binding of PG-S2 (at 2 μg/ml) to collagen coats was demonstrated (Fig. 7). Although the titer of the first antibody used was comparably high, these samples showed lower amounts of bound antibodies. This may indicate that sites for proteoglycan binding appear sparsely in the coats, or perhaps that a significant proportion of epitopes on the proteoglycan core protein are hidden when the molecule is bound to collagen.

In further experiments, coats of acid-extracted collagen I were incubated with solutions of matrix components at several concentrations. Binding of PG-S2 core protein was tested separately by using a solution of PG-S2 incubated with chondroitinase ABC (0.05 units/mg sample) for 60 min at 37 °C prior to binding assay. Curves illustrating binding saturation were obtained (Fig. 9). After subtraction of the low absorbance values corresponding to coats incubated with buffer containing no matrix components, these data were used for Scatchard-type plots (Fig. 9). The good correlations with straight lines suggest that this treatment of the data is appropriate. The linear nature of the plots also demonstrates the relative absence of lower affinity binding sites. It seems reasonable to assume that ΔA (see legend to Fig. 9) is proportional to fractional saturation, which is true when there is only a single class of sites. Thus, according to the data on binding of PG-S2 and the 59-kDa protein to coats of collagen, in each case

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![Fig. 4](image-url) The combined effects of the 59-kDa protein and PG-S2 on fibrillogenesis of acid-extracted collagen I. A 100-μg portion of collagen and various amounts of the 59-kDa protein and PG-S2 were combined in 1 ml of saline/phosphate buffer containing 30 mM TES, at 30 °C. Absorbance was monitored at 3-min intervals.

![Fig. 5](image-url) The combined effects of the 59-kDa protein and PG-S2 on fibrillogenesis of pepsin-extracted collagen II. A 500-μg portion of collagen and various amounts of the 59-kDa protein and PG-S2 were combined in 1 ml of saline/phosphate buffer, at 37 °C. Absorbance was monitored at 3-min intervals.

![Fig. 6](image-url) Binding of the 59-kDa protein to insolubilized collagens. Microtiter plates, coated with collagen (10 μg/ml at pH 7.3), were incubated overnight at 20 °C with samples of the 59-kDa protein separated by affinity-purified antiserum, anti-(rabbit IgG) antibodies conjugated to alkaline phosphatase, and p-nitrophenyl phosphate. Formation of product was measured as increase of absorbance at 405 nm.
FIG. 7. Binding of PG-S2 to insolubilized collagens. Microtiter plates, coated with collagen (10 µg/ml at pH 7.3), were incubated with samples of PG-S2 at 2 µg/ml in saline/phosphate buffer containing 0.05% (v/v) of Tween 20. Bound proteoglycans were detected by an enzyme-linked immunosorbent assay (see legend to Fig. 6).

FIG. 8. Binding capacity of acid-extracted collagen I for the 59-kDa protein and PG-S2. Collagen-coated wells were incubated with samples of the 59-kDa protein (top) or PG-S2 (bottom) at various concentrations, in saline/phosphate buffer containing 0.05% (v/v) of Tween 20. Analogously, binding to wells solely coated with bovine serum albumin, was measured. Proteoglycan solution was used either directly or after addition of chondroitinase ABC (0.05 units/mg of sample) and preincubation for 60 min at 37 °C, to yield equivalent amounts of core protein. ●, 59-kDa protein or PG-S2 bound to collagen coat; ○, PG-S2 core protein bound to collagen coat; ■, 59-kDa protein or PG-S2 bound to control coat; □, PG-S2 core protein bound to control coat.

FIG. 9. Titration data from Fig. 8 expressed as logarithmic and Scatchard-type plots. Absorbance-values of binding to control coats, subtracted from those measured for binding to collagen coats, were used to calculate specific absorbance change (ΔA). These values were expressed as logarithmic (a and c) and Scatchard-type (b and d) plots. Straight lines were fitted for Scatchard-plotted data on binding of the 59-kDa protein (b) and PG-S2 (d), by applying the least square method. Dissociation constants, calculated from the slopes of the lines, were 35 nM for the 59-kDa protein (R² = 0.96), 16 nM for PG-S2 (R² = 0.97), and 12 nM for PG-S2 core protein (R² = 0.97). ●, 59-kDa protein or PG-S2; ○, PG-S2 core protein.

there is one type of binding site to which almost all the bound molecules are tied. Alternatively there may be different types of binding sites having very similar dissociation constants. The dissociation constants calculated from the Scatchard-type plots in Fig. 9 were 16 nM for PG-S2, 12 nM for PG-S2 treated with chondroitinase ABC, and 35 nM for the 59-kDa protein.

Attempts were made to test the ability of one component to influence the binding of another in this system. Coats of collagen were incubated with solutions containing both the 59-kDa protein and PG-S2. PG-S2, when present at the high concentrations required for binding saturation (see Fig. 8), appeared to decrease binding of the 59-kDa protein (data not shown). However, control samples showed that high concentrations of bovine serum albumin, chondroitin sulfate, or large proteoglycans also affected binding as measured in this assay. Furthermore, the scatter of experimental data made the interpretation uncertain. Competition studies should probably be carried out by using a more direct method for the detection of bound components.

In addition, the ability of PG-S2 to bind to the 59-kDa protein coated on plastics, and vice versa, was tested using the described techniques. No interaction between the two components was demonstrated.

DISCUSSION

Information that supports the view that non-collagenous matrix proteins interact with collagen molecules and influence the organization of fibrils is limited (Piez, 1984). The best studied example of a collagen-binding protein is fibronectin. This large glycoprotein, present on surfaces of cells, promotes the attachment of fibroblasts to collagen. The binding site is
Located in a distinct region on the α1(I) chain (Dessau et al., 1978). The presence of fibronectin affects the rate of collagen fibril formation in vitro (Kleinman et al., 1981). Link proteins, present in cartilage and a number of other connective tissues, although primarily associated with proteoglycan aggregate formation (see Heinigård and Sommarin, 1987), appear in combination with proteoglycans to modulate collagen fibril formation in vitro (Chandrasekhar et al., 1984). A protein with the ability to bind to collagen II has been isolated from Swarm rat chondrosarcoma (Chandrasekhar et al., 1986). It is also capable of interfering with collagen self-assembly, but only in the presence of proteoglycans. The 54-kDa subunit of the CMP cartilage matrix protein is made up of two large domains of homologous amino acid sequences (Argraves et al., 1987), which also have been found in other proteins where they have been ascribed a collagen-binding activity (Argraves et al., 1987; Pytela, 1988).

A glycoprotein of similar size to the 59-kDa protein, also having high contents of leucine and acidic amino acid residues, has been identified in MgCl₂ extracts of tendon (Anderson, 1975). Interestingly this glycoprotein appears to affect collagen I fibrillogenesis in vitro (Anderson et al., 1977). It may represent a protein related to the 59-kDa protein.

Among techniques for the examination of the functional aspects of purified matrix components, the fibrillogenesis assay is very efficient in demonstrating interactions with collagen. During the process, collagen molecules at various levels of organization are exposed to the added component. Interference at any level of association can be sensitively monitored as an altered turbidity curve.

The shape of this curve is determined by a multistep process (Gelman et al., 1979a). During the lag-phase longitudinal growth predominates and the collagen appears as thin filaments. The increase in turbidity is associated with lateral assembly of filaments. The final absorbance level, reached at stability of collagen organization, is a result of the rates and durations of these two processes. Under certain conditions linear and lateral growth proceed simultaneously (Holmes et al., 1986). Stability is then reached faster.

Extremely low proportions of 59-kDa protein (0.2 µg/ml; 2 µg/mg collagen) decreased the final level of turbidity (Fig. 1). At somewhat higher proportions of the protein, the duration of the lag-phase was increased but the slope of the curve was constant. This probably indicates that the protein interaction interferes with early events of the lag-phase. At concentrations of 59-kDa protein exceeding 1 µg/ml (10 µg/ml collagen), a more general inhibition of turbidity development is seen. The concentration-dependent decrease of the turbidity change obviously reaches a maximum at molecular proportions [matrix protein]/[collagen] ~ 1:1, suggesting this ratio to be the stoichiometry of the reaction.

In this type of assay the measured turbidity depends on several parameters, e.g. quantity and structure of formed collagen fibrils. The recorded absorbance gives no direct information on the nature of formed fibrils, as pointed out previously (Vogel et al., 1984). Therefore, to estimate effects on the proportions of collagen incorporated into fibrils, measurement of hydroxyproline was included in the study. These values were affected much less by the presence of the 59-kDa protein than were the values of turbidity. This shows that fibrils with different optical properties, and thus dimensions, are formed in the presence of the 59-kDa protein.

Specific inhibition of collagen fibrillogenesis, similar to the effects of the 59-kDa protein discussed here, has previously been reported for the PG-S2 small dermatan sulfate proteoglycan of tendon (Vogel et al., 1984). In this case also the inhibition of turbidity development was more pronounced than the decrease of collagen incorporation into fibrils. Morphologically the fibrils formed in the presence of PG-S2 are significantly thinner, as revealed by electron microscopy (Vogel and Trotter, 1987). It remains to be shown if fibrils formed in the presence of 59-kDa protein are thinner.

The solid-phase assay allows reliable measurement of binding capability. In this assay system, the focus is on binding of protein to collagen, while the complex interplay between collagen molecules at various stages of aggregation should not play a role. Thus, binding to collagen I and II was demonstrated for both the 59-kDa protein and PG-S2. This corroborates observations using the fibrillogenesis assay that indicates binding to early stages in fibril formation. Titration experiments were done to obtain estimates of the dissociation constants. For this purpose a fluid-phase assay would be theoretically ideal. If the total amount of collagen in the system was known, the number of binding sites/collagen molecules could be estimated. Using data obtained with the solid phase assay, however, we could calculate some characteristics of the interactions. The dissociation constants for the binding of either molecule to collagen were very similar. On the basis of these data it is not possible to conclude whether the 59-kDa protein and PG-S2 bind to collagen by different mechanisms. Unfortunately experiments in which costs were incubated with mixtures of the components did not provide further information. Maybe a clue was provided by fibrillogenesis experiments in which the two components were present together. Thus, a mixture of the two components inhibited fibrillogenesis more efficiently than did either of them alone at corresponding molar concentrations (Figs. 4 and 5). One possible explanation is synergism of two different interactions.

The present results do not provide assessments of the exact specificities of the described interactions and the location of the interacting sites within the molecules. The results, however, indicate that in neither case do the nonhelical regions of collagen molecules participate in the binding. The observations accounted for here, made in vitro, suggest that the 59-kDa protein is one of the factors that regulate collagen organization in the tissue.

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REFERENCES
Collagen-binding Connective Tissue Matrix Components