Interactions between Chondroitin Sulfate Proteoglycan, Fibronectin, and Collagen

Received for publication, July 21, 1981

Ake Oldberg and Erkki Ruoslahti
From the Cancer Research Center, La Jolla Cancer Research Foundation, La Jolla, California 92037

A proteoglycan isolated from a rat yolk sac tumor and characterized as a chondroitin sulfate proteoglycan with a smaller amount of dermatan sulfate was studied with respect to complex formation with collagen and fibronectin. The proteoglycan co-precipitated with native collagen from neutral salt solutions at 6°C and 37°C. Addition of fibroin to such precipitation mixtures resulted in incorporation of fibronectin to the precipitate. Treatment of the proteoglycan with alkali to separate the glycosaminoglycan chains from the protein part and digestion of the protein part with papain greatly reduced the capacity of the proteoglycan to precipitate collagen and fibronectin. A defined extracellular matrix as represented by the complexes of collagen, proteoglycan, and fibronectin constructed here may be useful for studies on the biological effects of extracellular matrices. The multiple interactions of matrix macromolecules exemplified by these results may play a role in the formation of extracellular matrices and in the maintenance of their integrity.

It has been known for several years that proteoglycans and glycosaminoglycans interact with collagen (1-3). More recently it has become evident that other interactions between the components of extracellular matrix and basement membranes also occur. Fibronectin, a high molecular weight cell surface and extracellular matrix glycoprotein (4, 5), interacts with heparin, heparan sulfate, and hyaluronic acid (6-9), and with collagen (10, 11). Laminin, a newly discovered basement membrane glycoprotein (12, 13), also interacts with glycosaminoglycans (14).

It was recently demonstrated that glycosaminoglycans enhance the binding of fibronectin to collagen and that complexes formed by these components are insoluble (7, 15-17). Furthermore, it has been shown that fibronectin can be cross-linked to chondroitin sulfate proteoglycans at the cell surface of cultured fibroblasts (18). Based on these observations, it was postulated that extracellular matrix may be formed through self-assembly caused by multiple interactions of the matrix components and that proteoglycans, through their ability to interact with several of the other components, could play a central role in such self-assembly (4, 15). Since the studies leading to this model were primarily conducted with glycosaminoglycans and these, with the possible exception of hyaluronic acid, exist in tissues as proteoglycans in which the glycosaminoglycan chains are covalently linked to proteins (19), we thought it important to study the effect of proteoglycans on the interaction of collagen and fibronectin.

We have recently purified a proteoglycan from a rat yolk sac tumor (20). This proteoglycan has 5% protein and contains mainly chondroitin sulfate side chains with some dermatan sulfate. We report here on the interaction of this proteoglycan with collagen and its ability to co-precipitate with collagen and fibronectin.

EXPERIMENTAL PROCEDURES

Materials

Proteoglycan—The purification of the proteoglycan from ascites fluid of a rat yolk sac tumor (21) and its characterization as a chondroitin sulfate proteoglycan have been described elsewhere (20). Fibronectin and Collagen—Fibronectin was purified from rat or human plasma by affinity chromatography on gelatin-Sepharose (22). Rat tail tendon collagen was extracted with pepsin and purified by salt fractionation (23). Commercial calf skin collagen (Sigma type III) and human type I collagen, kindly provided by Dr. E. J. Miller, University of Alabama, were also used. Gel electrophoresis in the presence of sodium dodecyl sulfate and 2-mercaptoethanol revealed the typical polypeptides of type I collagen as the only components detected in these collagens. Papain (2 x crystallized) was from Sigma. The heparin preparation used was kindly provided by Dr. U. Lindahl, Swedish University of Agricultural Sciences, Uppsala, Sweden. Its characteristics have been described (24).

Methods

Uronic acid and protein were determined according to Bitter and Muir (25) and Lowry et al. (26), respectively. [125I] radioactivity was measured in a Beckman Gammma 4000 counter. Iodination of fibronectin, collagen, and proteoglycan was performed with the chloramine-T method as described (27). After iodination, the [125I]-fibroinectin and [125I]-proteoglycan were reisolated by chromatography on gelatin-Sepharose (22) and DEAE-cellulose (20), respectively. The [125I]-labeled rat and human fibronectins gave a single major band co-migrating with the corresponding purified plasma fibronectins in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The iodinated collagen was extensively dialyzed against 0.2 M acetic acid.

Digestion of proteoglycan with papain was carried out as previously described (28). Alkali treatment of the proteoglycan was performed in 0.5 M NaOH, 0.1 M NaBH4, for 24 h at room temperature (20).

Precipitation of Collagen, Fibronectin, and Proteoglycan—A method similar to that of Jilek and Hörnmann (16) was used. To study precipitation of collagen, indicated amounts of proteoglycan or glycosaminoglycan in 50 µl were mixed with 50 µl of [125I]-collagen solution (25 µg of collagen and 2 x 10⁶ cpm). Subsequently, 50 µl of buffer or 50 µl of buffer containing 10 µg of fibroinectin were added. Finally, 50 µl of a 1 mg/ml solution of bovine serum albumin were added to make an incubation volume of 200 µl. All solutions were made in NaCl/P, 1. The abbreviation used is: NaCl/P, phosphate-buffered saline (137 mM NaCl, 1.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.3). After incubation at 6°C or 37°C for 16-18 h, the samples were centrifuged at 2,000 × g for 15 min. The precipitates were washed once with 1 µl of 1/5 saturated ammonium sulfate and centrifuged at 2,000 × g for 10 min, and the radioactivity in the precipitates was determined. Centrifugation of the supernatants of 20,000 × g for 15 min did not substantially increase the total amount of radioactivity precipitated in these assays. Initial experiments also showed that the...
amount of radioactivity precipitated reached a maximum at 6 h and did not change thereafter when followed for 48 h. When denatured collagen was used, the collagen solution was heated to 56 °C for 1 h before mixing with proteoglycan or glycosaminoglycan.

To study the precipitation of fibronectin, indicated amounts of proteoglycan or glycosaminoglycan in 50 ml were mixed with 50 ml of buffer containing 25 μg of c-251 labeled collagen. Subsequently, 100 μl of solution containing 2.5 x 10^4 cpm of 125I-fibronectin and 10 mg/ml of bovine serum albumin were added. Similarly, to quantify the precipitation of proteoglycan, varying amounts of 125I-labeled proteoglycan were incubated in the presence of collagen and/or fibronectin. All solutions were in NaCl/P1, and the incubation mixtures were further treated as described above for precipitation of 125I-collagen, except that 1 ml of distilled water was used for the wash.

RESULTS

Precipitation of Collagen-Proteoglycan Complexes

Collagen Precipitation—To study proteoglycan-(glycosaminoglycan)-collagen-fibronectin interactions in solution, we first established the ability of the proteoglycan or glycosaminoglycan to precipitate 125I-labeled collagen. In order to dissociate the effects of collagen fibril formation from the effects of proteoglycan-collagen interaction, the experiments were carried out at 6°C unless otherwise specified. When proteoglycan was added to a solution of 125I-collagen, up to 70% of the collagen was insolubilized. The results were found to be independent of the species of origin of the collagen used; therefore, only the results for the calf skin collagen are shown in Fig. 1. At the optimal concentration for precipitation, the ratio of proteoglycan to collagen added to the reaction was about 2:1 by weight. Experiments carried out at 37°C gave results similar to those shown above for 6°C. Some insolubilization of 125I-collagen was observed at 37°C without added proteoglycan (Table I). This was probably due to formation of some insoluble collagen fibrils, even at the low concentrations used. Gels were obtained when the collagens were incubated at neutral pH at 37°C using concentrations of 1 mg/ml.

Effect of Chemical Modification of Proteoglycan—The collagen-precipitating activity of the proteoglycan was greatly reduced by treatment with alkali as shown in Fig. 1B. A similar reduction was seen after treatment of the proteoglycan with papain (not shown). The presence of fibronectin did not cause any significant increase in the amount of collagen precipitated in the presence of the intact or alkali-treated proteoglycan.

Precipitation of Collagen by Heparin—Heparin was 3–4 times more effective than the proteoglycan, based on the amount of uronic acid, in precipitating 125I-collagen (Fig. 1C). In the presence of fibronectin, the amount of insolubilized 125I-collagen was increased by about 20% at all concentrations of heparin.

Precipitation of Denatured Collagen—Denatured (56°C, 1 h) 125I-collagen did not precipitate when incubated with intact or papain-digested proteoglycan, and precipitated only slightly with heparin (Fig. 2). In the presence of fibronectin, the proteoglycan induced some insolubilization of denatured 125I-collagen. Fibronectin also caused a 2- to 3-fold increase in the amount of denatured 125I-collagen precipitated with heparin.

Proteoglycan Precipitation—Assays where 125I-labeled proteoglycan was mixed with unlabeled proteoglycan and collagen showed that some of the proteoglycan became incorporated in the precipitate (Fig. 3). The amount incorporated was not affected by the presence of fibronectin. Estimated from the amount of radioactivity in the precipitates, 4 μg of proteoglycan was insolubilized at the highest concentration of proteoglycan. This corresponded to the precipitation of 60% of the 25 μg of collagen as demonstrated in parallel assays with labeled collagen (see Fig. 1). Since the precipitated proteoglycan represented only a minor portion (1–2%) of the proteoglycan added to the precipitation mixture, we determined whether the proteoglycan remaining in the supernatant had retained the capacity to co-precipitate with collagen. It was found that addition of collagen to the supernatant of a previous precipitation reaction resulted in the insolubilization of an amount of radioactivity equivalent to that insolubilized in the first precipitation.

Incorporation of Fibronectin to Collagen-Proteoglycan Complexes

Precipitation of 125I-fibronectin—Proteoglycan, in the presence of collagen, induced insolubilization of 125I-fibronectin.
Proteoglycan and Fibronectin-Collagen Complexes

A slight increase in the insolubilization of the $^{125}$I-fibronectin over the background value was obtained with collagen alone, but this was greatly enhanced in the presence of proteoglycan or heparin. Neither one of these substances alone caused insolubilization of the $^{125}$I-fibronectin. At an optimal concentration of proteoglycan, 40–60% of the radioactive $^{125}$I-fibronectin was recovered in the precipitate in the presence of collagen. Only 2–5% precipitated in the absence of collagen. Addition of collagen to the supernatant from an experiment where 57% of the fibronectin radioactivity had precipitated resulted in precipitation of an additional 15% of the radioactivity, bringing the total radioactivity precipitable in this way to 72%. When $^{125}$I-IgG was used as a control, no radioactivity over the background level was found in the proteoglycan or heparin-induced collagen precipitates.

Alkali treatment of the proteoglycan abolished most of its activity in this assay also. Collagen precipitates obtained with heparin incorporated substantially less $^{125}$I-fibronectin than the proteoglycan-collagen complexes. Experiments carried out at 37 °C gave qualitatively similar results, but the amounts of fibronectin insolubilized were lower than at 6 °C (Table II).

Composition of Fibronectin-Proteoglycan-Collagen Complexes—The amount of fibronectin that could be incorporated into a precipitate of native collagen and proteoglycan was determined by adding increasing amounts of unlabeled rat fibronectin to a mixture of $^{125}$I-labeled rat fibronectin (about 10 ng of protein), 25 μg of rat tail collagen, and 50 μg of proteoglycan. The insolubilization of $^{125}$I-fibronectin was inhibited 50% from the maximal value by 3 μg of fibronectin, indicating that 1.5 μg of fibronectin was incorporated (Fig. 5). Based on the information described above on the precipitation of collagen and proteoglycan, the precipitate under these conditions contained about 15 μg of collagen, 0.8 μg of proteoglycan, and 1.5 μg of fibronectin. Similar results were obtained when increasing amounts of $^{125}$I-labeled human fibronectin were added.
Proteoglycan and Fibronectin-Collagen Complexes

Table II

| Insolubilization of $^{125}$I-fibronectin in the presence of rat type I collagen and proteoglycan at different temperatures |
|-------------------------------------------------------------------------------------------------------------------|
| $^{125}$I-labeled rat plasma fibronectin (35,000 cpm) was incubated for 16 h at the indicated temperatures in the presence of proteoglycan and/or rat tail collagen as described under "Methods." The percentage of $^{125}$I-fibronectin insolubilized was determined after correction for the amount of $^{125}$I-fibronectin insolubilized after incubation in NaCl/P.
| $^{125}$I-fibronectin insolubilized (%) |
| $4^\circ\text{C}$ | $23^\circ\text{C}$ | $37^\circ\text{C}$ |
| Proteoglycan (58 $\mu$g of uronic acid/ml) | 0 | 2 | 0 |
| Collagen (125 $\mu$g/ml) | 0 | 0 | 11 |
| Proteoglycan and collagen | 42 | 27 | 27 |

![Graph](description of graph)

**Fig. 5**. Inhibition of precipitation of $^{125}$I-rat fibronectin by rat fibronectin. $^{125}$I-fibronectin (approximately 10 $\mu$g, 50,000 cpm) was incubated with 25 $\mu$g of rat tail collagen, proteoglycan (12 $\mu$g of uronic acid), and unlabeled rat fibronectin. The fibronectin was diluted in steps of five from a starting amount of 108 $\mu$g.

Labeled to a low specific activity (3.63 x $10^6$ cpm/mg) were added to a mixture of 5 $\mu$g of calf skin collagen and 10 $\mu$g of proteoglycan. The precipitate formed was found to contain 0.3 $\mu$g of fibronectin at saturation.

**Discussion**

The availability of the purified proteoglycan from yolk sac tumor has made it possible to explore its biological properties. We decided to study the interaction of the proteoglycan with collagen and fibronectin because of the possible importance of such interactions in the formation of extracellular matrix (15). Our proteoglycan precipitated collagen, as has been previously shown for chondroitin sulfate-containing proteoglycans derived from cartilage (1-3).

The proteoglycan studied in the present work became much less active in precipitating collagen when the glycosaminoglycan chains were separated from protein by alkali treatment or when the protein part was destroyed by papain. The residual activity was probably due to the dermatan sulfate, since this glycosaminoglycan has a higher charge density than chondroitin sulfate (19), and high charge density has been shown to be important for interaction with collagen (29). Multiple interactions made possible by the presence of several glycosaminoglycan chains in the same molecule may be needed for effective interactions with collagen, and studies on glycosaminoglycans may give a misleading impression on their activities as proteoglycans. The proteoglycan alone did not affect the solubility of fibronectin, but fibronectin was insolubilized in the presence of proteoglycan and collagen.

Previous results have shown that the interaction of fibronectin and native collagen is weak (10, 16), but Kleinman et al. (30) have shown that fibronectin becomes incorporated in collagen fibrils formed in the presence of fibronectin. This was also apparent from our results. Significant amounts of the labeled fibronectin were insolubilized with collagen at temperatures where collagen fibril formation takes place. However, there was more precipitation of collagen and fibronectin in the presence of the proteoglycan under such conditions, and in the absence of spontaneous insolubilization of collagen, proteoglycan was essential for the precipitation of collagen and fibronectin. The insolubilization of collagen, fibronectin, and especially of proteoglycan was not complete; a large fraction of each component remained in solution. This is reminiscent of the situation in cell culture, where a large fraction of matrix components is found soluble in the culture media. Moreover, our proteoglycan is found in a soluble form in the ascites formed by the tumor that produces it (20). The inefficiency of the incorporation of the matrix components into insoluble structures may be due to low affinities of the interactions that lead to the formation of the complexes. This may be why more fibronectin was incorporated into the collagen-proteoglycan complexes in the cold (Table II), as the low temperature should favor low affinity interactions.

More fibronectin was incorporated into complexes of proteoglycan and native collagen than into similar heparin-induced complexes, and denatured collagen was precipitated only when both fibronectin and proteoglycan or heparin were present. These results suggest that the glycosaminoglycans and/or proteoglycan produced by a given cell as well as the amount of fibronectin produced may determine the composition and quantity of extracellular matrix deposited by the cell.

The proteoglycan-collagen-fibronectin complexes constructed here represent a defined extracellular matrix. Such matrices should prove useful for the understanding of the formation and biological properties of extracellular matrices. The three components we have used are likely to be present at the cell surface under conditions that are approximated by our model. Plasma fibronectin used in these experiments is abundant in vivo and is known to become incorporated into extracellular matrices in vivo (31) and in vivo (32). We also have immunological evidence for the presence of the chondroitin sulfate proteoglycan at the surface of all types of cells studied so far. However, since we used type I collagen truncated upon its solubilization from tissues by pepsin treatment, the model, when it comes to collagen, can only approximate the situation in extracellular matrices.

With the above reservations in mind, it seems reasonable to propose that proteoglycan-induced self-assembly of insoluble collagen-fibronectin-proteoglycan complexes could, along with collagen fibril formation, be of basic importance for the formation of extracellular matrix.

Another aspect of interest is the possible utility of the proteoglycan-collagen-fibronectin complexes as defined

1. A. Oldberg, E. G. Hayman, E. Ruoslahti, and C. Birdwell, unpublished results.
models for extracellular matrices. Matrices isolated from different tissues have been found to have profound effects on cellular growth and differentiation (33, 34). We have recently begun to study the effects of defined matrices on cellular behavior.

Acknowledgments—We thank Drs. Ulla Wewer and Reidar Albrechtsen for the rat yolk sac tumor cell line, and Drs. E. J. Miller and Eva Engvall for a collagen sample and helpful discussions.

REFERENCES

1. Greenwald, R. A., Schwartz, C. E., and Canlor, J. O. (1975) Bioch. J. 145, 601-605
2. Toole, B. P. (1976) J. Biol. Chem. 251, 885-897
3. Oegema, T. R., Laidlaw, J., Hascall, V. C., and Dziewiatkowski, D. D. (1975) Arch. Biochem. Biophys. 170, 698-709
4. Ruoslahti, E., Hayman, E., and Engvall, E. (1981) Collagen Relat. Res. 1, 95-128
5. Mosher, D. F. (1980) Prog. Hemostasis Thromb. 5, 111-151
6. Statidakis, N. E., and Mosesson, M. W. (1977) J. Clin. Invest. 60, 855-865
7. Ruoslahti, E., Pekkala, A., and Engvall, E. (1979) FEBS Lett. 107, 51-54
8. Hayashi, M., Schlesinger, D. H., Kennedy, D. W., and Yamada, K. M. (1980) J. Biol. Chem. 255, 10017-10020
9. Laterra, J., Arnbacher, R., and Culp, L. A. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 6692-6696
10. Engvall, E., Ruoslahti, E., and Miller, E. J. (1978) J. Exp. Med. 147, 1584-1596
11. Dessau, W., Adelmann, B. C., Timpl, R., and Martin, G. R. (1978) Biochem. J. 169, 55-69
12. Chung, A. E., Jaffe, R., Freeman, I. L, Vergnes, J.-P., Braginski, J. E., and Carlin, B. (1979) Cell 16, 277-287
13. Timpl, R., Rhode, H., Robey, P. G., Rennard, S. I., Foidart, J.-M., and Martin, G. R. (1979) J. Biol. Chem. 254, 9933-9937
14. Sakashita, S., Engvall, E., and Ruoslahti, E. (1980) FEBS Lett. 116, 243-246
15. Ruoslahti, E., and Engvall, E. (1980) Biochim. Biophys. Acta 631, 350-358
16. Jilek, F., and Hormann, H. (1979) Hoppe-Seyler’s Z. Physiol. Chem. 360, 587-603
17. Johansson, S., and Höök, M. (1980) Biochem. J. 187, 521-524
18. Perkins, M. E., Ji, T. H., and Hynes, R. O. (1979) Cell 16, 941-952
19. Roden, L. (1980) in The Biochemistry of Glycoproteins and Proteoglycans (Lennarz, W. J., ed) pp. 267-371, Plenum Publishing Co., New York
20. Oldberg, A., Hayman, E. G., and Ruoslahti, E. (1981) J. Biol. Chem. 256, 10847-10852
21. Wewer, U., Albrechtsen, R., and Ruoslahti, E. (1981) Cancer Res. 41, 1518-1524
22. Engvall, E., and Ruoslahti, E. (1977) Int. J. Cancer 20, 1-5
23. Chung, E., and Miller, E. J. (1974) Science (Wash. D. C.) 183, 1200-1201
24. Kjellen, L., Oldberg, Å., and Höök, M. (1980) J. Biol. Chem. 255, 10407-10413
25. Bitter, T., and Muir, H. M. (1962) Anal. Biochem. 4, 330-334
26. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
27. Ruoslahti, E., Vuonto, M., and Engvall, E. (1978) Biochim. Biophys. Acta 534, 210-218
28. Oldberg, Å., Höök, M., Öbrink, B., Pertot, H., and Rubin, K. (1977) Biochem. J. 164, 75-81
29. Lindahl, U., and Höök, M. (1978) Annu. Rev. Biochem. 47, 385-417
30. Kleinman, H. K., Wilkes, C. M., and Martin, G. R. (1981) Biochemistry 20, 2325-2330
31. Hayman, E. G., and Ruoslahti, E. (1979) J. Cell Biol. 83, 255-259
32. Oh, E., Fierschbacher, M., and Ruoslahti, E. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 3218-3221
33. Weiss, R. E., and Reddi, A. H. (1981) J. Cell Biol. 88, 630-636
34. Rojkind, M., Gatmaitan, Z., Mackensen, S., Giambrone, M.-A., Ponce, P., and Reid, L. M. (1980) J. Cell Biol. 87, 255-263
Interactions between chondroitin sulfate proteoglycan, fibronectin, and collagen.
A Oldberg and E Ruoslahti

*J. Biol. Chem.* 1982, 257:4859-4863.

Access the most updated version of this article at [http://www.jbc.org/content/257/9/4859](http://www.jbc.org/content/257/9/4859)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/257/9/4859.full.html#ref-list-1](http://www.jbc.org/content/257/9/4859.full.html#ref-list-1)