Biochem. J. (1987) 246, 121–129 (Printed in Great Britain)

Identification and partial characterization of two major proteins of M_r 47000 synthesized by bovine retinal endothelial cells in culture

Ann E. CANFIELD,* Ana M. SCHOR,† David C. WEST,‡ Seth L. SCHOR§ and Michael E. GRANT*

*Department of Biochemistry and Molecular Biology and §Department of Cell and Structural Biology, School of Biological Sciences, University of Manchester, Manchester M13 9PT, and †Cancer Research Campaign Department of Medical Oncology and ‡Department of Paediatric Oncology, Christie Hospital and Holt Radium Institute, Manchester M20 9BX, U.K.

Biosynthetic experiments with cultured bovine retinal endothelial cells have identified a glycoprotein of M_r 47000 (Gp47) as a major component secreted into the medium. Gp47 is a non-collagenous glycoprotein with a pI of 4.6–5.5, which does not bind to either gelatin–Sepharose or heparin–Sepharose but is retained by concanavalin A–Sepharose. The M_r of this species decreases to approx. 42000 in the presence of tunicamycin, indicating that it contains asparagine-linked oligosaccharides. A second protein of M_r 47000 (P47) is present in the cell layer/matrix of these cultured cells. The electrophoretic mobility of P47 remains unaltered when synthesized in the presence of tunicamycin. Peptide-mapping experiments using *N*-chlorosuccinimide and *Staphylococcus aureus* V8 proteinase demonstrate that Gp47 and P47 are distinct proteins, and are not related to colligin, a membrane-bound collagen-receptor protein of similar size, or to SPARC, a major secreted product of parietal endodermal cells and sparse cultures of aortic endothelial cells.

INTRODUCTION

Basement membranes found in different anatomical sites exhibit a considerable diversity in morphology and biochemical composition (Heathcote & Grant, 1981; Madri *et al.*, 1984). In spite of this variability, type IV procollagen, a number of specific glycoproteins (e.g. laminin, entactin and possibly fibronectin), and heparan sulphate proteoglycan appear to be common constituents of all basement membranes. The retinal capillary basement membrane, like the glomerular basement membrane, contains a multiplicity of components, with an M_r range of 11000–300000 (Duhamel *et al.*, 1983; Canfield & Grant, 1984; Canfield *et al.*, 1987). The characterization of these species by conventional separation techniques has been restricted because denaturing agents or digestion with proteolytic enzymes have to be used in order to obtain the required solubilization of basement-membrane components (Heathcote & Grant, 1981). These problems can be overcome in biosynthetic experiments, where it is possible to extract the newly synthesized native macromolecules before they become fully integrated within the insoluble basement-membrane matrix (Grant *et al.*, 1981).

Endothelial cells are believed to contribute to the synthesis of their basement membrane *in vivo*. We have found that endothelial cells isolated from the retinal microvasculature and cultured *in vitro* (Schor & Schor, 1986) retain their capacity to synthesize basement-membrane-related proteins (Canfield *et al.*, 1986). Early-passage retinal endothelial cells were found to synthesize basement-membrane (type IV) collagen, in addition to the high-M_r glycoproteins fibronectin and thrombospondin (Canfield *et al.*, 1986). Furthermore, we also noted the secretion into the medium of a major protein, of M_r 47000. A protein of similar M_r is also a major component of the cell layer/matrix of these cells. In this present study we describe the partial characterization of these two proteins of M_r 47000, and demonstrate that they are distinct species.

EXPERIMENTAL

Eagle's minimum essential medium, donor calf serum, sodium pyruvate, glutamine, non-essential amino acids, antibiotics and ultrapure urea (used for isoelectric focusing) were obtained from Gibco BRL, Paisley, Scotland, U.K. Ascorbic acid was obtained from BDH Chemicals, Poole, Dorset, U.K. Tunicamycin, *N*-chlorosuccinimide, β-aminopropionitrile fumarate, phenylmethanesulphonyl fluoride, *N*-ethylmaleimide, 2-mercaptoethanol, α-methyl D-mannoside, concanavalin A–Sepharose 4B (Con A–Sepharose) and Nonidet P40 were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Guanidinium chloride was obtained from Fluka Chemicals, Glossop, Derbyshire, U.K. *Staphylococcus aureus* V8 proteinase was purchased from Miles Laboratories, Slough, Bucks., U.K. Ampholine (pH 3.5–10.0) was purchased from LKB, South Croydon, Surrey, U.K., Pharmalyte (pH 3.0–10.0) from Pharmacia, Milton Keynes, Bucks., U.K., and Servalyte (pH 2.0–11.0) was obtained from Serva Uniscience, Cambridge, U.K.

L-[U-^{14}C]Proline (290 mCi/mmol), L-[^{35}S]methionine (1170 Ci/mmol and 1185 Ci/mmol), L-[6-^{3}H]fucose (16.7 Ci/mmol), D-[2,6-^{3}H]mannose (54 Ci/mmol) and [^{14}C]methylated standard protein mixture (M_r 14300–200000) were purchased from Amersham International, Amersham, Bucks., U.K. [^{35}S]Methionine-labelled colligin (Kurkinen *et al.*, 1984) was generously given by Dr. F. M. Watt and Mr. S. J. Hicks, Imperial Cancer Research Fund, Lincoln's Inn Fields, London W.C.1., U.K. [^{35}S]Methionine-labelled SPARC (Mason

Abbreviation used: Con A, concanavalin A.

et al., 1986*a*) was kindly provided by Dr. B. L. M. Hogan, National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K. Dr. S. R. Ayad, of the Department of Biochemistry and Molecular Biology, University of Manchester, provided the heparin–Sepharose.

Labelling of cell cultures and isolation of newly synthesized proteins

Bovine retinal endothelial cells were isolated and cultured on gelatin-coated Petri dishes as previously described (Schor & Schor, 1986; Canfield *et al.*, 1986). Cells were radiolabelled for 24 h at confluence with either [^{14}C]proline (3 μCi/ml) or [^{35}S]methionine (50 μCi/ml) in the presence of ascorbic acid (50 μg/ml) and β-aminopropionitrile fumarate (50 μg/ml) under the incubation conditions described previously (Canfield *et al.*, 1986). In studies where the influence of tunicamycin (0.5–2 μg/ml) was investigated, the cells were preincubated with this drug for 2 h before the addition of [^{35}S]methionine for a further 2 h (Kurkinen *et al.*, 1984). In some experiments the cells were radiolabelled for 24 h at 37 °C with [^{3}H]fucose (50 μCi/ml) or [^{3}H]mannose (250 μCi/ml) in Eagle's minimum essential medium containing 5% (v/v) donor calf serum, ascorbic acid (50 μg/ml), β-aminoproprionitrile fumarate (50 μg/ml), 2 mM-glutamine, 1 mM-sodium pyruvate, pencillin (100 units/ml), streptomycin (0.1 mg/ml) and non-essential amino acids.

The newly synthesized proteins secreted into the medium were collected and analysed immediately, or stored at −20 °C before analysis. In some experiments, the radiolabelled proteins were fractionated with $(NH_4)_2SO_4$. Briefly, the proteins from the medium were precipitated at 4 °C by the addition of $(NH_4)_2SO_4$ to 30% saturation and recovered by centrifugation (27000 *g* for 45 min). The labelled proteins remaining soluble at 30% $(NH_4)_2SO_4$ saturation were then precipitated by the further addition of $(NH_4)_2SO_4$ to 80% saturation (30–80% precipitate) and collected as described above. The $(NH_4)_2SO_4$ precipitates were resuspended in 0.1 M-Tris/HCl buffer, pH 7.4, containing 0.4 M-NaCl and proteinase inhibitors (2 mM-phenylmethanesulphonyl fluoride, 10 mM-*N*-ethylmaleimide, 25 mM-6-aminohexanoic acid and 25 mM-EDTA) and dialysed extensively against this solution at 4 °C. These samples were stored frozen at −20 °C until used for biochemical analysis.

Proteins present in the cell layer/matrix were extracted initially by the addition of cell-lysis buffer [1% (v/v) Nonidet P40, 0.15 M-NaCl, 50 mM-Tris/HCl, pH 8.0, 5 mM-EDTA] to the Petri dishes, and incubating the dishes for approx. 5 min at 4 °C as described by Kurkinen *et al.* (1984). The samples were decanted and centrifuged (11000 *g* for 2 min) to give supernatant (detergent-soluble) and pellet (detergent-insoluble) fractions. The material remaining on the dish after treatment with the lysis buffer was collected by scraping the dishes with a rubber policeman and combined with the detergent-insoluble pellet. This material was then extracted with 4 M-guanidinium chloride/50 mM-Tris/HCl, pH 7.4, for 24 h at 4 °C. Proteins solubilized by this treatment (guanidine extract) were recovered by centrifugation (27000 *g* for 45 min) and dialysed extensively against 0.5 M-acetic acid at 4 °C before storage at −20 °C. In some experiments, the total proteins present in the cell layer/matrix were extracted immediately with 4 M-guanidinium chloride/50 mM-Tris/HCl, pH 7.4, for 24 h at 4 °C and collected as described above.

Affinity chromatography of newly synthesized proteins

Newly synthesized proteins present in the medium and in the cell layer (detergent-soluble) fractions were tested for their abilities to bind to gelatin–Sepharose as described by Kurkinen *et al.* (1984). Proteins secreted into the medium were also tested for their abilities to bind to heparin–Sepharose by an adaptation of the above method. Samples (100–200 μl) of fresh medium were mixed immediately with 1 ml of Tris/saline buffer (0.15 M-NaCl/50 mM-Tris/HCl, pH 7.4), and 30 μl of a 50% (v/v) suspension of heparin–Sepharose in Tris/saline buffer was added. The samples were incubated for 2 h at 4 °C with end-over-end mixing. The heparin–Sepharose was collected by centrifugation (11000 *g* for 1 min) and washed with 2 × 1 ml of Tris/saline buffer and 2 × 1 ml of 10 mM-Tris/HCl, pH 6.8. Bound proteins were then released by incubation for 3 min at 100 °C in double-concentration electrophoresis sample buffer (Laemmli, 1970) and analysed by SDS/polyacrylamide-gel electrophoresis.

Proteins present in the medium and cell layer/matrix fractions were also tested for their affinities for Con A–Sepharose. Accordingly, samples (150 μl) of [^{35}S]-methionine-labelled proteins from the medium were mixed with 1 ml of Con A buffer (0.02 M-Tris/HCl, pH 7.4, containing 0.5 M-NaCl, 1 mM-$CaCl_2$ and 1 mM-$MnCl_2$). Samples of the detergent-soluble fraction and the guanidine extract of the cell layer/matrix were first dialysed against Con A buffer (16 h at 4 °C), and the volumes were then adjusted to approx. 1.2 ml with this buffer. A 50% (v/v) suspension (60 μl) of Con A–Sepharose in Con A buffer was then added, and the samples were incubated at 4 °C for 90 min with end-over-end mixing. The Con A–Sepharose was collected by centrifugation and washed with 4 × 1 ml of Con A buffer. Bound proteins were eluted from the Con A–Sepharose by the stepwise addition of α-methyl D-mannoside, in Con A buffer, at concentrations of 1, 10, 100 and 500 mM. Thus solutions of α-methyl D-mannoside (100 μl) at the appropriate concentrations were added, and the samples were incubated at 4 °C for 30 min. The samples were centrifuged (11000 *g* for 1 min), the supernatants collected, and the process was repeated twice. The proteins released at each stage were freeze-dried and analysed by SDS/polyacrylamide-gel electrophoresis. Proteins remaining bound to Con A–Sepharose after treatment with 500 mM-α-methyl D-mannoside were released by incubation for 3 min at 100 °C in double-concentration electrophoresis sample buffer (Laemmli, 1970) and analysed as above.

Electrophoretic analyses

Discontinuous SDS/polyacrylamide-gel electrophoresis was carried out on the medium and cell-layer/matrix fractions with or without reduction by 5% (v/v) 2-mercaptoethanol as described elsewhere (Canfield *et al.*, 1986). Newly synthesized proteins were detected by fluorography (Bonner & Laskey, 1974; Laskey & Mills, 1975). [^{14}C]Methylated M_r standards were myosin (M_r 200000), phosphorylase *b* (a doublet of M_r 100000 and 92500), bovine serum albumin (M_r 69000), ovalbumin (M_r 46000), carbonic anhydrase (M_r 30000) and lysozyme (M_r 14300).

Isoelectric focusing

Two-dimensional electrophoresis was performed in the ISO-DALT system (Anderson & Anderson, 1978; Tollaksen *et al.*, 1981) as modified by Jones *et al.* (1980). Double-distilled deionized water was used throughout. Samples were incubated in dissociation buffer [2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 50 mM-cyclohexylaminesulphonic acid, pH 9.5] for 3 min at 95 °C. Isoelectric focusing was performed in cylinder gels (14 cm × 1.5 mm) containing 4% (w/v) acrylamide, 8 M-urea, 1% (v/v) Nonidet P40 and 2% (w/v) ampholytes (a 1:1:1 mixture of Ampholine, pH 3.5–10.0, Pharmalyte, pH 3–10, and Servalyte, pH 2–11). The electrode buffers used were 8.67 mM-H_3PO_4 (anode) and 20 mM-NaOH (cathode). The gels were prefocused at 200 V for 30 min, and then samples (25 μl) were applied and focusing was continued at 650 V (constant) for 12000 V·h. The gels were then extruded into 5 ml of equilibration buffer (10% glycerol, 2% SDS, 0.125 M-Tris/HCl, pH 6.8) and stored at −70 °C. The pH gradient was determined from two control gels, run in parallel, in which the electrofocusing was conducted in the absence of sample (i.e. 25 μl of dissociation buffer alone). The gels were sliced into 1 cm pieces and each pair was equilibrated in freshly boiled double-distilled deionized water (0.5 ml) at 4 °C overnight. The extracts were allowed to come to room temperature and the pH was measured with a standard pH-meter. These values were used to construct a pH gradient, from which the pI values of the samples were determined. The proteins were separated in the second dimension by SDS/polyacrylamide-gel electrophoresis in a 10–20% (w/v) linear gradient polyacrylamide slab gel. The cylinder gels containing the electrofocused proteins were placed horizontally on this slab gel and fixed in place with 0.7% (w/v) agarose. M_r markers, incorporated into 5 mm slices of 0.7% agarose, were placed on either side of the cylinder gel. Electrophoresis was conducted overnight at 20 mA/gel. All the samples were electrophoresed concurrently, to allow direct comparison. Radioactive proteins thus separated were detected by fluorography as described above.

Peptide mapping

Peptide-mapping techniques were used to compare newly synthesized proteins present in the medium and cell layer/matrix. The radiolabelled proteins were denatured under reducing conditions, separated on 8%-polyacrylamide slab gels and detected by fluorography. Specific bands were excised and incubated for 30 min at room temperature with 0.15 M-*N*-chlorosuccinimide in urea/water/acetic acid (1 g/1 ml/1 ml) as described by Lischwe & Ochs (1982). The resultant peptides were separated by electrophoresis on 15%-polyacrylamide slab gels and detected by fluorography. In some experiments the protein bands were digested with *S. aureus* V8 proteinase by the techniques of Cleveland *et al.* (1977). In this case, the excised bands were swollen for 1 h in 0.125 M-Tris/HCl buffer, pH 6.8, containing 1 mM-EDTA, 0.1% SDS and 30% glycerol, and placed in adjacent slots of a 15%-polyacrylamide slab gel. Each gel slice was overlayered with 20 μl of this buffer containing 20% glycerol. Finally 10 μl of 0.125 M-Tris/HCl buffer, pH 6.8, containing 1 mM-EDTA, 0.1% SDS, 10% glycerol, Bromophenol Blue tracking dye and 10 μg of *S. aureus* V8 proteinase was layered over the sample. Electrophoresis was performed at 20 mA/gel until the dye front reached the separating gel, when the power was switched off for 1 h to allow enzyme digestion to occur. The resulting peptides were separated by continuing the electrophoresis in the usual manner.

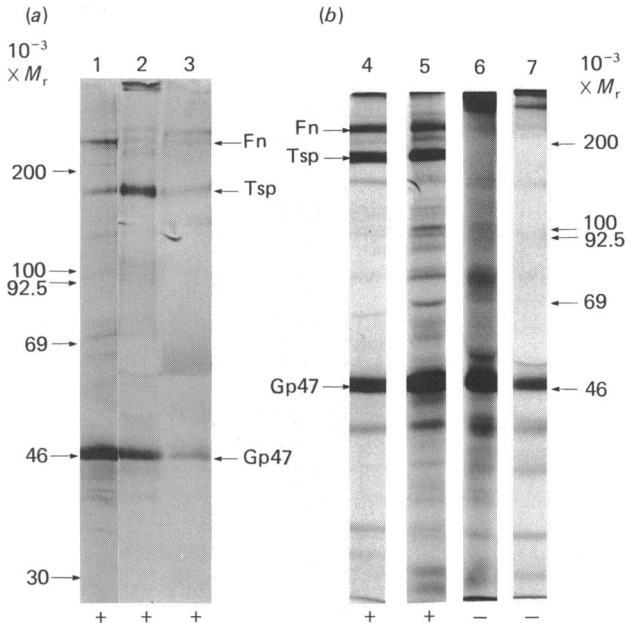

Fig. 1. Fluorograms of radiolabelled proteins secreted into the medium by retinal endothelial cells

Cells were cultured on gelatin-coated Petri dishes as previously described (Schor & Schor, 1986). At confluence, they were incubated with [^{35}S]methionine (50 μCi/ml), [^{3}H]fucose (50 μCi/ml), [^{3}H]mannose (250 μCi/ml) or [^{14}C]proline (3 μCi/ml) for 24 h at 37 °C. The medium was then decanted and either analysed immediately or fractionated with $(NH_4)_2SO_4$ as described in the Experimental section. The newly synthesized proteins were separated by electrophoresis on (*a*) 8%- and (*b*) 10%-polyacrylamide slab gels and detected by fluorography. Samples in tracks 1–7 are as follows: 1, 5 and 6, total [^{35}S]methionine-labelled proteins; 2, 30–80%-satd.-$(NH_4)_2SO_4$ precipitate of [^{3}H]fucose-labelled proteins; 3, total [^{3}H]mannose-labelled proteins; 4 and 7, 30–80%-satd.-$(NH_4)_2SO_4$ precipitate of [^{14}C]proline-labelled proteins. Samples were electrophoresed in the presence (+) or absence (−) of 2-mercaptoethanol. The migration positions of fibronectin (Fn), thrombospondin (Tsp), Gp47 and [^{14}C]methylated M_r standards are indicated.

RESULTS

Preliminary characterization of proteins of M_r 47000 present in the medium and in the cell layer/matrix

Cultured bovine retinal endothelial cells secrete a multiplicity of proteins into the medium. The major high-M_r proteins have previously been identified as fibronectin, type IV procollagen and thrombospondin (Canfield *et al.*, 1986). In addition, these cells synthesize several low-M_r proteins, one of which represents approx. 20% of the total newly synthesized [^{35}S]methionine-labelled proteins secreted into the medium (Fig. 1). This

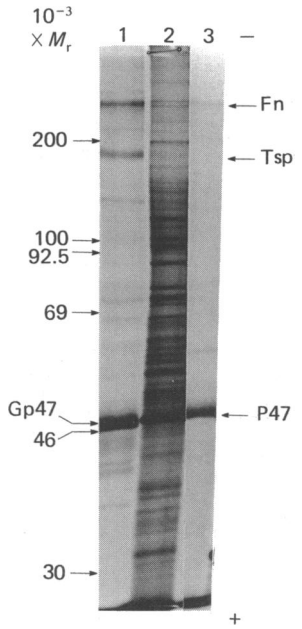

Fig. 2. Fluorogram of [^{35}S]methionine-labelled proteins present in the medium and cell layer/matrix

Endothelial cells, grown to confluence on gelatin-coated Petri dishes, were incubated with [^{35}S]methionine (50 μCi/ml) at 37 °C for 24 h as described in the text. The medium was decanted, centrifuged to precipitate any cell debris, and the proteins present were analysed without further fractionation. The newly synthesized proteins present in the cell layer/matrix were extracted initially with cell-lysis buffer (1% Nonidet P40, 0.15 M-NaCl, 50 mM-Tris/HCl, pH 8.0, 5 mM-EDTA) and then with 4 M-guanidinium chloride/50 mM-Tris/HCl, pH 7.4, as described in the Experimental section. Newly synthesized proteins present in the medium and cell-layer/matrix extracts were separated by electrophoresis on an 8%-polyacrylamide slab gel under reducing conditions, and detected by fluorography. Samples in tracks 1–3 are as follows: 1, proteins present in the medium; 2, proteins present in the detergent extract of the cell layer/matrix; 3, proteins present in the guanidine extract of the cell layer/matrix. The migration positions of fibronectin (Fn), thrombospondin (Tsp), Gp47, P47 and M_r markers are indicated.

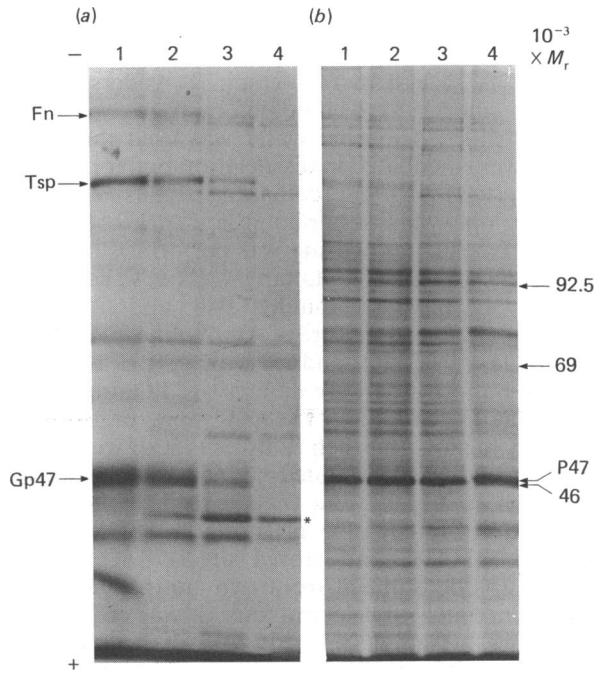

Fig. 3. Effect of tunicamycin on the synthesis of [^{35}S]methionine-labelled proteins by retinal endothelial cells

Endothelial cells were grown to confluence on gelatin-coated Petri dishes (35 mm diam.). Cultures were pre-incubated for 2 h in methionine-free Eagle's minimum essential medium (1 ml) containing 0–2 μg of tunicamycin as described in the text. At this time [^{35}S]methionine (50 μCi) was added and the incubation continued for a further 2 h. The medium was decanted, centrifuged to precipitate any cell debris and analysed by electrophoresis on 8%-polyacrylamide slab gels under reducing conditions, followed by fluorography. The cells were lysed by incubation for 5 min at 4 °C with cell lysis buffer as described in the text, centrifuged, and the supernatants were analysed by SDS/polyacrylamide-gel electrophoresis as described above. Total [^{35}S]methionine-labelled medium proteins are shown in (*a*), and proteins present in the cell lysate in (*b*). Track 1: control cultures incubated without tunicamycin. Track 2: cultures incubated with 0.5 μg of tunicamycin/ml. Track 3: cultures incubated with 1.0 μg of tunicamycin/ml. Track 4: cultures incubated with 2.0 μg of tunicamycin/ml. The migration positions of fibronectin (Fn), thrombospondin (Tsp), Gp47, P47 and M_r markers are indicated. The protein of M_r 42000, which represents an unglycosylated form of Gp47, is marked with an asterisk (*).

protein is concentrated in the 30–80%-satd.-$(NH_4)_2SO_4$ fraction of the medium and is of M_r approx. 47000, based on non-collagenous standards (Canfield *et al.*, 1986), and is referred to hereafter as 'Gp47'. When the cells were radiolabelled with [^{3}H]fucose and [^{3}H]mannose, bands with electrophoretic mobilities corresponding to Gp47 were detected in the medium (Fig. 1*a*). Experiments in which cells were incubated with [^{35}S]sulphate showed that Gp47 was not sulphated (results not shown). Gp47, labelled with either [^{14}C]proline or [^{35}S]methionine, migrated in the same position when it was electrophoresed under reducing (Fig. 1*b*; tracks 4 and 5) and non-reducing (Fig. 1*b*; tracks 6 and 7) conditions, suggesting that it does not form disulphide-bonded aggregates. This finding was confirmed by two-dimensional non-reduced/reduced SDS/polyacrylamide gel electrophoresis (Yasui *et al.*, 1984) (results not shown).

Gp47 synthesized in the presence of [^{14}C]proline did not contain hydroxy[^{14}C]proline as assessed by the method of Juva & Prockop (1966). This protein was completely digested by incubation with pepsin (100 μg/ml for 4 h at 4 °C), but not by highly purified bacterial collagenase (Canfield *et al.*, 1986), indicating that it is non-collagenous (results not shown).

Lysis of the endothelial cells with detergent released a range of [^{35}S]methionine-labelled proteins, representing intracellular proteins and cell-surface-associated proteins (Fig. 2, track 2). One of these proteins has a M_r of 47000. Subsequent extraction of the cell layer/matrix with 4 M-guanidinium chloride resulted in the solubilization of only a limited number of proteins, the major one of

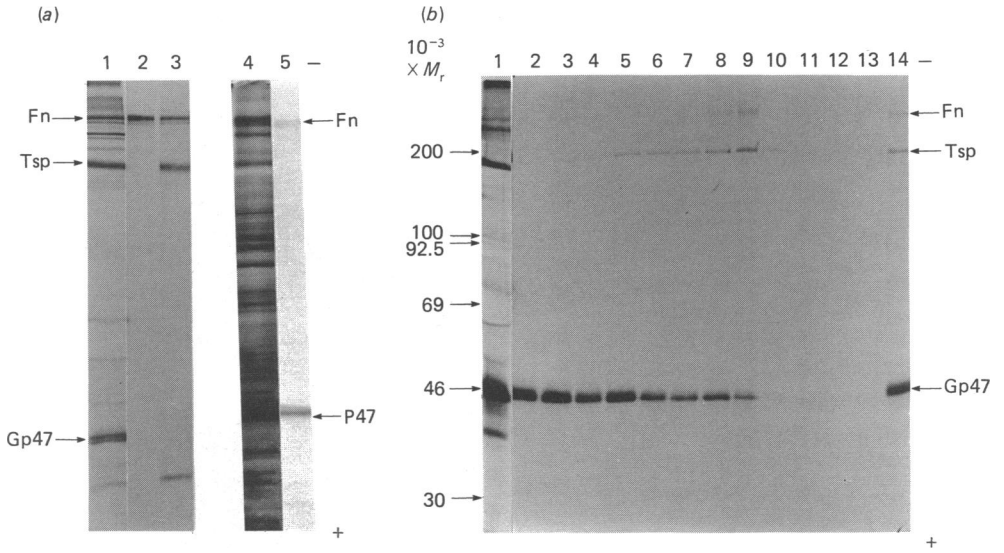

Fig. 4. Affinity chromatography of proteins Gp47 and P47

(*a*) Newly synthesized proteins were tested for their affinities for gelatin–Sepharose and heparin–Sepharose. Samples of freshly collected [^{14}C]proline-labelled medium (150 μl) and [^{35}S]methionine-labelled detergent-soluble cell-layer extracts (75 μl) were analysed as described in the text. Bound proteins were separated by electrophoresis on 6.5%-polyacrylamide slab gels under reducing conditions and detected by fluorography. Samples in tracks 1–5 are as follows: 1, total medium proteins; 2, medium proteins bound to gelatin–Sepharose; 3, medium proteins bound to heparin–Sepharose; 4, total proteins present in the detergent extract of the cell layer/matrix; 5, proteins present in the detergent extract which bound to gelatin–Sepharose. Note the presence of a gelatin-binding protein with a slightly slower electrophoretic mobility than P47. (*b*) [^{35}S]Methionine-labelled medium proteins precipitable at 30–80% saturation with $(NH_4)_2SO_4$ were tested for their affinities for Con A–Sepharose as detailed in the text. Bound proteins were analysed by electrophoresis on 8%-polyacrylamide slab gels under reducing conditions and detected by fluorography. Samples in tracks 1–14 are as follows: 1, total proteins present in the 30–80%-satd.-$(NH_4)_2SO_4$ precipitate of the medium; 2–4, proteins eluted from Con A–Sepharose by incubation with 1 mM-α-methyl D-mannoside; 5–7, proteins eluted from Con A–Sepharose by incubation with 10 mM-α-methyl D-mannoside; 8–10, proteins eluted from Con A–Sepharose by incubation with 100 mM-α-methyl D-mannoside; 11–13, proteins eluted from Con A–Sepharose by incubation with 500 mM-α-methyl D-mannoside; 14, proteins released from Con A–Sepharose by boiling for 3 min with double-concentration electrophoresis sample buffer. The migration positions of fibronectin (Fn), thrombospondin (Tsp), Gp47, P47 and M_r markers are indicated.

which also migrated with a M_r of 47000 (Fig. 2, track 3), and was not digested with bacterial collagenase (results not shown). When the cells were radiolabelled with either [^{3}H]fucose or [^{3}H]mannose, only a very faint band was detected in this position on SDS/polyacrylamide gels in both the cell lysates and the guanidine extracts (results not shown). These results indicate that the labelling patterns with [^{3}H]fucose and [^{3}H]mannose of the M_r-47000 protein present in the cell layer/matrix differ from that of Gp47 in the medium. Subsequent studies (shown below) demonstrate that these two proteins are in fact quite distinct: the M_r-47000 protein present in the cell layer/matrix is therefore referred to hereafter as 'P47'.

We have previously shown that antibodies raised against fibronectin and thrombospondin do not immunoprecipitate Gp47 or P47 from either the culture medium or the cell lysate (Canfield *et al.*, 1986). Although one cannot rule out the possibility that antigenic sites have been lost, it is therefore unlikely that proteins Gp47 and P47 are degradation products of these high-M_r glycoproteins. Furthermore, other antibodies raised against proteins of similar M_r, namely human articular-cartilage proteoglycan link protein (M_r 41000–48000; kindly provided by Dr. J. C. Anderson, Department of Biochemistry and Molecular Biology, University of Manchester) and human urokinase (kindly provided by Dr. Ian R. MacGregor, Scottish National Blood Transfusion Service, Edinburgh, U.K.) failed to immunoprecipitate Gp47 from the culture medium (results not shown).

Effect of tunicamycin on the synthesis of proteins Gp47 and P47

The ability of tunicamycin to inhibit the addition of asparagine-linked oligosaccharide side-chains (Kuo & Lampen, 1974) was used to identify the presence of such glycosylated chains in proteins Gp47 and P47. When the retinal cells were radiolabelled with [^{35}S]methionine in the presence of increasing amounts of tunicamycin (0–2 μg/ml), the amount of Gp47 secreted into the medium gradually decreased, and a protein of approx. M_r 42000 was generated (Fig. 3*a*). This latter protein is therefore likely to represent an unglycosylated form of Gp47. By contrast, the electrophoretic mobility of P47 (in the detergent-soluble fraction of the cell layer) was unaltered in the presence of the drug, indicating that this protein does not contain asparagine-linked carbohydrate (Fig. 3*b*).

Affinity chromatography of proteins Gp47 and P47

Experiments were conducted to determine the affinity of Gp47 and P47 for various ligands. Fig. 4(*a*) demonstrates that, of the numerous [^{14}C]proline-labelled

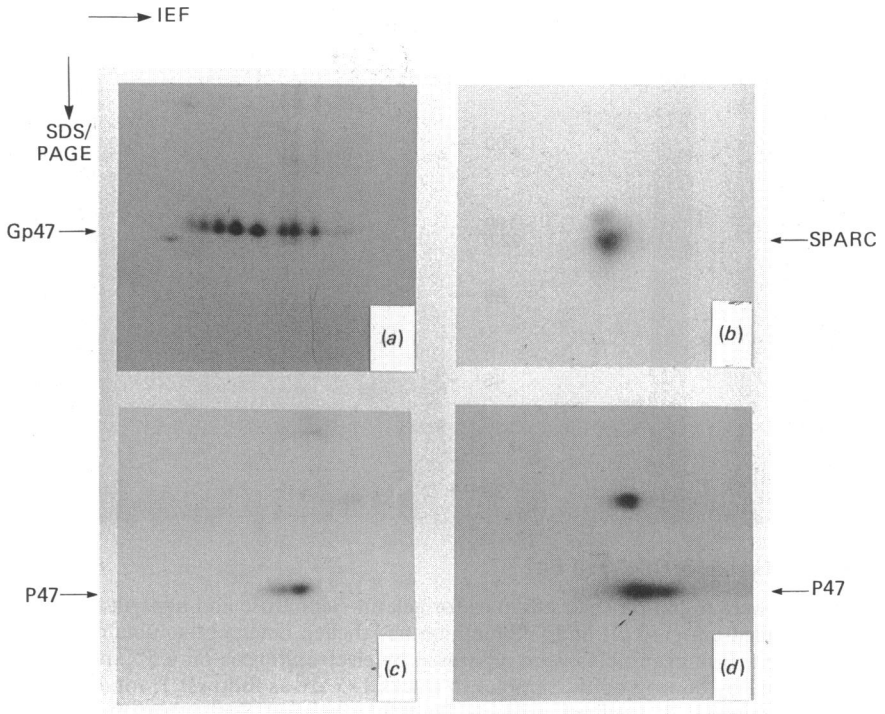

Fig. 5. Two-dimensional electrophoresis of radiolabelled proteins

Confluent cultures of cells were radiolabelled with [^{35}S]methionine or [^{14}C]proline for 24 h at 37 °C, and the newly synthesized proteins present in the medium and cell layer/matrix were collected as previously described. Samples were dialysed against distilled water at 4 °C, freeze-dried and incubated in dissociation buffer for analysis by isoelectric focusing (IEF) and SDS/polyacrylamide-gel electrophoresis (SDS/PAGE) as described in the Experimental section. The resulting spots were detected by fluorography. The samples and their calculated isoelectric points are as follows: (*a*) [^{35}S]methionine-labelled Gp47 (pI 4.6–5.5); (*b*) [^{35}S]methionine-labelled SPARC (pI 5.0); (*c*) [^{14}C]proline-labelled P47 in the detergent extract of the cell layer/matrix (pI 5.1 and 5.3); (*d*) [^{14}C]proline-labelled P47 in the guanidine extract of the cell layer/matrix (pI = 4.9 and 5.2). Only the relevant areas of the fluorograms containing the proteins of interest (arrowed) are shown.

proteins secreted into the medium by the endothelial cells, only the protein previously identified as fibronectin (Canfield *et al.*, 1986) bound to gelatin–Sepharose (Fig. 4*a*, track 2). Furthermore, when the medium proteins were incubated with heparin–Sepharose, only fibronectin, thrombospondin (Canfield *et al.*, 1986) and an unidentified protein of approx. M_r 40000 were bound (Fig. 4*a*, track 3). Therefore Gp47 showed no affinity for these ligands. The presence of two gelatin-binding proteins in the detergent-soluble fraction of the cell layer was noted: fibronectin and a species migrating just slower than P47 (Fig. 4*a*, tracks 4 and 5). Experiments in which the cells were incubated with [^{3}H]mannose suggest that this low-M_r gelatin-binding protein contains carbohydrate (results not shown). Studies are now required to determine whether this latter species is colligin, a mannosylated protein of similar M_r which has previously been localized in this fraction from parietal endodermal cells (Kurkinen *et al.*, 1984) and human keratinocytes (Taylor *et al.*, 1985). The protein P47 showed no affinity for gelatin–Sepharose (Fig. 4*a*).

It is interesting that Gp47 can be clearly resolved into two bands on the fluorogram (Fig. 4*a*, track 1). This closely spaced doublet was always observed when the fluorograms were exposed to X-ray film for short periods of time. By contrast, only one band was ever observed for P47.

The affinities of Gp47 and P47 for Con A–Sepharose were investigated. Both proteins bound to this lectin, but marked differences were noted in the conditions required for their subsequent elution. Between 80 and 90% of Gp47 could be released from Con A–Sepharose by the stepwise addition of α-methyl D-mannoside (1–500 mM), and the remaining 10–20% was eluted by boiling the samples with electrophoresis sample buffer (Fig. 4*b*). In contrast, only a small proportion (less than 10%) of P47 (present in both the detergent-soluble fraction and the guanidine extract of the cell layer) was eluted from the lectin with concentrations of α-methyl D-mannoside up to 500 mM. Thus most of the protein P47 was tightly bound to Con A–Sepharose and required boiling with electrophoresis sample buffer for its release (results not shown). In control experiments, it was demonstrated that the addition of 50 mM-α-methyl D-mannoside to the reaction mixture did not significantly inhibit the binding of P47 to this lectin, suggesting that P47 was not competing with α-methyl D-mannoside for the carbohydrate-binding site, but may be binding elsewhere (results not shown).

Isoelectric focusing

The proteins Gp47 and P47 were further analysed by two-dimensional high resolution electrophoresis, and were compared with SPARC, a major secreted product of parietal endodermal cells and sparse cultures of aortic endothelial cells (Sage *et al.*, 1984, 1986; Mason *et al.*,

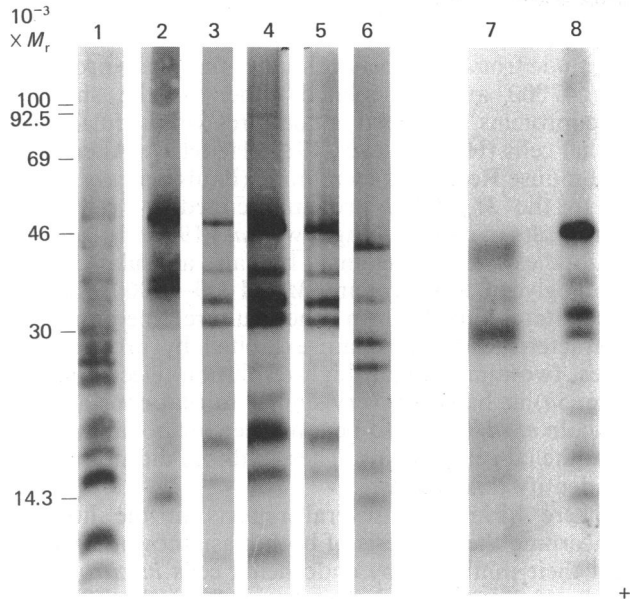

Fig. 6. Peptide maps of radiolabelled proteins obtained by using *N*-chlorosuccinimide

Confluent cultures of cells were incubated with [^{35}S]methionine (50 μCi/ml) for 2 h at 37 °C with or without tunicamycin (2 μg/ml) as described previously. Newly synthesized proteins present in the medium and cell-layer fractions were denatured under reducing conditions, separated on 8%-polyacrylamide gels and detected by fluorography. Bands corresponding to proteins Gp47 and P47 were excised from the gels and incubated for 30 min at room temperature with 0.15 M-*N*-chlorosuccinimide as described by Lischwe & Ochs (1982). The resulting peptides were separated by electrophoresis on 15%-polyacrylamide slab gels and detected by fluorography. Samples in tracks 1–9 are as follows: 1, [^{35}S]methionine-labelled colligin; 2, P47 present in the detergent-soluble fraction of the cell layer/matrix; 3, the upper band of the Gp47 doublet present in the medium; 4, the lower band of the Gp47 doublet present in the medium; 6, Gp47 present in the medium; 6, protein of M_r 42000 synthesized in the presence of tunicamycin and secreted into the medium; 7, [^{35}S]methionine-labelled SPARC; 8, Gp47 present in the medium. The samples in tracks 7 and 8 were electrophoresed on a different polyacrylamide gel. The migration positions of M_r markers are indicated.

1986*a*,*b*). Protein Gp47 yielded eight or nine distinct spots (isoelectric forms) with pI values from 4.6 to 5.5 (Fig. 5*a*). Such a charge chain is characteristic of sialylated proteins (Anderson *et al.*, 1979). Migrating slightly ahead of this charge chain was a minor protein of pI 4.5. In contrast, SPARC was resolved into a major spot (pI approx. 5) and a minor spot (Fig. 5*b*). P47, present in the cell layer/matrix, was resolved into two spots, with pI values of 5.1 and 5.3 (detergent-extracted P47; Fig. 5*c*), and 4.9 and 5.2 (guanidine-extracted P47; Fig. 5*d*). The relative proportions of these two spots (isoforms) varied in the two cell-layer extracts, indicating differences in their relative solubilities (cf. Figs. 5*c* and 5*d*).

Peptide-mapping studies

A series of peptide-mapping experiments using *N*-chlorosuccinimide and *S. aureus* V8 proteinase were conducted to determine the relationships between (*a*) the upper and lower bands of the Gp47 doublet, (*b*) Gp47 and the protein of M_r 42000 synthesized in the presence of tunicamycin, and (*c*) Gp47 in the medium, P47 in the cell layer/matrix, SPARC and colligin [a membrane-bound collagen-receptor protein of similar size (Kurkinen *et al.*, 1984)]. Fig. 6 shows the peptides produced by digestion of the excised protein bands with *N*-chlorosuccinimide. The peptide maps produced from the upper and lower bands of the Gp47 doublet are clearly identical (Fig. 6; cf. tracks 3–5), suggesting that the two bands correspond to the same protein which may have been glycosylated to slightly different extents. Furthermore, the fragmentation pattern of the M_r-42000 protein (secreted in the presence of tunicamycin) was

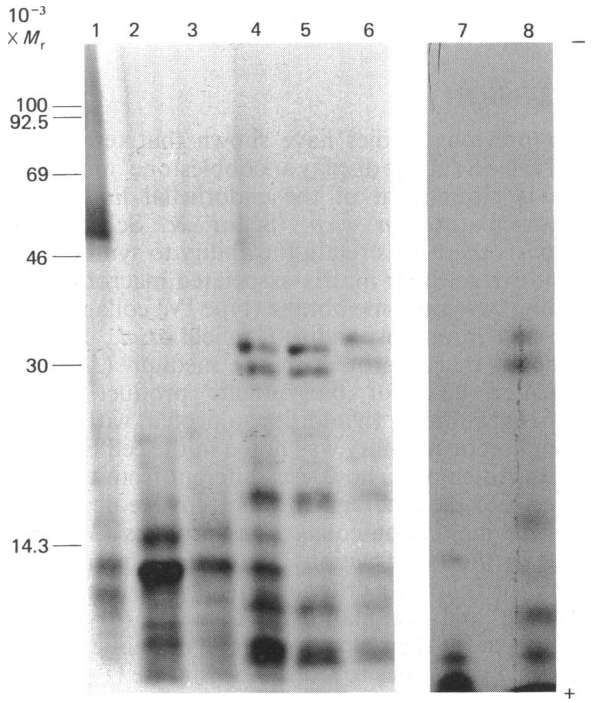

Fig. 7. Peptide maps of radiolabelled proteins obtained by using *S. aureus* V8 proteinase

[^{35}S]Methionine-labelled proteins were prepared as described in the legend to Fig. 5, separated on 8%-polyacrylamide slab gels and detected by fluorography. Bands corresponding to proteins Gp47 and P47 were excised from the gels and digested with *S. aureus* V8 proteinase (10 μg) for 30 min as described in the Experimental section. The resulting peptides were separated by electrophoresis on 15%-polyacrylamide slab gels and detected by fluorography. Samples in tracks 1–8 are as follows: 1, [^{35}S]methionine-labelled colligin; 2, P47 present in the detergent-extract of the cell layer/matrix; 3, P47 present in the guanidine extract of the cell layer/matrix; 4, Gp47 present in the medium; 5, the lower band of the Gp47 doublet present in the medium; 6, the upper band of the Gp47 doublet present in the medium; 7, [^{35}S]methionine-labelled SPARC; 8, Gp47 present in the medium. Samples in tracks 7 and 8 were electrophoresed on a different polyacrylamide gel.

virtually identical with that of Gp47, the only difference being a decrease in the M_r of the peptide fragments, presumably owing to the absence of carbohydrate (Fig. 6, cf. tracks 5 and 6). These results confirm that the M_r-42000 protein is an unglycosylated form of Gp47, and further suggest that carbohydrate side chains are distributed along the length of polypeptide Gp47. Fig. 6 also demonstrates conclusively that Gp47 and P47 are distinct proteins (cf. tracks 2 and 3), and, moreover, neither is related to colligin (cf. tracks 1–3) or to SPARC (tracks 7 and 8). It is also noteworthy that the fragmentation patterns produced by digestion of [^{14}C]proline-labelled Gp47 and P47 were also different (results not shown), but were identical with those produced from the corresponding [^{35}S]methionine-labelled proteins.

A parallel series of mapping experiments were conducted by using *S. aureus* V8 proteinase. The fragmentation patterns produced by digestion of Gp47 and P47 showed some similarities, but several peptide bands were definitely distinct (Fig. 7). The peptide maps of colligin and SPARC were again quite distinct from those of either Gp47 or P47 (Fig. 7).

DISCUSSION

Our previous studies have shown that retinal endothelial cells in culture display a 'cobblestone' morphology which is reminiscent of the endothelial lining of the microvasculature *in vivo* (Schor & Schor, 1986). Moreover, these cells retain the ability to synthesize and deposit extracellular matrix-associated macromolecules, including basement-membrane (type IV) collagen, fibronectin and thrombospondin (Canfield *et al.*, 1986). We also noted that a protein in the medium (M_r 47000) represented a major biosynthetic product of these endothelial cultures. In addition, a protein with a similar electrophoretic mobility was noted in the cell layer/matrix; this protein was, however, different from the species secreted into the medium. In this study we demonstrate that these major non-collagenous proteins (M_r 47000) present in the two fractions are distinct species on the basis of the following criteria: (*a*) labelling patterns with [^{3}H]fucose and [^{3}H]mannose (Fig. 1), (*b*) effect of tunicamycin on their electrophoretic mobilities (Fig. 3), (*c*) binding properties to Con A–Sepharose (Fig. 4*b*), (*d*) different isoelectric points (Fig. 5), and (*e*) peptide mapping with *N*-chlorosuccinimide (Fig. 6) and *S. aureus* V8 proteinase (Fig. 7). These species are therefore referred to as proteins Gp47 (found in the medium) and P47 (found in the cell layer/matrix).

It was important to determine whether these proteins were related to species of similar size which have been described in cultures of other vascular and non-vascular cells. On the basis of studies reported here, it is concluded that neither Gp47 nor P47 is related to SPARC, a glycoprotein of M_r 43000 (Sage *et al.*, 1984, 1986; Mason *et al.*, 1986*a*,*b*), which may be associated with cellular stress or injury *in vitro* (Sage *et al.*, 1986). Furthermore, peptide-mapping studies (Figs. 6 and 7) have clearly shown that neither Gp47 nor P46 is related to colligin, a membrane-associated glycoprotein which binds to native type IV collagen and gelatin (Kurkinen *et al.*, 1984). It is noteworthy, however, that a relatively minor protein with several properties similar to those of colligin is present in the cell lysate of retinal endothelial cells (see Fig. 4*a*), and experiments are required to determine whether it is the same protein. We can also conclude from our labelling studies that these proteins of M_r 47000 are not related either to the sulphated glycoproteins described in cultures of vascular endothelial cells (Heifetz *et al.*, 1982; Heifetz & Allen, 1982) and mouse Reichert's membrane (Paulsson *et al.*, 1985), or to the M_r-50000 protein secreted by rat dermal fibroblasts in culture (Raghow *et al.*, 1986). In addition, antibodies raised against human articular-cartilage proteoglycan link protein (M_r 41000–48000) failed to precipitate Gp47 from the culture medium. The characteristic charge chain exhibited by protein Gp47 after two-dimensional high-resolution electrophoresis (Fig. 5*a*) is, however, very similar to that considered by Lincoln *et al.* (1984) to be a marker for bovine vascular endothelial-cell proteins. These authors did not attempt to identify this species.

There have been several reports in the literature concerning the synthesis of both plasminogen activators and their inhibitors by endothelial cells *in vitro* (Laug, 1981; Gross *et al.*, 1982; Levin & Loskutoff, 1982; Booyse *et al.*, 1984; Loskutoff & Levin, 1984; van Mourik *et al.*, 1984). These reports have led to the proposal that endothelial cells may be important in mediating fibrinolysis *in vivo* (Loskutoff & Levin, 1984). It is unlikely that either Gp47 or P47 is a urokinase-type plasminogen activator, which are of M_r 52000–54000 (Booyse *et al.*, 1984; Loskutoff & Levin, 1984) because these enzymes do not bind to Con A–Sepharose (Gross *et al.*, 1982), and furthermore, antibodies raised against human urokinase failed to immunoprecipitate Gp47 from the culture medium. Proteins Gp47 and P47 also have significantly lower M_r values than do the tissue-type plasminogen activators (Loskutoff & Levin, 1984). However, the biochemical properties of Gp47 do show some similarities to those reported for an inhibitor of plasminogen activators (M_r approx. 50000) secreted by bovine aortic endothelial cells *in vitro* (van Mourik *et al.*, 1984; Pannekoek *et al.*, 1986), although another study demonstrated that the plasminogen-activator inhibitor (M_r 47000) synthesized by cultured human lung fibroblasts showed affinity for heparin–Sepharose (Laiho *et al.*, 1986), whereas Gp47 did not bind to this ligand (Fig. 4*a*).

At present, we do not know whether proteins Gp47 and P47 are associated with the capillary basement membrane *in vivo*. However, the facts that they are synthesized by cells which make basement-membrane proteins *in vitro*, and that proteins of M_r 30000–50000 are major constituents of retinal-vessel basement membranes (Duhamel *et al.*, 1983; Canfield & Grant, 1984; Canfield *et al.*, 1987), suggest that this may be a distinct possibility. Of course, other possibilities also remain: P47 may be associated with the cell membrane *in vivo* (indeed, some of its properties do show some resemblance to those of the cytoskeletal protein actin), and Gp47 may be present in the glycocalyx or may indeed be a plasma glycoprotein. These questions will be answered by raising antibodies to purified preparations of these proteins and using them to probe the locations and roles of these major species *in vivo*.

We thank Dr. B. L. M. Hogan and Dr. F. Watt for many helpful discussions. The financial support of the British Diabetic Association, the Wellcome Trust and the Medical Research Council is gratefully acknowledged.

REFERENCES

Anderson, N. L. & Anderson, N. G. (1978) Anal. Biochem. **85**, 341–354

Anderson, N. G., Anderson, N. L. & Tollaksen, S. L. (1979) Clin. Chem. **25**, 1199–1210

Bonner, W. M. & Laskey, R. A. (1974) Eur. J. Biochem. **46**, 83–88

Booyse, F. M., Osikowicz, G., Feder, S. & Scheinbuks, J. (1984) J. Biol. Chem. **259**, 7198–7205

Canfield, A. E. & Grant, M. E. (1984) Biochem. Soc. Trans. **12**, 1053–1054

Canfield, A. E., Schor, A. M., Schor, S. L. & Grant, M. E. (1986) Biochem. J. **235**, 375–383

Canfield, A. E., Schor, A. M., Schor, S. L. & Grant, M. E. (1987) in Renal Basement Membranes in Health and Disease (Price, R. G. & Hudson, B. G., eds.), pp. 165–180, Academic Press, London

Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) J. Biol. Chem. **252**, 1102–1106

Duhamel, R. C., Meezan, E. & Brendel, K. (1983) Exp. Eye Res. **36**, 257–267

Grant, M. E., Heathcote, J. G. & Orkin, R. W. (1981) Biosci. Rep. **1**, 819–842

Gross, J. L., Moscatelli, D., Jaffe, E. A. & Rifkin, D. B. (1982) J. Cell Biol. **95**, 974–981

Heathcote, J. G. & Grant, M. E. (1981) Int. Rev. Connect. Tissue Res. **9**, 191–264

Heifetz, A. & Allen, D. (1982) Biochemistry **21**, 171–177

Heifetz, A., Watson, C., Johnson, A. R. & Roberts, M. K. (1982) J. Biol. Chem., **257**, 13581–13586

Jones, M. I., Massingham, W. E. & Spragg, S. P. (1980) Anal. Biochem. **106**, 446–449

Juva, K. & Prockop, D. J. (1966) Anal. Biochem. **15**, 77–83

Kuo, S. C. & Lampen, J. O. (1974) Biochem. Biophys. Res. Commun. **58**, 287–295

Kurkinen, M., Taylor, A., Garrels, J. I. & Hogan, B. L. M. (1984) J. Biol. Chem. **259**, 5915–5922

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

Laiho, M., Saksela, O., Andreasen, P. A. & Keski-Oja, J. (1986) J. Cell Biol. **103**, 2403–2410

Laskey, R. A. & Mills, A. D. (1975) Eur. J. Biochem. **56**, 335–341

Laug, W. E. (1981) Thromb. Haemostasis **45**, 219–224

Levin, E. G. & Loskutoff, D. J. (1982) J. Cell Biol. **94**, 631–636

Lincoln, D. W., Braunschweiger, K. I. & Braunschweiger, W. R. (1984) J. Cell Sci. **72**, 135–145

Lischwe, M. A. & Ochs, D. (1982) Anal. Biochem. **127**, 453–457

Loskutoff, D. J. & Levin, E. (1984) in Biology of Endothelial Cells (Jaffe, E. A., ed.), pp. 200–208, Martinus Nijhoff, Boston

Madri, J. A., Pratt, B. M., Yurchenco, P. D. & Furthmayr, H. (1984) Basement Membranes and Cell Movement: Ciba Found. Symp. **108**, 6–24

Mason, I. J., Taylor, A., Williams, J. G., Sage, H. & Hogan, B. L. M. (1986*a*) EMBO J. **5**, 1465–1472

Mason, I. J., Murphy, D., Munke, M., Francke, U., Elliot, R. W. & Hogan, B. L. M. (1986*b*) EMBO J. **5**, 1831–1837

Pannekoek, H., Veerman, H., Lambers, H., Diergaarde, P., Verweij, C. L., van Zonneveld, A.-J. & van Mourik, J. A. (1986) EMBO J. **5**, 2539–2544

Paulsson, M., Dziadek, M., Suchanek, C., Huttner, W. B. & Timpl, R. (1985) Biochem. J. **231**, 571–579

Raghow, R., Gossage, D. & Kang, A. H. (1986) J. Biol. Chem. **261**, 4677–4684

Sage, H., Johnson, C. & Bornstein, P. (1984) J. Biol. Chem. **259**, 3993–4007

Sage, H., Tupper, J. & Branson, R. (1986) J. Cell. Physiol. **127**, 373–387

Schor, A. M. & Schor, S. L. (1986) Microvasc. Res. **32**, 21–38

Taylor, A., Hogan, B. L. M. & Watt, F. M. (1985) Exp. Cell Res. **159**, 47–54

Tollaksen, S. L., Anderson, N. L. & Anderson, N. G. (1981) Operation of the Iso-Dalt system, 6th edn., Argonne National Laboratory, Argonne, IL

van Mourik, J. A., Lawrence, D. A. & Loskutoff, D. J. (1984) J. Biol. Chem. **259**, 14914–14921

Yasui, N., Benya, P. D. & Nimni, M. E. (1984) J. Biol. Chem. **259**, 14175–14179

Received 2 February 1987/14 April 1987; accepted 6 May 1987