A 48 kDa collagen-binding phosphoprotein isolated from bovine aortic endothelial cells interacts with the collagenous domain, but not the globular domain, of collagen type IV

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We have identified collagen-binding proteins in detergent extracts of metabolically labelled bovine aortic endothelial cells (BAEC) by collagen type IV–Sepharose affinity chromatography. The major collagen type IV-binding protein identified by SDS/PAGE had a molecular mass of 48 kDa, which we term the ‘collagen-binding 48 kDa protein’ (CB48). The pI of CB48 was 8.0–8.3 in a two-dimensional gel system, running non-equilibrium pH gel electrophoresis in the first dimension and SDS/PAGE in the second dimension. Under these conditions CB48 separated into two major (a and b) and one minor isoform (c); a was the most basic of the three isoforms. Two-dimensional chymotryptic peptide maps derived from each individual isoform were virtually identical. The charge differences between the isoforms were due in part to differential H₃³²PO₄ incorporation by the protein. CB48 bound to intact collagen type IV and the collagenous region of collagen type IV, but not to the globular NC1 domain. Cell-surface labelling and indirect immunofluorescence experiments localized the bulk of CB48 intracellularly in the endoplasmic reticulum Golgi region, with a minor population of molecules on the cell surface. A specific rabbit polyclonal anti-CB48 serum did not inhibit the attachment or spreading of BAEC to collagen type IV in an ‘*in vitro*’ adhesion assay, suggesting that the cell-surface population of CB48 is not involved in BAEC adhesion. We conclude that CB48 is a collagen-binding phosphoprotein that interacts with the collagenous domain of collagen type IV and may be involved in intracellular transport of collagen molecules.

INTRODUCTION

Arterial endothelial cells are a mitotically inactive though highly metabolic population of cells (Fishman, 1982). These cells are polar and have their apical surfaces facing the lumen of the vessel and their basal surfaces in direct contact with a basement membrane. The interactions between endothelial cells and the individual components of the basement membrane are critical for maintaining the integrity of the vessel wall (Madri *et al.*, 1988). Identification of the receptor/binding protein(s) responsible for mediating these interactions is an important biological problem that has recently been addressed (Yannariello-Brown *et al.*, 1988).

Collagen type IV is distinct from other collagen types both structurally and functionally (Kuhn, 1982; Brodsky & Eikenberry, 1985) and is one of the major components of all basement membranes (Bornstein & Sage, 1980; Timpl & Martin, 1982). Large vessel (Madri *et al.*, 1988) and microvascular endothelial cells (Madri & Williams, 1983) interact with collagen type IV-containing basement membranes *in vivo* as well as isolated collagen type IV *in vitro*. A variety of other cell types, including hepatocytes (Rubin *et al.*, 1984), mouse-embryo parietal endoderm cells (Kurkinen *et al.*, 1984), both normal and malignant mesenchymal cells (Wayner & Carter, 1987)

and corneal epithelial cells (Sugrue, 1987) also interact with collagen type IV.

Cell-surface binding proteins/receptors thought to mediate the direct interaction between collagen and the individual cells have been isolated from cell and tissue extracts via gelatin, α -chain or native-collagen affinity chromatography. One collagen type IV-binding protein isolated from mouse parietal endoderm cells has been named ‘colligin’ (Kurkinen *et al.*, 1984). Colligin is a 47 kDa protein and binds native collagen type I, gelatin (denatured types I and III) as well as collagen types IV and V (Kurkinen *et al.*, 1984). A colligin-like 47 kDa collagen-binding phosphoprotein has also been isolated from chick embryos using gelatin affinity chromatography (Nagata & Yamada, 1986). The avian 47 kDa collagen binding protein is a novel heat-shock protein (hsp47) (Nagata *et al.*, 1986). Hsp47 mRNA levels and levels of phosphorylation are modulated during heat-shock and viral transformation of chick-embryo fibroblasts (Nagata *et al.*, 1988). A 47 kDa as well as a 70 kDa collagen type IV-binding protein has also been identified in extracts of corneal epithelial cells (Sugrue, 1987).

Using collagen type IV affinity chromatography we have isolated a 48 kDa collagen-binding protein (CB48) from detergent-solubilized bovine aortic endothelial-cell

Abbreviations used: BAEC, bovine aortic endothelial cells; NEPHGE, non-equilibrium pH gel electrophoresis; CB48, collagen-binding 48 kDa protein; hsp, heat-shock protein; DMEM, Dulbecco’s minimum essential medium; NP40, Nonidet P40; TBS, Tris-buffered saline (0.05 M-Tris/0.150 M-NaCl); PBS, phosphate-buffered saline, pH 7.2. (0.1 M-sodium phosphate/0.150 M-NaCl); BSA, bovine serum albumin.

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(BAEC) membranes that has similar biological and chemical properties to those of murine colligin and avian hsp47. We show that CB48 is a phosphoprotein that interacts with the collagenous domain of collagen type IV but not with the pepsin-sensitive NC1 globular domain. Surface iodination studies in conjunction with immunolocalization and functional studies suggest that most of the CB48 molecules are present intracellularly, with a minor population on the cells surface. 'In vitro' adhesion experiments suggest that cell-surface CB48 is not involved in mediating adhesion of BAEC to collagen type IV. A possible role for CB48 in collagen transport is discussed.

MATERIALS AND METHODS

Cell culture

BAEC were isolated from freshly obtained bovine aortae and cultured in Dulbecco's minimum essential medium (DMEM; Gibco), containing 10% (v/v) fetal-bovine serum as described previously (Yannariello-Brown *et al.*, 1988).

Type IV collagen isolation, purification and production of proteolytic fragments

Collagen type IV was isolated from the lathyrus Engelbreth-Holm-Swarm tumour as described by Yurchenco & Furthmayr (1984). The purity of the preparation was assessed by SDS/PAGE and e.l.i.s.a. as described previously (Madri & Williams, 1983). The pepsin-sensitive non-collagenous (NC1) domain and the pepsin-insensitive/collagenase-sensitive region of collagen type IV, gifts generously provided by Dr. Heinz Furthmayr (Department of Pathology, Yale University), were prepared as described by Foellmer *et al.* (1983).

Preparation of the affinity columns

All affinity columns were prepared by coupling the individual ligands to Sepharose 4B by the method of Roll *et al.* (1979), with the exception that the intact form of collagen type IV was coupled to Sepharose CL-4B in 0.1 M-calcium acetate, pH 9.0, plus 400 mM-NaCl. In all cases 1 mg of protein bound/ml of Sepharose.

One and two-dimensional gel electrophoresis

One-dimensional SDS/PAGE was performed by the method of Laemmli (1970) under reducing or non-reducing conditions according to the experiment. [³²P]Orthophosphate-labelled and unlabelled CB48 were analysed by two-dimensional gel electrophoresis using non-equilibrium pH gel electrophoresis (NEPHGE) in the first dimension and SDS 10%-(w/v)-PAGE in the second dimension. NEPHGE was performed by the method of O'Farrell *et al.* (1977), with the addition of CHAPS in place of Nonidet P40 (NP40) (Perdew *et al.*, 1983). Protein standards were used as reference points to estimate the pI of the proteins in question [cytochrome *c* (Sigma) and carbamoylated glucose-3-phosphate dehydrogenase (Pharmacia)]. The gels were stained with Coomassie Brilliant Blue 250, then silver-stained (Wray *et al.*, 1981). Autoradiography was performed by exposing the dried gels at -70 °C to Kodak X-Omat AR or RP film with the use of an enhancing screen. When analysing [³⁵S]methionine-labelled samples, enhancement of the signal was accomplished with either En³Hance (du

Pont) or Enlightning (New England Nuclear) before fluorography.

Preparation of membranes from cultured BAEC

BAEC membranes were prepared from postnuclear supernatants as described previously (Yannariello-Brown *et al.*, 1988). Briefly, BAEC were grown to confluency on 471 cm² tissue-culture-grade trays (Nunc). Cell layers were harvested by scraping them off, then disrupting them with a Dounce homogenizer fitted with an A pestle in 10 mM-Tris (pH 8.0)/5 mM-EGTA/2 mM-MgCl₂ containing a proteinase-inhibitor cocktail (2 mM-di-isopropyl fluorophosphate, 1 mM-phenylmethanesulphonyl fluoride and 1 mM-N-ethylmaleimide; Sigma). Nuclei and intact cells were removed by differential centrifugation, and the membrane-containing supernatants were centrifuged at 100 000 *g* for 60 min at 5 °C. Membranes were stored in 10 mM-Tris (pH 8.0)/5 mM-EDTA/20% (v/v) glycerol and a proteinase-inhibitor cocktail at -70 °C for up to 6 months without any appreciable loss of collagen-binding activity.

Large-scale preparation of bovine CB48 using collagen type IV-Sepharose affinity chromatography

Cell membranes isolated from 20–30 471 cm² trays were solubilized in 3 vol. of Tris-buffered saline (TBS) (pH 8.0)/1.0% NP40/5 mM-EDTA/proteinase-inhibitor cocktail with stirring for 30 min in an ice bath. The suspension was then centrifuged for 60 min at 100 000 *g* at 5 °C to remove insoluble material. The detergent-soluble fraction was removed, and the pellet was re-extracted as described above. After the supernatants were pooled, the NP40 and protein concentrations were decreased to 0.1% and 300–500 µg/ml respectively by diluting the mixture with TBS (pH 7.2)/5 mM-EDTA/proteinase-inhibitor cocktail. The extract was added to 5 ml of collagen type IV-Sepharose, previously washed three times in the same buffer, and incubated with end-over-end mixing overnight at 4 °C. At the end of the incubation period the affinity matrix was washed four times with 10 vol. of buffer to remove unbound material, then eluted with 6 M-urea containing 50 mM-Tris, pH 8.0, 5 mM-EDTA and the proteinase-inhibitor cocktail. The eluate was dialysed into 100 mM-ammonium acetate and the resultant precipitate freeze-dried.

Cell-surface iodination and metabolic labelling of BAEC

In preparation for cell-surface labelling, BAEC were grown to confluency in tissue-culture flasks, treated with trypsin and washed in phosphate-buffered saline, pH 7.2 (PBS). Cell number was adjusted to 0.5 × 10⁶ cells/ml in sterile DMEM containing 5% (w/v) BSA, transferred to a spinner flask and incubated in an air/CO₂ (23:2) environment for 2 h to allow cells to recover. The cells were harvested by centrifugation, washed in PBS and cell viability was determined by Trypan Blue dye exclusion; BAEC were used only if their viability was more than 95%. The cell surface was iodinated by the lactoperoxidase/glucose oxidase method (Hynes, 1976). Briefly, (1–2) × 10⁷ BAEC were suspended in 1 ml of cold PBS plus 5 mM-MgCl₂. Then 5 units of lactoperoxidase (320 units/mg), 3.65 units of glucose oxidase (146 000 units/g), 250 µg of D-glucose, 2 mCi of Na¹²⁵I and 1 mM-di-isopropyl fluorophosphate (Sigma) were added to the cells and incubated on ice for 15 min with

gentle mixing every 30–60 s. PBS (10 vol. containing 2 mM-tyrosine and a proteinase-inhibitor cocktail) was added and the cells were washed three times in 40 vol. of the above buffer to remove residual free radioactivity.

In order to label BAEC metabolically, cell monolayers were incubated for 16 h at 37 °C in an air/CO₂ (19:1) atmosphere, with either phosphate-free medium 199E (Gibco) containing H₃³²PO₄ (0.5 mCi/10⁶ cells; 300 µCi/ml) or Medium 199 plus Earle's salts, containing 1 µg of methionine/ml and 100 µCi of [³⁵S]methionine/ml. At the end of the incubation period the monolayers were washed three times with PBS to remove any residual radioactivity.

Affinity-chromatographic isolation of CB48 from radiolabelled BAEC extracts

BAEC labelled with H₃³²PO₄ or [³⁵S]methionine were harvested by scraping them into TBS containing a proteinase-inhibitor cocktail. In the case of the ¹²⁵I-labelling experiments, cells in suspension were harvested by centrifugation at 800 rev./min for 5 min. After harvesting, the cells were solubilized in 5 vol. of TBS plus 1 % NP40, 5 mM-EDTA and proteinase-inhibitor cocktail, with constant stirring in an ice bath for 30 min. Insoluble material was sedimented in an Eppendorf Microfuge in the cold at 10000 g, and the insoluble pellet was re-extracted as described above and the supernatants pooled. Affinity chromatography was performed by adding 2 × 10⁶ cell equivalents to 50 µl of the appropriate affinity matrix in 1 ml of TBS/0.1 % NP-40/5 mM-EDTA/proteinase-inhibitor cocktail. The mixture was incubated with end-over-end mixing overnight at 4 °C, then the affinity matrices were washed three times in 10 vol. of the above buffer and once in 10 mM-Tris/HCl, pH 6.8. CB48 was eluted with SDS/PAGE sample buffer and the samples were processed for either one- or two-dimensional gel electrophoresis.

Two-dimensional chymotryptic-peptide-map analysis of CB48 isoforms, hsp47 and collagen type IV

Two-dimensional chymotryptic peptide mapping was performed on the three major isoforms of CB48, hsp47 (kindly provided by Dr. Shinsuke Saga, Dr. Kazuhiro Nagata and Dr. K. Yamada, National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A.) and collagen type IV as described by Speicher *et al.* (1982).

Production and characterization of polyclonal rabbit anti-(bovine CB48) sera

Electrophoretically purified CB48 was isolated by excising the Coomassie Blue-stained bands from two-dimensional gels. The gel slices were washed exhaustively in water before being homogenized in Freund's complete or incomplete adjuvant (Difco). Female New Zealand White rabbits were immunized three times over a 6-week period with 30–10 µg of CB48 by using a combination of intradermal and subcutaneous injections. The animal was bled 2–3 weeks after the last injection. Collected antiserum was stored at –70 °C.

Collagen type IV and BAEC lysates were transferred to 0.1 µm-pore-size nitrocellulose paper (BA85; Schleicher and Schuell, Keene, NH, U.S.A.) as described by Towbin *et al.* (1979), with the following modifications: 0.1 % SDS was added to the transfer buffer and the transfer continued for a minimum of 80 V · h. The nitro-

cellulose sheets were subsequently blocked in TBS, pH 7.4, plus 5 % BSA and 0.1 % NaN₃, for 48 h at 37 °C. Primary antibodies were added in TBS, pH 7.4, containing 1 % BSA and 0.1 % NaN₃, at various dilutions and incubated for 2 h at room temperature with constant agitation. After washing in the same buffer, ¹²⁵I-*Staphylococcus aureus* protein A (Pharmacia) was added to the strips and incubated for 1 h at room temperature. The nitrocellulose strips were washed in TBS, pH 7.4, plus 0.05 % Triton X-100, dried and processed for autoradiography.

BAEC attachment and spreading on collagen type IV in the presence of anti-CB48 serum

BAEC were grown to confluence on tissue-culture plastic, harvested by trypsin treatment, washed in PBS and allowed to recover by incubating in DMEM + 5 % BSA for 1 h with gentle stirring. The cells were adjusted to a concentration of 20000 cells/ml in PBS containing Ca²⁺ and Mg²⁺ and either preimmune sera or anti-CB48 sera (1:5 dilution). The cells were preincubated on ice for 15 min before adding to collagen type IV-coated dishes (Madri & Williams, 1983). To assess the effects of anti-CB48 on BAEC attachment, the cells were added in 1 ml volumes to collagen type IV-coated 24-well bacteriological plastic plates (Costar), incubated for 1 or 4 h, washed three times in PBS to remove unattached cells, and the percentage of cells attached was determined by enzymic determination of acid phosphatase (Connolly *et al.*, 1986). Briefly, 200 µl of buffer, containing 0.1 M-sodium acetate, pH 5.5, 0.1 % Triton X-100 and 10 mM-*p*-nitrophenyl phosphate (Sigma), were added/well. The cluster dishes were placed in a 37 °C incubator for 2 h. The reaction was stopped by the addition of 20 µl of 1 M-NaOH, and the yellow colour produced was monitored at 405 nm. Standard curves of attached BAEC were generated with each assay in order to determine cell number.

To determine the effect of anti-CB48 on cell spreading, BAEC were pretreated with either polyclonal anti-CB48 or preimmune sera before the cells were added to collagen type IV-coated 35 mm-diameter dishes. The cells were allowed to attach and spread at 37 °C in the presence of the antibodies for 1 or 4 h. At the end of the incubation period the cells were washed in PBS, fixed in 1.0 % glutaraldehyde, then stained with haematoxylin. The degree of spreading (in µm²) was determined by manually tracing cell perimeters on a digitizing tablet (MacTablet; Summagraphics) and determining the area (in µm²) of the cell covering the substratum using Macmeasure software (Yale University shareware).

Immunofluorescence localization of CB48 in bovine aortic-tissue sections and cultured BAEC

Bovine aortae were obtained fresh from the local abattoir and the tissue was cut into 1–2 mm pieces before fixing in paraformaldehyde/lysine/periodate (McLean & Nakane, 1974). The tissue pieces were dehydrated, embedded in paraffin, and 5 µm-thick sections were prepared. Sections were deparaffinized in successive xylene baths, and the tissue was stained with the anti-CB48 antisera or control preimmune sera (NRS), followed by a goat anti-(rabbit Ig) secondary antibody conjugated with rhodamine isothiocyanate (Organon-Teknika-Cappell). Slides were viewed with a Zeiss microscope equipped for epifluorescence. Cultured

Fig. 1. Identification of collagen type IV-binding proteins in BAEC using affinity chromatography

BAEC membranes were detergent-solubilized and incubated with a collagen type IV-Sepharose affinity column as described in the Materials and methods section. Lane a is the detergent-soluble membrane fraction used as starting material. Lanes b and c represent the eluted binding proteins analysed by SDS/10%-PAGE under reducing conditions and stained with Coomassie Blue (b) and under non-reducing conditions and silver-stained (c). The 48 kDa collagen type IV-binding protein can be detected by fluorography when it is isolated from BAEC that were metabolically labelled with [35 S]methionine (lane d). Molecular-mass standards used were as follows: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), catalase (60 kDa), ovalbumin (43 kDa), lactate dehydrogenase (36 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and ferritin (18.5 kDa).

BAEC were washed three times in PBS, pH 7.2, to remove residual serum-containing media. The cells were fixed in paraformaldehyde/lysine/periodate for 90 min at 4 °C, then quenched overnight in PBS containing 5% (w/v) BSA and 0.1% NaN₃. The cell layers were incubated with the anti-CB48 sera before or after permeabilization with 0.2% Triton X-100 in PBS for 20 min at 4 °C. Antigen detection was carried out as described for the aortic sections.

RESULTS

Identification of BAEC collagen type IV binding proteins using affinity chromatography

BAEC membranes were extracted in the non-ionic detergent NP40, and the soluble fraction was incubated with collagen type IV-Sepharose. When the bound proteins were eluted and then analysed by SDS/PAGE (Fig. 1), a major band was observed migrating with a molecular mass of approx. 48 kDa under both reducing (lane b) and non-reducing (results not shown) conditions, indicating the absence of intra- or inter-molecular disulphide bonds. A more-sensitive silver stain (lane c) of a similar preparation run under non-reducing conditions also reveals a major band migrating with a molecular mass of approx. 48 kDa. To determine whether this

Fig. 2. Determination of the isoelectric point of CB48 by two-dimensional gel electrophoresis

CB48 was isolated by collagen type IV-Sepharose affinity chromatography from detergent-solubilized BAEC membranes and analysed in the first dimension by NEPHGE, followed by SDS/10%-PAGE. (a) Shows CB48 (pI 8.0–8.3), the carbamylated glucose-6-phosphate dehydrogenase standards (pI of the fastest-migrating isoform, 8.3) and cytochrome *c* (pI 10.2). (b) Shows a close-up of CB48, demonstrating the possibility of three isoforms (a, b and c). (c) When 100 ng of CB48 was analysed by two-dimensional gel electrophoresis, then silver-stained, at least three isoforms (a, b and c) can be detected in this gel system.

molecule was synthesized by the cells or sequestered from the serum-containing medium, BAEC were cultured in the presence of [35 S]methionine for 18 h. NP40 extracts of the labelled BAEC were incubated with collagen type IV-Sepharose. The major binding protein at 48 kDa incorporated the label, indicating that the cells are actively synthesizing this protein (Fig. 1, lane d). Since the 48 kDa band was the major collagen type IV-binding protein detected in BAEC lysates and it was a metabolic product of the cells, we characterized it further and term this protein 'collagen-binding 48 kDa protein' (CB48).

Two-dimensional gel analysis of CB48

CB48 isolated from solubilized BAEC membranes by collagen type IV-Sepharose affinity chromatography was analysed by two-dimensional gel electrophoresis, running NEPHGE in the first dimension and SDS/10%-PAGE in the second dimension. CB48 migrated on the acidic side of the most basic glucose-3-phosphate dehydrogenase spot (pI 8.3). CB48 was composed of multiple spots or isoforms, which we have designated 'a', 'b' and 'c'; a was the most basic of the three (Figs. 2b and 2c).

Fig. 3. Two-dimensional chymotryptic-peptide-map analysis of CB48a, CB48b and CB48c

CB48 was isolated by collagen type IV-Sepharose affinity chromatography and separated by two-dimensional gel electrophoresis. The individual spots, a, b and c, were excised from the gel, digested with chymotrypsin and iodinated. The resulting peptides were then analysed by high-voltage electrophoresis in the first dimension, followed by ascending chromatography in the second dimension. The peptide patterns of the individual isoforms, as determined by autoradiography, are shown for comparison: a (a), b (b) and c (c) and total CB48, including equal parts of a, b and c (d).

The question raised by this experiment was whether the multiple spots represent (i) multiple charge isoforms of CB48 or (ii) an unrelated protein with an identical molecular mass but a slightly more acidic pI.

Two-dimensional peptide map analysis of CB48a, CB48b and CB48c, avian hsp47 and collagen type IV

To determine whether the individual 48 kDa spots were isoforms of CB48a, a 20 μ g portion was separated by two-dimensional gel electrophoresis then subjected to proteolysis by chymotrypsin. Two-dimensional peptide maps of the resulting chymotryptic peptides of CB48a, CB48b and CB48c, as well as a peptide map of the total (a + b + c), were virtually identical (Fig. 3).

Collagen type IV is actively synthesized by BAEC in culture (Madri & Stenn, 1982) and is present in the membrane fractions used to isolate CB48 (J. Yannariello-Brown, unpublished work). Since collagen type IV self-associates under the conditions used during the isolation of CB48, the possibility existed that fragments of collagen type IV derived from BAEC were bound to the collagen affinity matrix so that CB48 is actually a degradation product of collagen type IV. To rule out this possibility, we compared two-dimensional chymotryptic peptide maps of the isolated $\alpha 1$ chains of collagen type IV [$\alpha 1$ (IV)]

and $\alpha 2$ chains of collagen type IV [$\alpha 2$ (IV)], the intact γ form of type IV (trimer) and CB48. The two-dimensional chymotryptic peptide map of CB48 resembled neither the map of intact trimeric collagen type IV nor those of the $\alpha 1$ (IV) or $\alpha 2$ (IV) chain (results not shown). Schematic diagrams of CB48 and $\alpha 2$ (IV) maps are overlaid in Fig. 4(a) to emphasize the dissimilar patterns. Chymotryptic peptide maps of bovine CB48 were compared with

Fig. 4. Comparison of the two-dimensional chymotryptic peptide maps of CB48 with those of the $\alpha 2$ chain of collagen type IV and avian hsp47

The $\alpha 2$ chain of collagen type (IV) was excised from a one-dimensional SDS/PAGE gel, digested with chymotrypsin, iodinated and the resulting peptides were analysed as described for CB48 in Fig. 3. Autoradiograph analyses of the resulting labelled peptides and comparisons with CB48 made. A schematic drawing of the overlay of ($\oplus$) $\alpha 2$ (IV) on CB48 ($\odot$) peptides is shown in (a). hsp47 was excised from one-dimensional gels before iodinating and digesting with chymotrypsin. The chymotryptic peptides were then analysed by two-dimensional peptide mapping as described in Fig. 3 and compared with CB48. A schematic overlay of CB48 ($\odot$) on hsp47 ($\oplus$) is presented in (b).

Fig. 5. Isolation of CB48 after [^{32}P]orthophosphate labelling of cultured BAEC

CB48 was isolated from BAEC lysates by collagen type IV-Sepharose affinity chromatography after an overnight labelling of the cells with [^{32}P]orthophosphate. CB48 was analysed on two-dimensional gels, silver-stained, and ^{32}P incorporation was analysed by autoradiography. CB48 resolves into at least three separate isoforms labelled a, b and c, and ^{32}P incorporation is detected in isoforms b and c only.

peptide maps of avian hsp47. General peptide patterns can be identified for both proteins. However, precise overlap is not seen in the schematic map overlay shown in Fig. 4(b). This most likely reflects species differences. A more complete comparison awaits sequence analysis of both proteins.

$\text{H}_3^{32}\text{PO}_4$ incorporation into CB48

Near-confluent BAEC were labelled with $\text{H}_3^{32}\text{PO}_4$ for 18 h. The cells were harvested as described in the Materials and methods section, and CB48 was isolated and analysed by two-dimensional gel electrophoresis. Autoradiography of the dried gels demonstrated the incorporation of ^{32}P into CB48b and CB48c (Fig. 5). Comparison of the autoradiographic and silver-stained patterns indicated that isoform b was the most heavily labelled of the three isoforms, and a, which was the major isoform represented, did not incorporate any detectable label.

CB48 binds to the collagenous region of collagen type IV

Collagen type IV is comprised of a large triple-helical collagenous domain and a smaller C-terminal non-collagenous globular domain referred to as 'NC1' (Kuhn *et al.*, 1985). Since these two domains are molecularly distinct, we investigated whether CB48 could bind to each domain. Affinity columns were prepared by using intact collagen type IV, the pepsin-sensitive globular NC1 domain or the pepsin-insensitive collagenous domain by linking each to Sepharose. When detergent extracts of [^{35}S]methionine-labelled BAEC were incubated with the individual affinity matrices, CB48 bound to intact collagen type IV (Fig. 6, lane c) and the collagenous domain of collagen type IV (lane a), but not to the non-collagenous globular NC1 domain (lane b).

Characterization of the polyclonal rabbit anti-(bovine CB48) sera

When BAEC detergent extracts were separated by SDS/PAGE, then transferred to nitrocellulose and probed with the polyclonal rabbit anti-CB48 sera (1:200 dilution), a single immunoreactive band was detected at 48 kDa (Fig. 7b, lane e). Two additional immunoreactive bands were detected at 70 and 90 kDa (Fig. 7b, lanes f-b) at a lower dilution of the antisera (1:25). Since CB48 was

Fig. 6. CB48 binds exclusively to the collagenous region of collagen type IV and not to the globular NC1 domain

Solubilized [^{35}S]methionine-labelled BAEC lysates were incubated with either intact collagen type IV, the non-collagenous NC1 domain or the collagenous region of collagen type IV linked to Sepharose. The bound proteins were eluted in sample buffer and analysed by SDS/10%-PAGE. The fluorogram of the gel demonstrates that CB48 binds to the collagenous region of type IV collagen (a) and intact collagen type IV (c), but not to the non-collagenous globular NC1 domain of type IV collagen (b).

isolated for injection by extraction from two-dimensional gels, it is unlikely that the 70 and 90 kDa polypeptides were contaminating the CB48 preparations used for immunization. The two cross-reacting polypeptides could be CB48 precursors or other proteins structurally related to CB48. Rabbit anti-CB48 did not cross-react with collagen type IV as measured by e.l.i.s.a. (results not shown) or by Western-blot analysis (Fig. 7a).

Paraffin-embedded bovine aortic sections were immunostained with the anti-CB48 in order to examine the presence of the antigen *in situ* (Fig. 8). CB48 was distributed throughout the cells in a diffuse pattern. Figs. 8(c) and 8(d) show a section where the endothelium was separated from the intima, which confirms that the immunodetectable CB48 was associated with the endothelium and not the subendothelial matrix.

Immunofluorescence analysis with anti-CB48 sera was performed on permeable or intact cultures of confluent BAEC. Intact cells did not show significant immunoreactivity above background on their dorsal surfaces (Fig. 9a). However, cells stained after permeabilization showed a lacy cytoplasmic and perinuclear pattern of immunoreactivity (Figs 9b and 9c). Preimmune-sera controls gave negative results (not shown).

Isolation of CB48 after ^{125}I surface labelling of BAEC

BAEC were surface-labelled with Na^{125}I , extracted with NP40, and the soluble fraction was incubated with

Fig. 7. Immunoblotting of collagen type IV (a) and BAEC lysates (b) with polyclonal anti-CB48

(a) Purified collagen type IV was transferred to nitrocellulose and allowed to react with either preimmune sera (lane a) or anti-CB48 (lane b) at a 1:25 dilution, followed by ^{125}I -*Staphylococcus aureus* Protein A. Autoradiographs of the strips do not show any reactivity of either sera with the transferred collagen type IV, even with long-exposure autoradiography. (b) Detergent-solubilized BAEC membranes were subjected to SDS/10%-PAGE, transferred to nitrocellulose, then probed with preimmune sera at a 1:25 dilution (lane a) and anti-CB48 at 1:25 (lane b), 1:50 (lane c), 1:100 (lane d) and 1:200 (lane e) dilution followed by ^{125}I -*Staphylococcus aureus* Protein A. The preimmune sera does not react with the lysates. A 48 kDa band can be detected at all dilutions with the anti-CB48 sera, and additional higher-molecular-mass bands at 70 and 90 kDa become evident at higher concentrations of the antisera.

collagen type IV-Sepharose in the presence or absence of bivalent cations. The bound proteins were eluted, then analysed by SDS/PAGE under both reducing and non-reducing conditions (Fig. 10). Lane b is the autoradiograph of BAEC surface proteins bound to collagen type IV in the absence of bivalent cations analysed by

SDS/PAGE under non-reducing conditions. CB48 incorporated the label in addition to three other polypeptides at 95, 110 and 130 kDa, which were not readily detected on silver-stained gels (results not shown). If 5 mM- Ca^{2+} and 5 mM- Mg^{2+} were added to the binding buffer (lane a), the autoradiographic pattern was not

Fig. 8. Immunolocalization of CB48 in bovine aortic-tissue sections

Fresh bovine aortae were washed, fixed in paraformaldehyde/lysine/periodate then embedded in paraffin. The sections were dehydrated in xylene, quenched with BSA, then stained with anti-CB48 (b and d). (a) is a phase-contrast micrograph of a cross-section of bovine aorta and (b) is the immunofluorescence micrograph of the same section stained with anti-CB48. (c) is a phase-contrast micrograph of a cross-section of bovine aorta in which the endothelium was displaced, and (d) is the immunofluorescence micrograph of the same section stained with anti-CB48. Preimmune controls were negative (not shown).

Fig. 9. Immunolocalization of CB48 in permeabilized or non-permeabilized cultured BAEC

Culture monolayers of BAEC were washed, fixed in paraformaldehyde/lysine/periodase, then either (1) stained directly with anti-CB48, followed by the appropriate rhodamine-conjugated secondary antibody to reveal any surface-bound CB48 or (2) permeabilized in 0.2% Triton X-100 in PBS, then stained with anti-CB48 in order to reveal both surface and internal protein. (a) represents non-permeable BAEC stained with the anti-CB48 antibodies. Note the relative lack of immunoreactivity on these non-permeabilized cells. (b) and (c) represent cells that had been permeabilized with detergent before staining with the antisera. The pattern is reminiscent of an intracellular endoplasmic-reticulum staining.

appreciably changed, indicating that binding is not dependent on bivalent cations. After electrophoresis in the presence of 2.5% β -mercaptoethanol, as shown in lane c, a band at approx. 200 kDa was also detected, and the 130 and the 95 kDa bands migrate at 120 and 88 kDa respectively. Migration of CB48 and the 110 kDa band was unaffected by the reducing agent. The migration shift of the 130 and 95 kDa polypeptides was revealed by titrating the concentration of the reducing agent in the SDS/PAGE gels (results not shown). These experiments indicate that a population of CB48 molecules can be detected on the surface of BAEC with cell-surface [125 I]-iodination.

Antibodies to CB48 do not inhibit the attachment or spreading of BAEC on collagen type IV

To test whether the cell-surface population of CB48 played a role in BAEC adhesion, the effect of anti-CB48 on BAEC attachment and spreading on collagen type IV was investigated. BAEC were pretreated with either anti-CB48 antisera or the preimmune sera at a 1:5 dilution before seeding on to collagen type IV-coated bacteriological plastic plates. The percentage attachment was determined by the acid phosphatase assay described in the Materials and methods sections. Neither the anti-CB48 antisera nor preimmune sera had any effect on the rate or extent of BAEC attachment to collagen type IV; in each case approx. 2×10^4 cells/well had attached by 4 h.

BAEC attached and spread on collagen type IV-coated dishes in the presence of either anti-CB48 or the preimmune sera. In the presence of the preimmune sera the mean area spread per cell was 1327 ± 68 (S.E.M.) at 1 h and $1817 \pm 117 \mu\text{m}^2$ after 4 h. In the presence of anti-

Fig. 10. Collagen type IV-binding proteins isolated after surface-labelling live BAEC

Live BAEC were surface-labelled in suspension with Na^{125}I by using the lactoperoxidase-glucose oxidase method. The cells were detergent-extracted and CB48 was isolated by collagen type IV-Sepharose affinity chromatography. The eluted fractions were then analysed by SDS/10%-PAGE under reducing or non-reducing conditions and in the presence or absence of bivalent cations as indicated; the gels were then processed for autoradiography. Lanes a and b: CB48 appears to incorporate the label as well as a group of higher-molecular-mass bands which migrate at 95, 110 and 130 kDa under non-reducing conditions. The presence of bivalent cations (M^{2+}) during the affinity chromatography does not effect binding. Lane a: binding in the presence of 5 mM- $\text{Ca}^{2+}/\text{Mg}^{2+}$; lane b: binding in the absence of bivalent cations and in the presence of 5 mM-EDTA; lane c: when analysed by SDS/10%-PAGE under reducing conditions (2.5% β -mercaptoethanol, ME), the 95 and the 130 kDa bands shift their migrations to 88 and 120 kDa respectively. Broken lines indicate the shifts in mobilities of the individual bands upon reduction.

CB48 sera, the mean area spread per cell was $1363 \pm 58 \mu\text{m}^2$ after 1 h and $1846 \pm 100 \mu\text{m}^2$ after 4 h. Thus cell-surface CB48 does not appear to mediate directly BAEC adhesion to collagen type IV.

DISCUSSION

Affinity chromatography was used to identify and isolate collagen type IV-binding proteins from detergent-solubilized BAEC membranes. The major protein eluted from collagen type IV-Sepharose was a 48 kDa phosphoprotein synthesized by the endothelial cells which we term 'collagen-binding 48 kDa protein' (CB48). Complete elution of CB48 from the affinity matrix required the strong chaotropic agent urea. By using these harsh conditions we confirmed that CB48 was not a degradative fragment of collagen type IV on the bases of two criteria: (1) two-dimensional chymotryptic peptide maps of CB48 were distinct from those of the $\alpha 1$ and $\alpha 2$ chains of collagen type IV; and (2) a specific polyclonal antisera directed against CB48 did not cross-react with collagen type iV in an e.l.i.s.a. or in Western-blotting experiments.

A relationship between bovine CB48 and other

47 kDa collagen-binding proteins reported previously (i.e. murine colligin, avian hsp47 and the avian cornea 47 kDa protein) has not been unequivocally established. However, on the basis of similar protein chemistries and similar functional properties, it is likely that they represent the same protein from three species. This group of proteins appears to bind multiple types of collagen. CB48 can bind to, and be eluted from, collagen type IV and gelatin affinity columns (J. Yannariello-Brown, unpublished work). Colligin has the ability to bind both denatured and native collagen type I, and colligin also binds collagen type V (Taylor *et al.*, 1985). The ability to bind more than one collagen type is consistent with our observation that CB48 binds to the collagenous region of collagen type IV, but not the globular NC1 domain. The collagenous region is the area of greatest sequence similarity between the individual collagen types. Similarly, hepatocytes adhere to intact collagen molecules or to synthetic peptides with collagen-like sequences, but not to *N*-terminal propeptides (Rubin *et al.*, 1981). Whether CB48 might bind to the short helical domains of the *N*-terminal propeptides of collagen types I and III is unknown at this time. Binding of CB48 may be restricted to collagenous proteins, since we have not detected binding of CB48 to laminin (Yannariello-Brown *et al.*, 1988) or fibronectin (J. Yannariello-Brown, unpublished work) affinity columns. The full range of collagen types that CB48 will bind has yet to be determined.

CB48 was resolved into three individual spots (a, b and c) with basic pI values ranging from 8.0 to 8.3 when analysed by two-dimensional gel electrophoresis. Since the two-dimensional chymotryptic peptide maps of each polypeptide were virtually identical, the differences in pI must have been due to charge differences. ³²P label was selectively incorporated only into the b and c isoforms of CB48; isoform a did not incorporate ³²P during the 18 h labelling period. Since the more acidic isoforms of CB48 were phosphorylated, it is possible that phosphorylation differences regulate the isoforms. Although these observations are consistent with the charge differences seen between the isoforms, they do not rule out other possible explanations, such as degradative deamination or glycosylation. Avian hsp47 is also a phosphoprotein and its phosphorylation is altered during transformation of cultured cells with Rous sarcoma virus (Nagata *et al.*, 1988). Although those authors noted a minor spot trailing hsp47 in their two-dimensional gel system, they did not report differential phosphorylation of the multiple isoforms. Since we detect the presence of multiple charged isoforms, it is possible that only a subpopulation of CB48 molecules are being modified, and that some function of CB48 may be regulated by alternating cycles of phosphorylation-dephosphorylation. The functional significance of this post-translational modification is presently unknown.

The topography of the 47 kDa collagen-binding proteins within a cell is presently an unresolved issue. Kurkinen *et al.* (1984) detected colligin on the cell surface after surface iodination of intact cells. This finding was very surprising in light of the fact that colligin bears exclusively high-mannose-type carbohydrate, characteristic of many proteins resident in the endoplasmic reticulum (Hughes *et al.*, 1987). Embryonic chicken corneal epithelial 47 kDa protein is also readily iodinated, indicating a cell-surface localization (Sugrue, 1987). Saga

et al. (1987), however, have localized avian hsp47 exclusively to the endoplasmic reticulum of chick-embryo fibroblasts using immunoelectron microscopy with specific poly- and mono-clonal antibodies. To address this issue in BAEC, we examined the intracellular localization of CB48 using both cell-surface labelling and immunofluorescence techniques. Whereas CB48 was iodinated on live BAEC, it was not detected immunologically on non-permeabilized cells with a polyclonal antiserum. One possible explanation is that CB48 may be present on the cell surface in low copy numbers and not readily detected by immunolocalization. This possibility is supported by the observation that, even though the surface-iodinated higher-molecular-mass bands (i.e. 95, 110 and 1230 kDa) were not readily visible on silver-stained gels and CB48 was very prominent, the incorporation of radiolabel into CB48 was relatively inefficient when compared with the level of incorporation into these higher-molecular-mass bands (see Fig. 1). In addition, immunofluorescence detection of surface CB48 may have been reduced by the presence of collagen molecules synthesized by cultured BAEC (Madri & Stenn, 1982). The collagen molecules may be bound to BAEC, sterically hindering antibody binding.

When the relative migration of the surface-iodinated high-molecular-mass bands (95, 110 and 130 kDa) was compared with or without reduction, the higher-molecular-mass bands at 130 and 95 kDa shifted their mobilities to values consistent, but not identical, with that noted for the Integrin (CSAT Ag) protein family (Hynes, 1987). We have recently identified three polypeptides in solubilized BAEC membranes with almost identical molecular masses that could be eluted from a laminin-Sepharose affinity matrix and have speculated on their relationship to the Integrin family of proteins (Yannariello-Brown *et al.*, 1988). Members of the Integrin supergene family have been shown to bind to fibronectin and laminin (Horwitz *et al.*, 1985), collagen type I (Shadle *et al.*, 1984) and also collagen type IV (Wayner & Carter, 1987; Takada *et al.*, 1988).

To discern whether cell-surface CB48 mediated BAEC adhesion to collagen type IV, anti-CB48 sera was used in an attempt to inhibit the adhesion of BAEC to collagen type IV in an '*in vitro*' assay. Even at high concentrations, anti-CB48 did not block the attachment or spreading of BAEC on collagen type IV. This situation is similar to that seen with the class III extracellular-matrix receptors described by Wayner & Carter (1987). Antibodies to this class of receptors do not inhibit the adhesion of cells to the appropriate collagen substrate. Thus the authors concluded that this class of receptors was not the primary mediators of cell adhesion.

The observations presented here argue that CB48 does not mediate BAEC adhesion to collagen type IV, but may have an alternative function within the cell. Hughes *et al.* (1987) have proposed that the function of colligin is to bind collagen monomers, preventing premature self-association during transport through the cell. Our findings support this hypothesis. We have localized CB48 predominately inside the cell, which is consistent with a role in intracellular transport. The observation that CB48 binds the collagenous region of collagen type IV, but not to the NC1 globular domain, would be expected of a protein capable of binding multiple collagen types and of a protein whose role may be to prevent the lateral association of collagen. It is possible that CB48 sterically

inhibits the association of the individual monomers by binding along the length of their collagenous regions. Dissociation of CB48 from the collagens could possibly occur via acidification of the transporting vesicle en route to the Golgi or the cell surface. If CB48 remains associated with the transporting vesicle en route to the cell surface, it would be detected when surface labelling the cells, explaining the iodination results presented in our studies and those of others (Sugrue, 1988; Kurkinen *et al.*, 1984). Further experimentation will determine if the 47–48 kDa collagen-binding proteins are indeed collagen-transport molecules.

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