

## Binding of the basement-membrane glycoprotein laminin to glycosaminoglycans

### An affinity-chromatography study

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The binding of the basement-membrane glycoprotein laminin to glycosaminoglycans (aggregating and non-aggregating subsets of heparan sulphates and dermatan sulphates, as well as heparin, chondroitin sulphates and hyaluronic acid) was studied by affinity chromatography. Partially periodate-oxidized chains of glycosaminoglycans were coupled to adipic acid dihydrazide-substituted agarose. Co-polymeric glycosaminoglycans reveal high affinity for laminin, whereas hyaluronic acid does not. Competitive-release experiments indicate that glycosaminoglycans share a common binding site on the laminin molecule.

The basal lamina, a complex extracellular material that closely conforms to the contours of the basal surface of epithelial cells, is believed to participate in maintaining the morphology of normal epithelial organs. Collagenous and non-collagenous glycoproteins and proteoglycans constitute both basement membranes and pericellular matrix (Kefalides, 1978). A four-stranded non-collagenous glycoprotein composed by two disulphide-bonded polypeptide chains (mol.wt. 200 000–440 000) isolated from a basement-membrane-producing mouse tumour, the Engelbreth-Holm-Swarm sarcoma (Timpl *et al.*, 1979), has been named laminin. Antibodies against laminin react with a variety of basement membranes (eye, blood vessels, kidney, skin), suggesting that laminin is a basement-membrane-specific glycoprotein (Chung *et al.*, 1979; Foidart *et al.*, 1980). Kanwar & Farquhar (1979) have demonstrated the presence of heparan sulphate in the glomerular basement membrane, and the isolation from basal lamina of a heparan sulphate-rich proteoglycan has been reported by Gordon & Bernfield (1980) and Hassel *et al.* (1980). Laminin binds to heparin and heparan sulphate (Sakashita *et al.*, 1980), indicating that this interaction could be involved in the organization of basement membrane itself. Evidence has accumulated about the interactions among fibronectin, collagens and glycosaminoglycans (Engvall & Ruoslahti, 1977; Jilek & Hormann, 1979; Stathakis & Mosesson, 1977) suggesting that the properties of pericellular matrix might ensue from such interactions. Looking to basement membrane as to a structurally ordered array of pericellular ground substance, we studied by affinity chromatography the molecular interactions between

laminin and glycosaminoglycans, which could play an important role in ordering complex macromolecules of pericellular matrices.

### Experimental

#### *Sources of glycosaminoglycans coupled to agarose*

Aggregating and non-aggregating dermatan sulphates (from pig skin), aggregating and non-aggregating heparan sulphates (from heparin by-products), chondroitin 6-sulphate (from human nucleus pulposus) and chondroitin 4-sulphate (from bovine nasal cartilage) were kindly provided by Dr. Lars Åke Fransson (Department of Physical Chemistry II, University of Lund, Lund, Sweden). Self-aggregating subfractions of heparan sulphate, referred to as heparan sulphate I and heparan sulphate II, were obtained from the total pool of heparin by-products by stepwise precipitation with cetylpyridinium chloride in the presence of decreasing concentrations of NaCl, as described in detail by Fransson *et al.* (1980). Hyaluronic acid was of commercial origin (Sigma Chemical Co., St. Louis, MO, U.S.A.), as also was heparin (Glaxo Operations, Runcorn, Cheshire, U.K.).

#### *Glycosaminoglycans used in the elution buffers*

Heparan sulphate was purified from heparin by-products by the procedure of Rodén *et al.* (1972). Dermatan sulphate was obtained as the copper salt from the same procedure, and then transformed into the sodium salt. Heparin was a gift from Laboratorio Derivati Organici (Vercelli, Italy). Hyaluronic acid and chondroitin sulphates were of commercial origin (Sigma Chemical Co.).

### Protein labelling

Laminin, purified from the Engelbreth-Holm-Swarm sarcoma, was kindly given by Professor Rupert Timpl (Max-Planck-Institut für Biochemie, München, Germany). The protein, electrophoresed under non-reducing conditions on 3.5% polyacrylamide gels in the presence of 2% sodium dodecyl sulphate (Furthmayr & Timpl, 1971), gave a single band barely penetrating the gel. The amino acid composition, determined on a JEOL analyser (JEOL, Tokyo, Japan), showed the absence of both 3- and 4-hydroxyproline. Laminin was radio-iodinated by the chloramine- $\tau$  method essentially as described by Adelman *et al.* (1973). Iodination was performed in 0.1 M-Tris/HCl buffer, pH 7.9, and was stopped by the addition of  $\text{Na}_2\text{S}_2\text{O}_5$ . The solution was dialysed for 48 h against the same buffer. The resulting specific radioactivity was 41 000 d.p.m./ $\mu\text{g}$  of protein.

### Immobilization of glycosaminoglycans on agarose

Glycosaminoglycans were immobilized on Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) activated with CNBr and substituted with adipic acid dihydrazide, by the procedure of Fransson *et al.* (1981): chondroitin 4-sulphate, chondroitin 6-sulphate and hyaluronic acid were partially periodate-oxidized for 5 min at 37°C in 20 mM/ $\text{NaIO}_4$ /50 mM-sodium phosphate buffer, pH 7.0; heparin, heparan sulphates and dermatan sulphates were oxidized in 20 mM- $\text{NaIO}_4$ /50 mM-sodium formate buffer, pH 3.0, at 4°C for 5 min. Both reactions were stopped by the addition of 10% (w/v) mannitol. The partially periodate-oxidized chains were dialysed and then coupled to adipic acid dihydrazide-substituted agarose; the resulting aldimines were stabilized by reduction with  $\text{NaBH}_4$ . The incubation mixture (5 mg of glycosaminoglycan/ml of agarose) was washed in distilled water, and amino sugars were measured in an amino acid analyser (JEOL). The binding efficiency was 90%.

### Affinity chromatography

Studies of binding of laminin to glycosaminoglycan-substituted gels were performed at room temperature on columns (10 mm  $\times$  150 mm) containing 12 ml of gel. The columns were equilibrated with 0.1 M-Tris/HCl buffer, pH 7.9, containing 0.1% bovine serum albumin.  $^{125}\text{I}$ -labelled laminin (10  $\mu\text{g}$  in 1 ml of the same buffer) was applied and drained into the column; 5 ml (void volume of the column) was then eluted in order to distribute laminin throughout the whole bed of the gel. Then 2 h later the column was washed with the same buffer at a rate of 40 ml  $\cdot$  cm $^{-2}$   $\cdot$  h $^{-1}$ . A 50 ml total of washing buffer (5 column volumes) was eluted until radioactivity decreased to the background value. The effluent was analysed for radioactivity in a Packard Tri-Carb 460

CD liquid-scintillation counter with automatic quench correction, Bray's (1960) mixture (1.8 ml of sample and 7.5 ml of liquid) being used as scintillator. Elution was then performed with a linear gradient of 0.0–1.5 M-NaCl in 0.1 M-Tris/HCl buffer, pH 7.9, containing 0.1% albumin, at a flow rate of 40 ml  $\cdot$  cm $^{-2}$   $\cdot$  h $^{-1}$ ; the slope of the gradient was checked by conductivity measurements.

### Competitive-dissociation experiments

Laminin bound to dermatan sulphate- and heparan sulphate-substituted gels was eluted with different concentrations of each glycosaminoglycan, after the columns had been washed with 50 ml of the starting buffer as described above. The flow rates were the same as described above. A final wash with 2.0 M-NaCl was used to remove retained material, and fractions were checked for radioactivity as described above.

### Results

Fig. 1 shows the binding profiles of labelled laminin on agarose columns substituted with various glycosaminoglycans. The results demonstrate no binding for hyaluronic acid, and all the other glycosaminoglycans differ with respect to both the amount of bound  $^{125}\text{I}$ -labelled laminin and the NaCl requirement to obtain complete detachment. Both values, as well as NaCl molarity corresponding to the peak of laminin elution, are reported in Table 1. Non-aggregating heparan sulphate demonstrates the highest affinity for laminin (38%) among the various glycosaminoglycans, but the percentage binding does not vary widely for other co-polymeric glycosaminoglycans; a smaller percentage binding is observed with chondroitin 4-sulphate and chondroitin 6-sulphate (17% and 14.5% respectively). A control column, where adipic acid dihydrazide-substituted agarose was treated with formaldehyde as described in the Experimental section, gave a binding of 15% (the values reported in the present paper have been corrected for that control value). The NaCl concentration required to obtain the elution of laminin denotes the strength of binding: the interaction is stronger for non-aggregating heparan sulphate, heparin and dermatan sulphates; it is weaker for aggregating subfractions of heparan sulphates and chondroitin sulphates (Table 1). It is noteworthy that the electrophoretic properties under non-reducing conditions on 3.5% polyacrylamide gels in the presence of 2% sodium dodecyl sulphate and the amino acid compositions of both retained and non-retained material were the same (results not shown).

Competitive-release experiments with standard glycosaminoglycans were performed to check the specificity of binding and to reveal the existence of

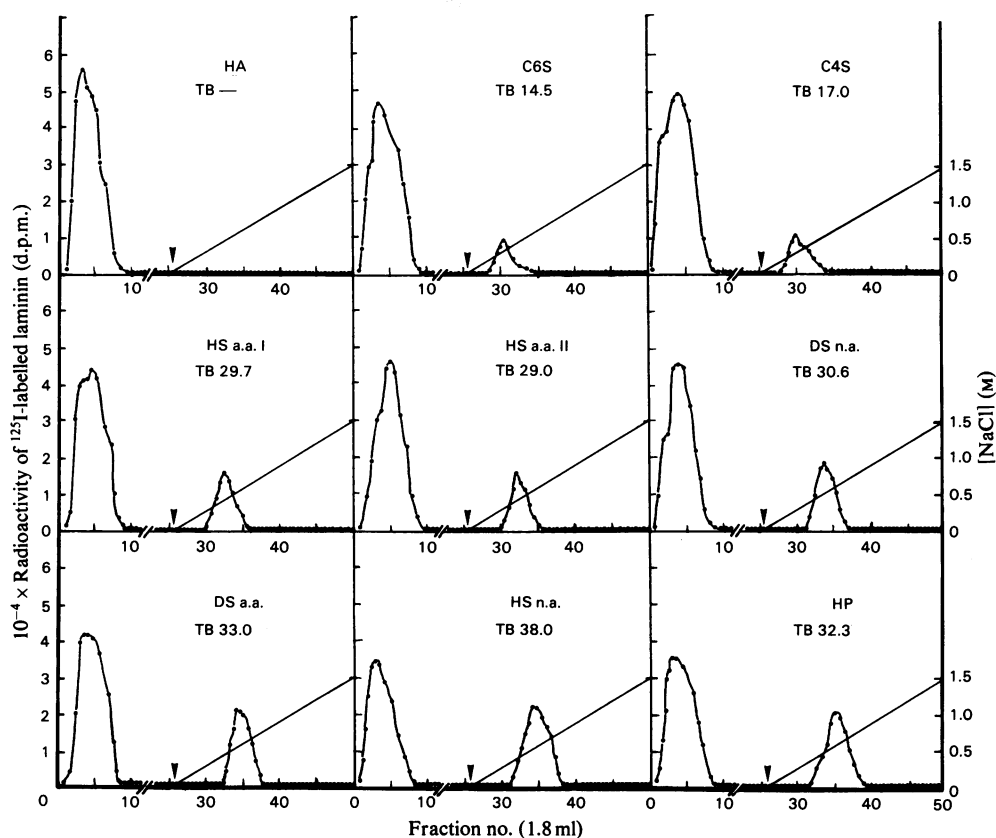


Fig. 1. Affinity-chromatography profiles of laminin on glycosaminoglycan-substituted agarose. Abbreviations used: HA, hyaluronic acid; C6S, chondroitin 6-sulphate; C4S, chondroitin 4-sulphate; HS n.a., non-aggregating heparan sulphate; HS a.a. I, first subfraction of aggregating heparan sulphate; HS a.a. II, second subfraction of aggregating heparan sulphate; HP, heparin; DS n.a., non-aggregating dermatan sulphate; DS a.a., aggregating dermatan sulphate; TB, total binding. The affinity-chromatography experiments are described in detail in the text. The arrows indicate the beginning of the NaCl gradient.

Table 1. Affinity of the binding of laminin to glycosaminoglycans  
For experimental details see the text.

Glycosaminoglycan	Percentage of bound laminin	Range of NaCl concn. required to elute all bound laminin (M)	NaCl concn. corresponding to the peak of elution (M)
Hyaluronic acid	—	—	—
Chondroitin 6-sulphate	14.5	0.22–0.60	0.34
Chondroitin 4-sulphate	17.0	0.22–0.60	0.33
Heparan sulphate (aggregating) I	29.7	0.28–0.63	0.38
Heparan sulphate (aggregating) II	29.0	0.28–0.63	0.38
Dermatan sulphate (non-aggregating)	30.6	0.32–0.70	0.45
Dermatan sulphate (aggregating)	33.0	0.37–0.71	0.49
Heparan sulphate (non-aggregating)	38.0	0.33–0.90	0.49
Heparin	32.3	0.35–0.91	0.50

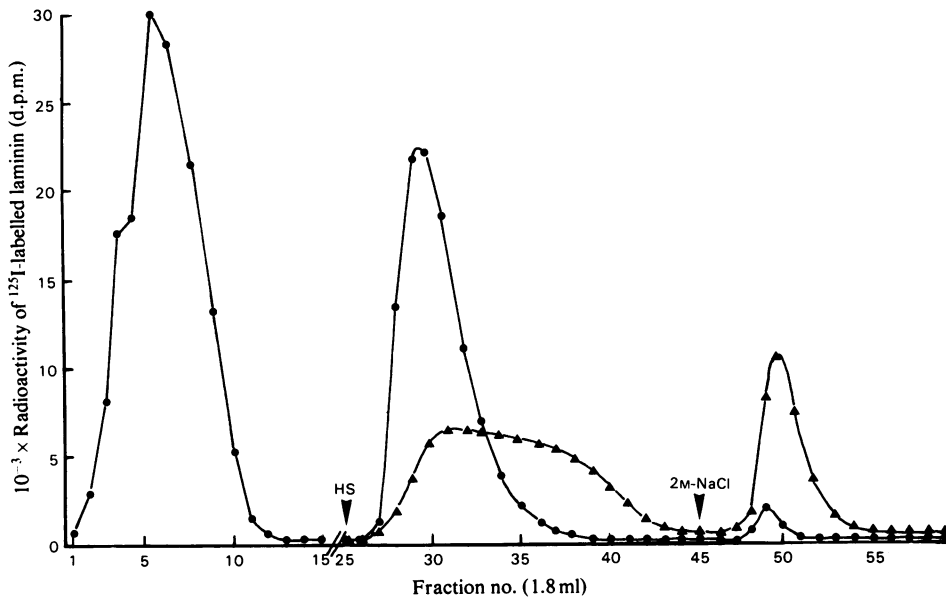


Fig. 2. Competitive dissociation experiment of laminin bound to heparan sulphate (non-aggregating)-substituted agarose by heparan sulphate-containing elution buffer

●. 1.0 mg of heparan sulphate/ml in the elution buffer; ▲. 100 µg of heparan sulphate/ml in the elution buffer. For experimental details see the text. The arrows indicate the beginning of elution by heparan sulphate (HS) and by 2.0 M-NaCl.

Table 2. Competitive-dissociation experiments on heparan sulphate (non-aggregating)- and dermatan sulphate (non-aggregating)-substituted agarose gels loaded with laminin

Heparan sulphate (non-aggregating) (control)	% of laminin unbound	% of laminin detached by NaCl gradient (0.0–1.5 M)	% of laminin sensitive to 2.0 M-NaCl
	62	38	0
Glycosaminoglycan of the elution buffer		% of laminin eluted	
Hyaluronic acid	100 µg/ml and 1 mg/ml	0	38.0
Chondroitin 6-sulphate	100 µg/ml	0.5	37.5
	1 mg/ml	2.2	35.8
Dermatan sulphate	100 µg/ml	5.9	32.1
	1 mg/ml	31.2	6.8
Heparan sulphate	100 µg/ml	25.5	12.5
	1 mg/ml	36.1	1.9
Heparin	100 µg/ml	33.8	4.2
	1 mg/ml	36.2	1.8
Dermatan sulphate (non-aggregating) (control)	% of laminin unbound	% of laminin detached by NaCl gradient (0.0–1.5 M)	% of laminin sensitive to 2.0 M-NaCl
	67	33	0
Glycosaminoglycan of the elution buffer		% of laminin eluted	
Hyaluronic acid	100 µg/ml and 1 mg/ml	0	33.0
Chondroitin 6-sulphate	100 µg/ml	1.7	31.3
	1 mg/ml	8.1	24.9
Dermatan sulphate	100 µg/ml	26.7	6.3
	1 mg/ml	29.9	3.1
Heparan sulphate	100 µg/ml	26.6	6.4
	1 mg/ml	28.7	4.3
Heparin	100 µg/ml	29.7	3.3
	1 mg/ml	31.4	1.6

common or different glycosaminoglycan-binding sites on the laminin molecule. Fig. 2 shows the extent of competitive dissociation of a laminin-heparan sulphate complex by different concentrations of heparan sulphate in the elution buffer: the effect is dose-dependent. Table 2 shows the results obtained by competitively dissociating  $^{125}\text{I}$ -labelled laminin bound on both heparan sulphate (non-aggregating)- and dermatan sulphate (non-aggregating)-substituted agarose by two different concentrations of all glycosaminoglycans (100  $\mu\text{g}/\text{ml}$  and 1  $\text{mg}/\text{ml}$ ) in the elution buffer. After elution with glycosaminoglycan-containing buffers, columns were washed with a single step of 2.0M-NaCl, as described in the Experimental section, to elute retained material. Washing the gels with 8.0M-urea did not afford any further elution. Hyaluronic acid is unable to affect the binding of laminin to both heparan sulphate- and dermatan sulphate-substituted agarose, whatever its concentration in the buffer. Chondroitin 6-sulphate is hardly effective, and the ability to detach bound laminin from both gels increases progressively for dermatan sulphate, heparan sulphate and heparin. These results reflect the relative affinity for laminin of various co-polymeric glycosaminoglycans in terms of NaCl requirement to obtain elution of bound laminin. These results suggest that co-polymeric glycosaminoglycans share a common binding site on laminin.

### Conclusions

Little information is yet available on the specific function and interactions of basement-membrane macromolecules. Laminin and heparan sulphate-rich proteoglycans have been described as components of the lamina lucida (Foidart *et al.*, 1980), and type IV collagen has been found in the closest counterpart in connective tissue (Yaoita *et al.*, 1978). Our results confirm the affinity of laminin for heparan sulphate and heparin (Sakashita *et al.*, 1980), and extend the data to all the members of the glycosaminoglycan family. In the present study we approached the problem by coupling glycosaminoglycans to agarose through a spacer arm of adipic acid dihydrazide after a brief mild periodate oxidation; by this method only a few uronic acid residues per molecule (Fransson *et al.*, 1981) are engaged in forming a Schiff base with the *N*-terminal group of adipic acid, and molecules are conceivably exposed to the hydrophilic environment in an availability similar to that existing physiologically. Experiments were performed at pH 7.9 in 0.1M-Tris/HCl buffer because both a lower pH or a decrease of salt molarity cause precipitation of laminin. Although the conditions used in our affinity experiments are well below saturation of the columns (see the Experimental section), there are always peaks of

non-binding laminin: rechromatography of such non-bound material on the same column shows a binding that is proportional to the percentage binding of the material from the previous elution (results not shown). This suggests that binding kinetics are slow and exclude a non-binding due to heterogeneity of laminin. Thus we decided to calculate the total binding after a single chromatography in order to reproduce homogeneous conditions for various compounds. In the present study the lack of affinity of laminin for hyaluronic acid is a consistent finding, and the affinity of other glycosaminoglycans is shown to decrease in the following order: heparin, non-aggregating heparan sulphate, dermatan sulphates, aggregating heparan sulphates and chondroitin sulphates (Tables 1 and 2). The binding seems to be specific and to occur on a single binding site of laminin, as indicated by competitive-release experiments. However, since heparan sulphate is much more effective in displacing laminin from dermatan sulphate that is dermatan sulphate in displacing laminin from heparan sulphate (Table 2), it is possible that heparan sulphate and dermatan sulphate have separate binding sites on laminin: heparan sulphate could induce a conformational change that converts laminin from a high-affinity form into a low-affinity form for dermatan sulphate. The differential affinity of various glycosaminoglycans for laminin revealed in the present study suggests a possible role of these compounds in regulating the adhesiveness of the basal side of epithelial-cell layers to the lamina lucida. It is known that cells undergoing neoplastic transformation show a decrease of co-polymeric glycosaminoglycans and a simultaneous increase of hyaluronic acid and/or chondroitin sulphates (Chiarugi *et al.*, 1978): this change of glycosaminoglycan surface composition could disrupt recognition of the border of basement membrane that is characteristic of normal homeostatic epithelial cells.

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### References

- Adelmann, B. C., Gentner, G. J. & Hopper, K. J. (1973) *Immunol. Methods* **3**, 319-336
- Bray, G. A. (1960) *Anal. Biochem.* **1**, 279-285
- Chiarugi, V. P., Vannucchi, S., Cella, C., Fibbi, G., Del Rosso, M. & Cappelletti, R. (1978) *Cancer Res.* **38**, 4717-4721

- Chung, A. E., Jaffe, R., Freeman, J. L., Vernes, J.-P., Braginski, J. E. & Carlin, B. (1979) *Cell* **16**, 277-287
- Engvall, E. & Ruoslahti, E. (1977) *Int. J. Cancer* **20**, 1-5
- Foidart, J. M., Bere, E. W., Yaar, M., Rennard, S. I., Gullino, M., Martin, G. R. & Katz, S. J. (1980) *Lab. Invest.* **42**, 336-342
- Fransson, L. Å., Nieduszynski, I. A. & Sheehan, J. K. (1980) *Biochim. Biophys. Acta* **630**, 287-300
- Fransson, L. Å., Havsmark, B. & Sheehan, J. K. (1981) *J. Biol. Chem.* in the press
- Furthmayr, H. & Timpl, R. (1971) *Anal. Biochem.* **41**, 510-516
- Gordon, J. R. & Bernfield, M. R. (1980) *Dev. Biol.* **74**, 118-135
- Hassel, J. R., Robey, P. G., Barrach, H.-J., Wilczek, J., Rennard, S. J. & Martin, G. R. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4494-4498
- Jilek, F. & Hormann, H. (1979) *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 597-603
- Kanwar, Y. S. & Farquhar, M. G. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1303-1307
- Kefalides, N. A. (1978) in *Biology and Chemistry of Basement Membranes* (Kefalides, N. A., ed.), pp. 215-228, Academic Press, New York
- Rodén, L., Baker, J., Cifonelli, J. A. & Mathews, M. B. (1972) *Methods Enzymol.* **28**, 73-140
- Sakashita, S., Engvall, E. & Ruoslahti, E. (1980) *FEBS Lett.* **116**, 243-246
- Stathakis, N. E. & Mosesson, W. M. (1977) *J. Clin. Invest.* **60**, 855-865
- Timpl, R., Rohde, H., Robey, P. G., Rennard, S. I., Foidart, J.-M. & Martin, G. R. (1979) *J. Biol. Chem.* **254**, 9933-9937
- Yaoita, H., Foidart, J. M. & Katz, S. I. (1978) *J. Invest. Dermatol.* **70**, 191-193