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# Isolation and characterization of pepsin fragments of laminin from human placental and renal basement membranes

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The presence of laminin in authentic basement membranes was examined at the level of a large pepsin-resistant fragment P1. This strongly antigenic fragment has been recently isolated from a mouse tumour basement membrane. By using antibodies to mouse laminin P1 for identification it was possible to isolate a homologous fragment P1 ($M_r$ about 250000) and a related component Pa ($M_r$ about 70000–90000) from pepsin digests of human placenta and kidney. The fragments were rich in half-cystine (90–130 residues/1000) and carbohydrate and showed strong binding to concanavalin A. Reduction of disulphide bonds produced several smaller peptide chains, indicating a complex pepsin cleavage. Immunological assays demonstrated partial antigenic identity between laminin fragments obtained from mouse and human tissue, and suggested that fragment Pa may originate from a protein not completely identical with laminin. The results showed that laminin is an abundant component of tissues rich in basement membranes, which has been previously suggested by immunohistological studies.

Basement membranes are ubiquitous extracellular structures that consist mainly of collagenous and non-collagenous glycoproteins (Kefalides, 1973; Vracko, 1974). They serve as major filtration barriers between different compartments of the body, such as between blood and urinary tract in the kidney and maternal and foetal circulation in placenta. Owing to the limited knowledge of the structural components in these matrices, their architecture and functional properties are still poorly characterized. Laminin has been identified as a major non-collagenous protein in a mouse tumour basement membrane (Timpl *et al.*, 1979). It differed from other constituents such as type IV collagen and fibronectin in various chemical and immunological properties. Immunohistological studies indicated that laminin is an abundant structural component (Rohde *et al.*, 1979; Foidart *et al.*, 1980; Alitalo *et al.*, 1980*a*,*b*; Madri *et al.*, 1980), and was found, for example, in all of the renal and placental basement membranes. Laminin production could be demonstrated in the morula (16–32-cell) stage of development (Leivo *et al.*, 1980) and preceded tubule formation in embryonic kidney (Ekblom *et al.*, 1980), suggesting a crucial role for laminin in the formation of basement membranes.

The characterization of laminin in the tumour (EHS-sarcoma) basement membrane was facilitated by the fact that about 80% of the protein could be extracted in an intact native state by neutral buffer (Timpl *et al.*, 1979). This approach will not be applicable to most of the authentic basement membranes, since their constituents are largely insoluble, owing to a variety of covalent and non-covalent interactions (Kefalides, 1973). We had previously shown that most of the disulphide bonds and antigenic determinants of laminin are found in a large fragment P1 resisting proteolytic digestion (Rohde *et al.*, 1979, 1980). In the present paper we describe the application of pepsin digestion to characterize laminin in human tissues.

## Materials and methods

### *Materials*

Pre-washed human placenta and human plasma fibronectin were gifts from Behringwerke (Marburg, W. Germany). Human kidneys were obtained at autopsy and kindly supplied by Dr. K. Remberger (Institute of Pathology, University of Munich, Munich, W. Germany). Pepsin (Boehringer, Mannheim, W. Germany) and bacterial collagenase (form CLSPA; Worthington, Freehold, NJ, U.S.A.) were of commercial origin.

### *Purification of laminin fragments*

Pepsin digests were prepared at 6–8°C or 15°C from human placenta or kidney as described



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previously (Risteli *et al.*, 1980*b*). Large proteins were precipitated from these digests with 1.2M-NaCl at pH 2.5. Precipitated material was dissolved in 50mM-Tris/HCl buffer, pH 7.4, containing 0.2M-NaCl, dialysed overnight against the same buffer (6–8°C) and reprecipitated by raising the concentration of NaCl to 2M and then to 4M. At both concentrations precipitates were obtained containing both collagen and laminin fragments. They were separated from each other on a DEAE-cellulose column (2.5cm × 25cm) equilibrated in 50mM-Tris/HCl buffer, pH 8.6, containing 2M-urea, and eluted with a linear (1600ml/1600ml) NaCl gradient (0–0.3M). The fractions containing laminin fragments were dialysed against 0.2M-ammonium bicarbonate buffer, pH 7.9, digested with collagenase (enzyme/substrate ratio 1:200; 37°C; 1h) and after being freeze-dried chromatographed on a column (3.0cm × 85cm) of Sepharose CL-4B equilibrated in 5mM-Tris/HCl buffer, pH 8.6, containing 8M-urea. Samples were then dialysed against water, pH 8–9, and freeze-dried. If required, the fragments were further purified on a Sephacryl S-200 column (1.5cm × 110cm) equilibrated in 0.2M-ammonium bicarbonate buffer, pH 8.5, or by binding to a concanavalin A–Sepharose 4B (Pharmacia, Uppsala, Sweden) column (1cm × 5cm) equilibrated in 50mM-Tris/HCl buffer, pH 7.4, containing 0.15M-NaCl and 1mM-$CaCl_2$ (protein load 5–10mg). Material bound to the lectin was displaced by elution with 0.4M-α-methyl mannoside, and the column was regenerated by elution with 50% (v/v) ethylene glycol in the same buffer (Anttinen *et al.*, 1977). All chromatographic operations were performed at room temperature. The presence of fragments of human laminin in the various fractions was monitored by gel diffusion with antiserum to mouse laminin P1. Fragment P1 from soluble and insoluble mouse laminin was prepared as described previously (Rohde *et al.*, 1980).

*Chemical methods*

The amino acid composition was determined on a Durrum D-500 analyser after hydrolysis of the samples with 6M-HCl (110°C; 16h) under $N_2$. Hexoses were determined by the orcinol reaction (François *et al.*, 1962), with galactose as standard. Individual sugars were identified, after hydrolysis (4M-HCl; 110°C; 3h), by t.l.c. (Müller & Sinn, 1977). Polyacrylamide-gel electrophoresis was performed in the presence of 2% (w/v) sodium dodecyl sulphate (Furthmayr & Timpl, 1971) before or after reduction with 5% (v/v) 2-mercaptoethanol. The runs were calibrated for molecular-weight determination with reduced mouse laminin ($M_r$ 220000, 440000), fibronectin ($M_r$ 450000), immunoglobulin G ($M_r$ 140000), serum albumin ($M_r$ 68000), ovalbumin ($M_r$ 45000) and cytochrome *c* ($M_r$ 13000).

*Immunological assays*

Rabbit antisera against fragments P1 and Pa of human and mouse laminin were prepared by following a previously described schedule (Rohde *et al.*, 1979). They were analysed by radioimmunoassay and by gel diffusion in 1% agarose by using established procedures (Rohde *et al.*, 1979).

## Results

*Isolation of laminin peptides P1 and Pa from human placenta and kidney*

Pepsin digestion of human tissues solubilized fragments that showed, in gel diffusion, partial cross-reaction with antibodies to fragment P1 of mouse tumour laminin (Fig. 1*a*). By using these antibodies as an analytical tool, it was possible to purify two related fragments P1 and Pa from these digests. Both fragments were separated from collagen on DEAE-cellulose (Fig. 2*a*), and were separated from each other by molecular-sieve

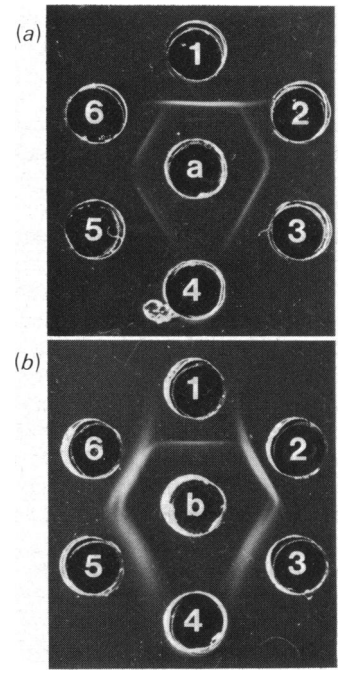


Fig. 1. *Gel-diffusion reactions of antisera against laminin fragments P1 from mouse tumour (a) and from human placenta (b)*

Antigens (0.5mg/ml) in the peripheral wells were (1) mouse tumour fragment P1, (2) human placenta fragment P1, (3) human placenta fragment Pa, (4) human serum, (5) human kidney fragment Pa and (6) human kidney fragment P1. For further details see the text.

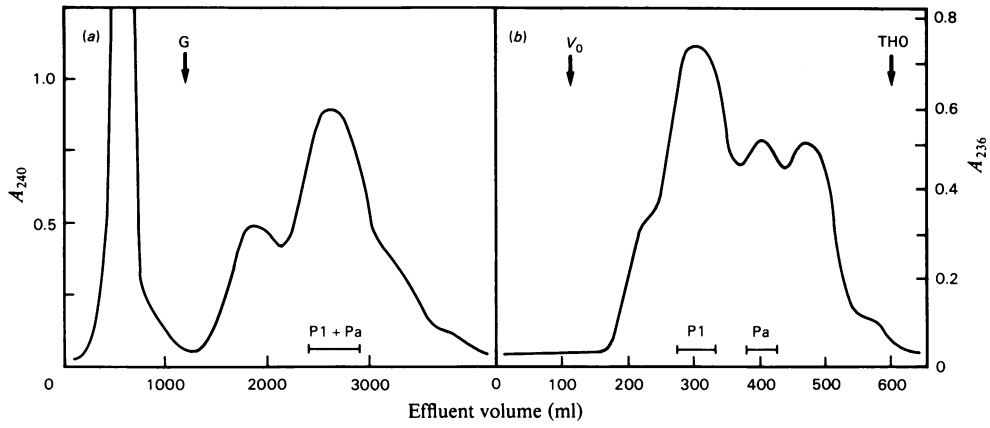


Fig. 2. *Purification of fragments P1 and Pa of human placenta obtained from a pepsin digest of placenta by chromatography on DEAE-cellulose (a) and on Sepharose CL-4B (b)*

The DEAE-cellulose column (2.5 cm × 25 cm) was equilibrated in 50 mM-Tris/HCl buffer, pH 8.6, containing 2 M-urea and was eluted with a linear gradient of 0–0.3 M-NaCl. The Sepharose column (3.0 cm × 85 cm) was equilibrated in 5 mM-Tris/HCl buffer, pH 8.6, containing 8 M-urea. The arrows denote the start of the gradient (G) and the void volume ($V_0$) and total volume (THO) of the column. For further details see the text. Most of the collagenous protein did not bind to DEAE-cellulose (Risteli *et al.*, 1980*b*) and could be removed from laminin fragments.

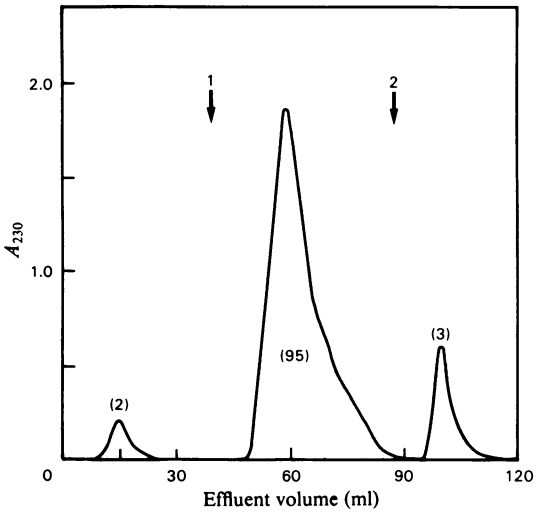


Fig. 3. *Binding of laminin fragment P1 to a column of concanavalin A–Sepharose 4B and elution with 0.4 M-α-methyl mannoside (1) followed by 50% ethylene glycol (2)*

The column (1 cm × 5 cm) was equilibrated in 50 mM-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl and 1 mM-$CaCl_2$ and loaded with 6 mg of protein dissolved in the same buffer. Numbers in parentheses refer to the relative amounts (in %) of fragment P1 as determined by radioimmunoassay. For further details see the text.

chromatography (Fig. 2*b*) after brief digestion with collagenase in order to destroy residual collagen. Both fragments showed strong affinity for concanavalin A, which was useful for final purification (Fig. 3). The final yields from human placenta were about 10–15 mg of fragment P1 and 5 mg of fragment Pa per kg wet wt. of tissue. Similar fragments could be prepared from human kidney in about 5-fold lower yields.

### *Chemical properties*

Fragments P1 and Pa from human placenta both had similar amino acid compositions, which were characterized by a high cysteine content (Table 1). They closely resembled those of laminin P1 from the mouse tumour (Rohde *et al.*, 1980). A similar composition was found for fragments P1 and Pa from human kidney (Table 1), except that cysteine values were lower. The orcinol reaction demonstrated 10–12% hexoses in fragment P1 of human placenta, and these were identified as glucose, mannose and galactose by t.l.c. A similar hexose content was indicated for the other fragments by their binding to immobilized concanavalin A and a positive periodate–Schiff reaction after electrophoresis.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 4) of fragments P1 from mouse tumour and human placenta showed similar broad bands migrating with a mobility between those of both subunits of laminin. In spite of the somewhat

Table 1. *Amino acid composition of pepsin-digest fragments of laminin*
For details see the text. Values for threonine and serine have been corrected by factors of 1.08 and 1.21 respectively. Data for mouse fragment P1 were taken from Rohde *et al.* (1980).

| | Amino acid composition (residues/1000 residues) | | | | |
|---|---|---|---|---|---|
| | Fragment P1 | | | Fragment Pa | |
| | Mouse tumour | Human placenta | Human kidney | Human placenta | Human kidney |
| Cystine (half) | 150 | 130 | 88 | 117 | 90 |
| Aspartic acid | 114 | 110 | 112 | 113 | 113 |
| Threonine | 55 | 55 | 57 | 58 | 57 |
| Serine | 77 | 69 | 78 | 81 | 86 |
| Glutamic acid | 105 | 106 | 108 | 114 | 112 |
| Proline | 70 | 75 | 68 | 61 | 70 |
| Glycine | 123 | 125 | 137 | 104 | 118 |
| Alanine | 46 | 49 | 57 | 46 | 62 |
| Valine | 37 | 34 | 47 | 42 | 47 |
| Methionine | 1 | 5 | 8 | 9 | 7 |
| Isoleucine | 13 | 20 | 27 | 35 | 28 |
| Leucine | 46 | 50 | 52 | 53 | 56 |
| Tyrosine | 26 | 22 | 28 | 23 | 25 |
| Phenylalanine | 30 | 33 | 31 | 28 | 26 |
| Histidine | 33 | 30 | 23 | 16 | 21 |
| Lysine | 32 | 32 | 36 | 49 | 38 |
| Arginine | 42 | 55 | 43 | 51 | 44 |

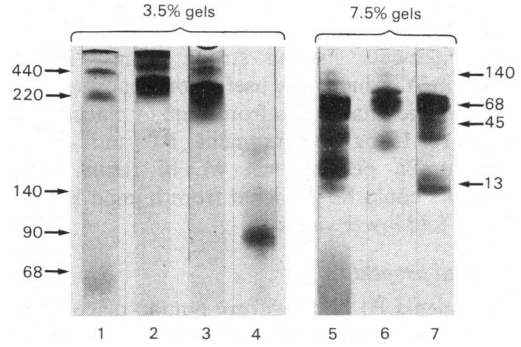


Fig. 4. *Comparison of laminin fragments by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate*
The samples were (1) reduced mouse laminin, (2) mouse fragment P1, (3) human placenta fragment P1 and (4) human placenta fragment Pa. The same samples were examined after reduction with 5% mercaptoethanol in a gel of higher porosity: (5) mouse fragment P1, (6) human placenta- fragment P1 and (7) human placenta fragment Pa. The arrows denote the migration positions of appropriate molecular-weight markers ($10^{-3} \times M_r$). For further details see the text.

heterogeneous appearance of mouse fragment P1, it was found to be homogeneous in the ultracentrifuge, with $M_r$ 290000 (Rohde *et al.*, 1980). A slightly smaller molecular weight of about 250000 was estimated for the human fragment P1 on the basis of electrophoretic mobility. As expected, fragment Pa was distinctly smaller and showed a main band with $M_r$ 70000–90000. Reduction of these fragments with mercaptoethanol produced a complex band pattern that was related to but not identical with that found with fragment P1 of mouse laminin (Fig. 4). This indicated differences in pepsin-susceptibility of mouse and human laminins.

### *Immunochemical characterization*

Antisera against fragment P1 of mouse laminin showed a line of partial antigenic identity with corresponding fragments from human placenta and kidney in gel diffusion (Fig. 1*a*). This finding was corroborated by radioimmunoassays showing for the human fragments partial inhibitory activity not exceeding 30–40% of the total reaction (Fig. 5*a*). If the same antiserum, however, was reacted against labelled human peptide P1 (Fig. 5*b*) then both mouse and human antigens showed complete inhibition, with slight differences in affinity. The data indicate that the various fragments resemble homologous portions of the same protein, and the incomplete cross-reaction is presumably due to interspecies variations in certain structural features.

Fragments P1 and Pa from human placenta were also good immunogens in rabbits, and produced strong precipitin reactions (Fig. 1*b*) and binding in

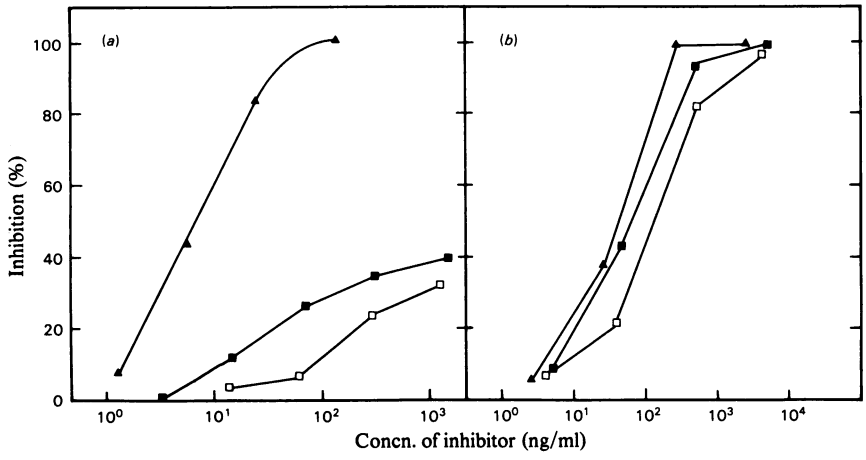


Fig. 5. *Cross-reaction of antiserum to fragment P1 from mouse laminin with fragments of human laminin*
The $^{125}$I-labelled test antigens were mouse fragment P1 (*a*) and human fragment P1 (*b*). Inhibitors of the reactions were fragments P1 from mouse tumour (▲) and human placenta (■) and fragment Pa (□) from human placenta. For further details see the text.

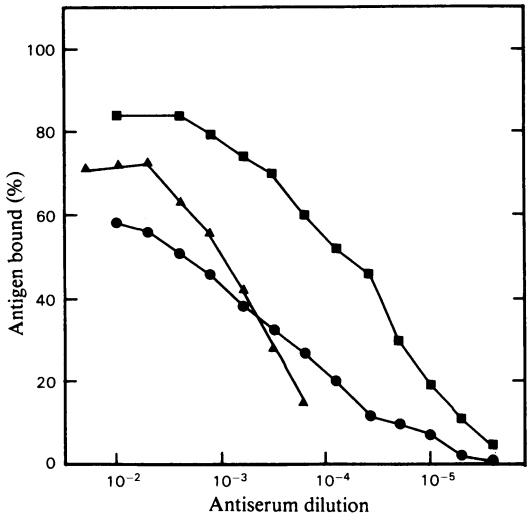


Fig. 6. *Binding profiles in radioimmunoassay of an antiserum against fragment P1 of human laminin*
The $^{125}$I-labelled antigens (1ng/tube) were fragments P1 (■) and Pa (●) from human placenta and fragment P1 from mouse laminin (▲). For further details see the text.

radioimmunoassay (Fig. 6). No large differences were noted between fragments P1 and Pa from human placenta and kidney when they were examined in both assays with antisera to fragment P1 (Figs. 1*b* and 7*a*). Antisera to fragment Pa could distinguish between fragments Pa and P1 (Fig. 7*b*), indicating that the former peptide possessed additional antigenic determinants not shared with fragment P1.

The antisera against human laminin showed no cross-reaction with human fibronectin (Fig. 7) but a distinct although weak cross-reaction with mouse laminin fragment P1 (Figs. 1, 6 and 7). Antigenicity was largely destroyed by reduction and alkylation of disulphide bonds in the presence of 8M-urea (Fig. 7). It was also possible to detect in human serum antigenic material related to the fragments of laminin by radioimmunoinhibition assay (results not shown). The concentrations found in serum were in the range 10–50ng/ml. These low concentrations explain why no positive reaction was observed in the less-sensitive gel-diffusion assay (Fig. 1).

## Discussion

Laminin has so far been isolated only as an intact molecule from a solid mouse tumour (Timpl *et al.*, 1979) and as chain constituents from a mouse teratocarcinoma cell line (Chung *et al.*, 1979). Cell-culture studies have demonstrated that human tissues should possess a protein with similar subunits (Alitalo *et al.*, 1980*a*,*b*). Preliminary studies on neutral-salt extracts prepared from human placenta showed only negligible amounts of laminin when judged by radioimmunoassay (L. Risteli & R. Timpl, unpublished work). This may be due to the small amount of basement membrane and/or to the low

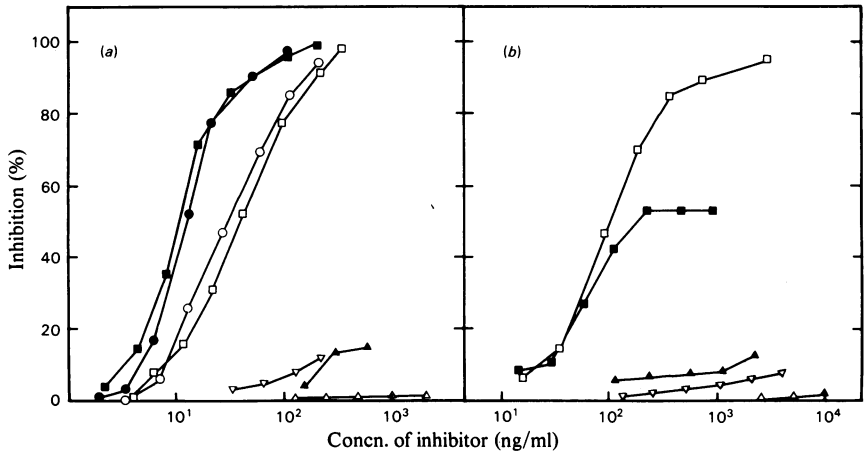


Fig. 7. *Inhibition of the reaction of antiserum to fragment P1 and labelled fragment P1 (a) and of antiserum to fragment Pa and labelled fragment Pa (b), both from human placenta*

Inhibitors of these reactions were fragments P1 from human placenta, (■), human kidney (●) and mouse tumour (▲), fragments Pa from human placenta (□) and kidney (○), and reduced and alkylated fragments P1 from human placenta (▽) and human plasma fibronectin (△). For further details see the text.

solubility of laminin in placenta. A substantial portion of the protein could, however, be solubilized by pepsin digestion in the form of fragments P1 and Pa, which are highly resistant to proteinases. Similar procedures have been previously used to solubilize other basement-membrane components from placenta, such as type-IV collagen fragments (Glanville *et al.*, 1979; Bailey *et al.*, 1979; Sage *et al.*, 1979; Kresina & Miller, 1979) and 7S collagen (Risteli *et al.*, 1980*b*).

Fragments P1 from human tissue were similar in several of their chemical and immunological properties to a homologous fragment prepared from mouse tumour laminin by pepsin digestion. Fragments obtained from placenta and kidney showed, in spite of some compositional differences, identical antigenic activity. This suggests that different organs contain the same laminin. A smaller fragment Pa was related to but not identical with fragment P1. It is possible that fragment Pa represents a further degradation product of fragment P1 or that it originates from a different protein. Several fragments similar in size to peptide Pa have been isolated from a pepsin digest of a tumour basement membrane (Rohde *et al.*, 1980). The results were interpreted as indicating that these smaller fragments are derived from laminin-like proteins and not from laminin.

The procedure described in the present paper should be useful for isolating laminin fragments from a variety of other tissues. It was possible, for example, to prepare small amounts of a similar fragment from bovine lens capsule, which is another basement membrane (L. Risteli & R. Timpl, unpublished work). If antibodies are not available to identify the fragments, other characteristic properties, such as electrophoretic mobility before and after reduction, the high cystine content and strong affinity for concanavalin A, may be useful for monitoring purification. Binding of concanavalin A to the glomerular basement membrane *in situ* has been demonstrated (Shibata *et al.*, 1977) and may be due to interaction with laminin.

Fragments of mouse and human laminins showed partial immunological cross-reactions in the range 15–40%. Similar interspecies differences have been noted with antibodies against intact laminin when used in tissue absorption studies (Rohde *et al.*, 1979). Antibodies raised against mouse fragment P1 showed complete cross-reaction with intact laminin, indicating that the release of the fragment is not accompanied by substantial structural changes (Rohde *et al.*, 1979, 1980). Thus antibodies against similar human fragments described here should be useful, as in previous studies, to analyse the tissue distribution of laminin in normal (Rohde *et al.*, 1979; Foidart *et al.*, 1980; Madri *et al.*, 1980) and pathological (Wick *et al.*, 1979; Hahn *et al.*, 1980) states and during embryonic development (Leivo *et al.*, 1980; Ekblom *et al.*, 1980). Small amounts of laminin antigen could also be detected in the circulation, and this is presumably derived from the endothelium lining the vascular wall. With the aid of radioimmunoassays it should be possible to detect pathological changes by monitoring the serum concentration of laminin (Risteli *et al.*, 1980*a*).

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## References

Alitalo, K., Kurkinen, M., Vaheri, A., Krieg, T. & Timpl, R. (1980*a*) *Cell* **19**, 1053–1062

Alitalo, K., Vaheri, A., Krieg, T. & Timpl, R. (1980*b*) *Eur. J. Biochem.* **109**, 247–255

Anttinen, H., Myllylä, R. & Kivirikko, K. J. (1977) *Eur. J. Biochem.* **78**, 11–17

Bailey, A. J., Sims, T. J., Duance, V. C. & Light, N. D. (1979) *FEBS Lett.* **99**, 361–366

Chung, E. A., Jaffe, R., Freeman, I. L., Vergnes, J. P., Braginski, J. E. & Carlin, B. (1979) *Cell* **16**, 277–287

Ekblom, P., Alitalo, K., Vaheri, A., Timpl, R. & Saxen, L. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 485–489

Foidart, J. M., Bere, E. W., Yaar, M., Rennard, S. I., Gullino, M., Martin, G. R. & Katz, S. I. (1980) *Lab. Invest.* **42**, 336–342

François, C., Marshall, R. D. & Neuberger, A. (1962) *Biochem. J.* **83**, 335–341

Furthmayr, H. & Timpl, R. (1971) *Anal. Biochem.* **41**, 510–516

Glanville, R. W., Rauter, A. & Fietzek, P. P. (1979) *Eur. J. Biochem.* **95**, 383–389

Hahn, E., Wick, G., Pencev, D. & Timpl, R. (1980) *Gut* **21**, 63–71

Kefalides, N. A. (1973) *Int. Rev. Connect. Tissue Res.* **6**, 63–104

Kresina, T. F. & Miller, E. J. (1979) *Biochemistry* **18**, 3089–3097

Leivo, I., Vaheri, A., Timpl, R. & Wartiovaara, J. (1980) *Dev. Biol.* **78**, 100–114

Madri, J. A., Roll, F. J., Furthmayr, H. & Foidart, J. M. (1980) *J. Cell. Biol.* **86**, 682–687

Müller, H. & Sinn, M. (1977) *Clin. Chim. Acta* **78**, 17–21

Risteli, J., Risteli, L., Rohde, H. & Timpl, R. (1980*a*) *Fresenius Z. Anal. Chem.* **301**, 122

Risteli, J., Bächinger, H. P., Engel, J., Furthmayr, H. & Timpl, R. (1980*b*) *Eur. J. Biochem.* **108**, 239–250

Rohde, H., Wick, G. & Timpl, R. (1979) *Eur. J. Biochem.* **102**, 195–201

Rohde, H., Bächinger, H. P. & Timpl, R. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 1651–1660

Sage, H., Woodbury, R. G. & Bornstein, P. (1979) *J. Biol. Chem.* **254**, 9893–9900

Shibata, S., Nagasawa, T. & Miura, K. (1977) *Biochim. Biophys. Acta* **499**, 392–403

Timpl, R., Rohde, H., Gehron Robey, P., Rennard, S. I., Foidart, J. M. & Martin, G. R. (1979) *J. Biol. Chem.* **254**, 9933–9937

Vracko, R. (1974) *Am. J. Pathol.* **77**, 314–338

Wick, G., Hönigsmann, H. & Timpl, R. (1979) *J. Invest. Dermatol.* **73**, 335–338