A Novel Endothelial-specific Membrane Protein Is a Marker of Cell–Cell Contacts

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Abstract. mAbs were raised in mice against cultured human endothelial cells (EC) and screened by indirect immunofluorescence for their ability to stain intercellular contacts. One mAb denoted 7B4 was identified which, out of many cultured cell types, specifically decorated cultured human EC. The antigen recognized by mAb 7B4 is bound at the appositional surfaces of cultured EC only as they become confluent and is stably expressed at intercellular boundaries of confluent monolayers. EC recognition specificity was maintained when the antibody was assayed by immunohistochemistry in tissue sections of many normal and malignant tissues and in blood vessels of different size and type. The antigen recognized by 7B4 was enriched at EC intercellular boundaries similarly in vitro and in situ. In vitro, addition of mAb 7B4 to confluent EC increased permeation of macromolecules across monolayers even without any obvious changes of cell morphology. In addition, when EC permeability was increased by agents such as thrombin, elastase, and TNF/γIFN, its distribution pattern at intercellular contact rims was severely altered. mAb 7B4 immunoprecipitated a major protein of 140 kD from metabolically and surface-labeled cultured EC extracts which appeared to be an integral membrane glycoprotein. On the basis of its distribution in cultured cells and in tissues in situ, 7B4 antigen is distinct from other described EC proteins enriched at intercellular contacts. NH_2-terminal sequencing of the antigen, immunopurified from human placenta, and sequencing of peptides from tryptic peptide maps revealed identity to the cDNA deduced sequence of a recently identified new member of the cadherin family (Suzuki, S., K. Sano, and H. Tanihara. 1991. *Cell Regul.* 2:261–270.) These data indicate that 7B4 antigen is an endothelial-specific cadherin that plays a role in the organization of lateral endothelial junctions and in the control of permeability properties of vascular endothelium.

THE structural and functional integrity of the endothelium is an essential requirement for its property of permselective barrier between the blood stream and the underlying tissues. The maintenance of a continuous endothelial cell (EC)[1] monolayer of tightly apposed cells is also central for preventing the vessel wall from platelet deposition and thrombus formation. Morphological studies in vitro and in vivo have shown the presence of tight junctions and gap junctions between adjacent EC. (Schneeberger and Lynch, 1984; Franke et al., 1988).

In other cell types, cell–cell adhesion structures have been extensively studied and specific molecules of both the calcium dependent and independent cell adhesion mechanisms have been identified (Cunningham, 1990; Takeichi, 1990) but, in spite of the importance of cell–cell junctions in the maintenance of the endothelial functional properties, little is known about their structural and molecular organization.

1. *Abbreviation used in this paper*: EC, endothelial cell.

So far, a few integral membrane proteins have been described in the EC intercellular domain: PECAM-1 (Newman et al., 1990), also called CD31 (Simmons et al., 1990; Müller et al., 1989) or endo-CAM (Albelda et al., 1990); V-cadherin in bovine EC (Heimark et al., 1990) and the integrins $\alpha_5\beta_1$ and $\alpha_2\beta_1$ (Lampugnani et al., 1991). Among these proteins, however, only V-cadherin was expressed exclusively in EC from bovine vessels. PECAM-1 was detected also in monocytes, polymorphonuclear cells, and platelets while $\alpha_5\beta_1$ and $\alpha_2\beta_1$ integrins have a widespread distribution. While the distribution and localization of the above proteins are well described, their role in the maintenance of EC intercellular contacts is not yet clear. Recently, PECAM-1 has been shown to mediate cell–cell adhesion of full-length PECAM-1 cDNA transfected cell (Albelda et al., 1991).

To characterize the structures involved in EC intercellular junctions we raised mouse mAbs against cultured human umbilical vein EC. The resulting antibodies were screened by indirect immunofluorescence for their ability to stain intercellular boundaries of cultured human EC in monolayer.

Using this selective approach a mAb, denoted 7B4, was obtained that recognized an apparently novel antigen that was strictly distributed at intercellular EC boundaries not only in vitro but also in endothelia lining intact vessels within tissues.

Materials and Methods

Antibodies

mAbs to cultured human EC from umbilical vein were raised in mice as extensively described by Pigott et al., 1991. The isotype of both 7B4 and 9G11 mAbs was IGgl. Purified antibodies were prepared from hybridoma supernatants by affinity chromatography on Prosep-A (Bioprocessing Ltd., Consett, England). Fab fragments were obtained by papain digestion (Pierce Chemical Co., Rockford, IL). mAb 9G11 recognizes the antigen PECAM-1/CD31 (Simmons et al., 1990). As negative control either ascitic fluid containing the isotype matched mAb to CD2 lymphocyte antigen (OKT11, Ortho Diagnostic System Inc., Raritan, N.J.), which is not expressed by EC, or purified nonimmune mouse IgG (Calbiochem, La Jolla, CA) were used as indicated.

Rabbit anti-pan-cadherin serum (Geiger et al., 1990) was kindly provided by Dr. B. Geiger (Weizmann Institute, Israel). Rabbit serum to N- or P-cadherin were a kind gift of Dr. Vestweber (MPI für Immunobiologie, Freiburg, Germany). These two antibodies were raised against bacterial fusion proteins containing, respectively, the human P-cadherin coding sequence from bp 759 to the natural stop codon and the mouse N-cadherin coding sequence from bp 1755 to 3 amino acids before the natural stop. mAb (Ec6C10) and rabbit polyclonal (6422) antibodies to V-cadherin were kindly provided by Dr. R. L. Heimark (ICOS Corporation, Bothell, WA).

Cells

Human EC were isolated from umbilical vein (Barbieri et al., 1981) by treatment with collagenase (0.1% from *Clostridium histolyticum*, Boehringer Mannheim, Germany) for 20 min at 37°C. Cells were cultured in medium 199 (Gibco Laboratories, Grand Island, NY) with 20% newborn calf serum (NCS; Gibco Laboratories). 100 μg/ml endothelial cell growth supplement (ECGS, prepared from bovine brain), 100 μg/ml heparin (from porcine intestinal mucosa; Sigma Chemical Co., St. Louis, MO, USA), 50 U/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml fungizone at 37°C. Cells up to 10 in vitro passages were used.

Human femoral artery smooth muscle cells were a kind gift of Dr. G. Gabbiani (Department of Pathology, University of Geneva, Geneva, Switzerland) and cultured in DME (Gibco Laboratories) with 10% fetal calf serum (Gibco Laboratories). Human epidermal keratinocytes were kindly provided by Dr. M. De Luca (IST, Genova, Italy) and cultured as described by Marchisio et al. (1991).

Human dermal fibroblasts were cultured in medium 199 with 20% NCS.

Human blood polymorphonuclear cells (PMN) (Bazzoni et al., 1991), platelets (Del Maschio et al., 1990) and monocytes (Colotta et al., 1984) were isolated and washed as previously described in the related references.

Cell Treatments

Purified human α-thrombin (1,665 U/mg) kindly donated by Dr. J. W. Fenton II (State University of New York at Albany) and human elastase (875 U/mg; from human sputum, ECP, Elastin Products, Owensville, MO) were used. They were added in serum free culture medium to confluent EC at the indicated doses and time intervals. Treatment with tumor necrosis factor-α (TNF, 100 U/ml human recombinant, Basf-Knoll, Germany) and γ-interferon (human recombinant γIFN, 200 U/ml, Hoffman La Roche, Nutley, NJ) was started 24 h after cell seeding and continued for 72-96 h. When 7B4 either intact IGgs or Fab fragments were used in permeability experiments they were added in medium 199 with 5% NCS. 5 mM EGTA in serum-free culture medium was used to decrease extracellular Ca^{2+} concentration (Heimark et al., 1990) in permeability and immunofluorescence experiments for the time intervals indicated. Neuraminidase (2 U/ml, from *Clostridium perfringens*, type X; Sigma Chemical Co.) was added to confluent ^{125}Iodine surface-labeled EC (see below), in Ca^{2+} and Mg^{2+} PBS, pH 5.3, for 45 min at 37°C before cell processing for immunoprecipitation as described below. Treatment of ^{125}Iodine-labeled EC with trypsin (0.01%, Sigma Type III, 13,900 u/mg) was in Ca-Mg free Hank's buffer with either 1 mM Ca^{2+} or 1 mM EGTA for 20 min at 37°C. Trypsin was stopped with soybean trypsin inhibitor (0.05% Sigma, 1 mg inhibiting 1.6 mg trypsin with 10,000 U/mg) before cell processing for immunoprecipitation. Control incubations were in Hank's with 1 mM EGTA or 1 mM Ca^{2+} and 1 mM Mg^{2+}. Phosphatidylinositol (PI)-specific phospholipaseC (PLC, 2 μg/ml Boehringer Mannheim) (Ploug et al., 1991) in serum-free culture medium was given to ^{125}Iodine-labeled EC for 20 min at 37°C before cell extraction for immunoprecipitation. Treatment with EDTA (50 μM) after ^{125}Iodine surface labeling of confluent EC was for 15 min at 37°C (Müller et al., 1989). Cells were then processed for immunoprecipitation as described below.

Cell Labeling and Extraction

[^{35}S]Methionine Metabolic Labeling. EC which had grown to confluency, unless otherwise indicated, on gelatin-coated 10-cm^2 well plates were washed twice with methionine-free RPMI (Gibco Laboratories) and cultured overnight in methionine-free RPMI with 20% NCS ECGS, and heparin (see above) containing 60 μCi/ml [^{35}S]methionine (Amersham International, Buckingham, UK). Cell layers were then washed three times with serum-free medium 199 and extracted with 280 μl of 10 mM NaCl, 10 mM Tris-HCl, 0.5% sodium deoxycholate, 1% Tween 40, 1 mM PMSF, 20 U/ml aprotinin, pH 7.4. Cell extracts were centrifuged for 5 min in an Eppendorf centrifuge at 13,000 rpm. The supernatant was stored at −20°C for immunoprecipitation analysis.

^{125}Iodine-Surface Labeling. EC which had grown to confluency, unless otherwise indicated, on gelatin-coated 10 cm^2 well plates were washed three times with Ca^{2+}-Mg^{2+} Dulbecco's PBS. Cells were labeled using the glucose oxidase-lactoperoxidase method (Labien et al., 1982). Briefly, one well was incubated with 500 μl Ca^{2+}-Mg^{2+} PBS containing 20 μg/ml lactoperoxidase, 0.25 U/ml glucose oxidase, 200 μCi/ml ^{125}Iodine (NEN, DuPont, Dreieichenhain, Germany). 2.5 mM glucose for 5 min on ice. Glucose, 2.5 mM, was added again and the reaction continued for 5 more min. The reaction was stopped with 3 ml of medium 199 containing 0.02% azide. The cell layer was washed three more times with medium 199 containing 0.02% azide and extracted as described above. In a few experiments, cell extraction was with 150 mM NaCl, 10 mM Tris HCl, 1 mM PMSF, 20 U/ml aprotinin, and either 1% Triton X-100 (TX-100) or 1% Triton X-114 (TX-114). TX-114 buffer and detergent phase were separated as described by Bordier (1981).

Immunoprecipitation

Protein G-Sepharose (recombinant; Pharmacia, Uppsala, Sweden) was washed three times with TBS (150 mM NaCl, 10 mM Tris-HCl, pH 7.4). For each sample, the resin pelleted from 500 μl of a 10% suspension was used. Coupling with the antibodies (either 500 μl undiluted hybridoma conditioned medium for 7B4 and 9G11 or 25 μg/ml of purified IgGs or 300 μl TBS containing a 1:200 dilution of the nonrelevant IgG isotype matched CD2 ascitic fluid) was for 60 min at room temperature under continuous mixing. The resin was then washed with TBS containing 1% Tween 40, 1 mM PMSF, 20 U/ml aprotinin, and 1 mM $CaCl_2$, $MgCl_2$, and $MnCl_2$, respectively (immunoprecipitation buffer). Incubation of cell extracts with protein G-Sepharose was in a total volume of 300–500 μl immunoprecipitation buffer for 1 h at room temperature under continuous mixing. The presence of divalent cations during this phase was mandatory to effective immunoprecipitation with mAb 7B4. In some experiments, as indicated, samples were precleared by incubating them for 1 h with uncoupled protein G-Sepharose. The supernatant was collected and used in immunoprecipitation. The resin was washed three times with immunoprecipitation buffer (1 ml a time). Sample buffer 2× (50 μl of 20% Tris-HCl 0.5 M, pH 6.8, 5% SDS, 20% glycerol, 0.25 mg/ml bromophenol in water; when indicated 5% 2-mercaptoethanol was added as a reducing agent) was added to the resin pellet which was boiled for 3 min. Supernatant was analyzed by electrophoresis on a 7.5% polyacrylamide gel in the presence of SDS. In each separation, ^{14}C-molecular weight standard proteins (Amersham International) were run in parallel to the samples. Dryed gels were exposed for autoradiography on X-AR film (Eastman Kodak Co., Rochester, NY) at −70°C. Gels with [^{35}S]methionine labeled samples were equilibrated with EN^{3}HANCE (NEN) before drying and exposure. Quantification of the band was made after digital image analysis of the film (RAS 3000 Loats System; Amersham International).

Lectin Binding

Cell extracts from ^{125}Iodine-labeled EC were incubated with Con A

Sepharose (Pharmacia LKB) in TBS with 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$ (binding buffer) either in the absence or in the presence of 0.5 M methyl α-D mannopyranoside (Sigma Chemical Co.) for 1 h at room temperature. The unbound fraction (supernatant) was stored. The resin was washed with binding buffer and bound material (eluant) eluted, incubating the resin with the competing sugar methyl-α-D mannopyranoside (0.5 M) for 1 h at room temperature. Both the supernatant and the eluant fractions were subjected to immunoprecipitation. Binding to wheat germ agglutinin (WGA) agarose (Pharmacia) was identical to the binding to Con A except that *N*-acetyl-D-glucosamine (0.5 M; Sigma Chemical Co.) was used as competing sugar.

Immunoblotting

After separation by SDS-electrophoresis, samples were electrotransferred onto nitrocellulose (Bio-Rad Laboratories, Richmond, CA) blocked with 3% BSA (fatty acid free type; Sigma Chemical Co.) in PBS and incubated overnight with a 1:100 dilution of either a rabbit anti-pan-cadherin serum or rabbit serum to N- or P-cadherin (see previous "Antibodies" section). The immunoreactive bands were revealed by ^{125}Iodine-labeled protein A (Amersham International) followed by autoradiography.

Immunofluorescence

Cells to be examined by immunofluorescence microscopy were grown on glass coverslips. Glass coverslips (13-mm diameter) were coated overnight at 4°C with either 1.5% gelatin (Difco) or 7 μg/ml fibronectin purified from human plasma (Engvall and Ruoslahti, 1977) or 7 μg/ml vitronectin purified from human serum (Yatohgo et al., 1988), then rinsed with serum-free medium 199 before seeding 3.5×10^4 cells/coverslip in 0.4 ml culture medium. Cells were grown to confluency for 72 h, unless otherwise specified. Coverslip-attached cells were then fixed with paraformaldehyde and processed for immunofluorescence microscopy as previously described in detail (Lampugnani et al., 1991). Briefly, the primary antibody was layered on fixed and permeabilized cells and incubated in a humid chamber for 30 min. After rinsing in 0.1% BSA in TBS, coverslips were incubated in the appropriate rhodamine-tagged second antibody (Dakopatts, Glostrup, Denmark) for 30 min at 37°C in the presence of 0.2 μg/ml of fluorescein-labeled phalloidin (F-PHD; Sigma Chemical Co.). Coverslips were then mounted in Mowiol 4-88 (Hoechst, Frankfurt, Germany).

Observations were carried out in a Zeiss Axiophot photomicroscope equipped for epifluorescence. Fluorescence images were recorded on Kodak T-Max 400 films exposed at 1000 ISO and developed in Kodak T-Max developer for 10 min at 20°C.

Immunohistochemistry

Fragments of human normal kidney (2), heart (2), lung (3), thymus (3), lymph nodes (5), thyroid (2), parathyroid (1), salivary gland (1), stomach (2), small and large intestine (2), testis (1), bone marrow (1), placenta (1) lung carcinoma (3), and intestinal carcinoma (2) were obtained at surgery or at autopsy. Tissue fragments were embedded in OCT compound (Ames Division, Miles Laboratories, Elkhart, IN), snap frozen in liquid nitrogen, and stored at −80°C until sectioning. Other fragments were formalin-fixed and paraffin-embedded for conventional histology. Cryostat sections were fixed in acetone for 10 min at room temperature and were immunostained with anti-PECAM-1/CD31 (mAb 9G11) or mAb 7B4 using avidin-biotin-peroxidase complex technique. Sections were preincubated with normal horse serum to prevent nonspecific binding, and then incubated with an optimal dilution of the primary antibody (1/10) for 30 min. The slides were sequentially incubated with biotin-conjugated horse anti-mouse Ig antibodies followed by avidin-biotin-peroxidase complex (PR 4002; Vector Laboratories, Burlingame, CA). Each incubation step lasted 30 min with 5-min TBS washes between each step. The sections were finally incubated with 0.03% H_2O_2 and 0.06% 3,3′ DAB (Sigma Chemical Co.) for 3–5 min. Slides were then washed for 5 min in running tap water, counterstained with hematoxylin for 5 min, and mounted in Canada balsam. Endogenous peroxidase was generally visualized only in eosinophils.

Measure of EC Barrier Properties

The Transwell TM cell culture chambers (polycarbonate filters, 0.4 μm pore size, Costar, Cambridge, MA) were used as described by Lampugnani et al. (1991). The polycarbonate filters were coated with 10 μg/ml human fibronectin for 1 h at room temperature, rinsed with serum-free medium before seeding 2×10^4 cells in 100 μl culture medium in the upper compartment. 600 μl of culture medium filled the lower compartment. Culture was continued for 5 d with daily refeeding. Before the experiment, the culture medium of both the upper and lower compartments was replaced with medium 199 either serum free or with 5% NCS as indicated in the "Cell treatments" paragraph. HRP (0.126 μM; HRP, VI-A type, 44,000 mol wt, 1,280 U/mg; Sigma Chemical Co.) was added to the upper compartment. After 1 h at 37°C, the medium in the lower compartment was collected and kept on ice until the enzymatic activity of HRP was assayed. To assay HRP enzymatic activity (Ortiz de Montellano et al., 1988), 60 μl of the culture medium, collected from the lower compartment, was added to 860 μl of a reaction buffer (50 mM NaH_2PO_4 with 5 mM guaiacol) and the reaction was started by adding 100 μl H_2O_2 (0.6 mM in H_2O, freshly made solution). The reaction was allowed to proceed for 25 min at room temperature before measuring the adsorbance at 470 nm.

Affinity Chromatography

Human placentae were minced, lysed with 0.005% digitonin (Calbiochem, San Diego, CA) in TBS with 1 mM Ca^{2+}, 1 mM Mg^{2+} and 1 mM Mn^{2+} ($Ca^{2+}/Mg^{2+}/Mn^{2+}$ TBS), 1 mM PMSF, and 20 U/ml aprotinin for 15 min at 4°C followed by 10 min centrifugation at 5,000 *g* at 4°C. Pellets were extracted with 1% Tx-100 (Bio-Rad Laboratories) in $Ca^{2+}/Mg^{2+}/Mn^{2+}$ TBS, 1 mM PMSF, and 20 U/ml aprotinin for 1 h at 4°C. The extract was centrifuged for 30 min at 10,000 *g* at 4°C. The supernatant was stored at −70°C before processing.

mAb 7B4 purified IgGs were covalently coupled to CNBr-Sepharose (Pharmacia), according to the manufacturer's instructions. Placenta extract was passed through a 7B4-CNBr-Sepharose column. The column was washed with $Ca^{2+}/Mg^{2+}/Mn^{2+}$ TBS and the antigen eluted with 0.1 M acetic acid, pH 2.5. Samples were stored at −70°C before further processing.

Protein Sequencing

Proteins and peptides were sequenced on a Sequenator (Model 477A; Applied Biosystems) using chemicals and protocols supplied by the manufacturer. For direct NH_2-terminal sequencing, samples of 100–200 μl were dried down on Protein support filters (Porton Instruments) and placed in the sequenator reaction cartridge.

For sequencing of proteins after SDS-PAGE, gels were subjected to overnight preelectrophoresis in 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 20 mM mercaptopropionic acid with a current of 5 mA/gel. Samples were boiled in sample buffer and subjected to electrophoresis in 25 mM Tris, 0.2 M glycine, 0.1% SDS, pH 8.3. After SDS-PAGE the proteins were electroblotted onto polyvinyl difluoride membranes using 25 mM Tris, 0.2 M glycine, pH 8.3 as transfer buffer. The membranes were stained with Coomassie R 250 in 40% MeOH and destained in 40% MeOH. After extensive washing with water individual bands were cut out and subjected to sequencing.

Peptide Mapping

Samples were dissolved in 100 μl of 0.1 M phosphate buffer, pH 7.5, containing 20% MeOH and TPCK-Trypsin (Worthington) was added (enzyme/substrate ratio 1:100 [wt/wt]). After incubation overnight at 37°C, a second aliquot of trypsin was added and digestion was continued for 2 h at 37°C. The reaction was terminated by addition of trifluoroacetic acid (TFA) to 1% (vol/vol) and samples were chromatographed on a 0.2 cm × 20 cm RP-C18 column (5 μm particles) using a model 130 A HPLC system (Applied Biosystems) and linear gradient from 0.1% TFA to 70% CH_3CN, 0.085% TFA over 50 min (flow: 275 μl/min). Individual peaks were collected and subjected to automated sequence analysis as described above.

Results

Localization of 7B4 Antigen in Cultured Human Endothelial Cells

Cultured EC monolayers were examined by immunofluorescence microscopy. As reported in Fig. 1 *a*, mAb 7B4 reacted with an antigen concentrated at the boundaries between closely apposed cells. The staining pattern consisted of a thin and sharp continuous line highlighting the margins of each cell. Notably, a bright signal was detectable in a cell com-

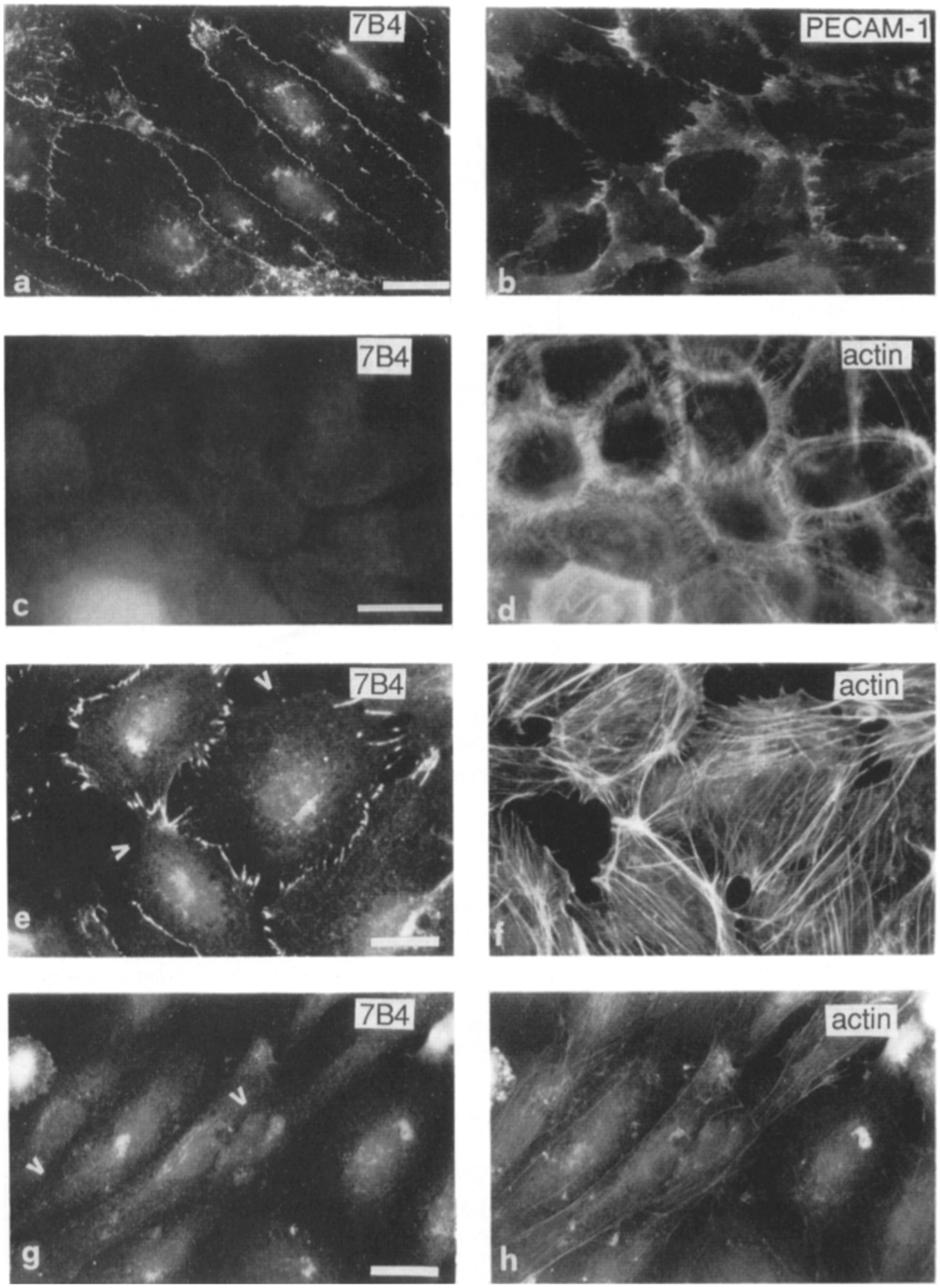

Figure 1. Immunofluorescence distribution of 7B4 antigen and PECAM-1 in confluent EC stained with mAb 7B4 (*a*) and mAb 9G11 to PECAM-1 (*b*). Immunofluorescence distribution of 7B4 antigen in colonies of human epidermal keratinocytes that are negative to mAb 7B4 (*c*). The F-actin pattern of the same cells is shown in *d*. *e* shows the distribution of 7B4 antigen in immunofluorescence of nonconfluent EC. Cell-free margins are negative to 7B4 (*arrows*) and reactivity is restricted to the areas of established cell contacts. Cells were double stained for F-actin with F-PHD (*f*). In *g* the effect of EGTA (5 mM for 20 min) on the distribution of 7B4 antigen in confluent EC is shown. No localized reactivity could be detected even at the areas of apparent residual cell contact (*arrows*). F-actin distribution in the same cells is shown in *h*. Bars: (*a*, *b*, *c*, *d*, *g*, *h*) 5 μm; (*e* and *f*) 3 μm.

partment that may be identified with the Golgi area (see also Fig. 1, *e* and *g*). When confluent monolayers of EC were decorated with a PECAM-1/CD31 mAb a different pattern of staining was observed (Fig. 1 *b*). The fluorescence signal along cell margins was more discontinuous and in many areas more diffuse than with mAb 7B4.

The distribution of mAb 7B4 antigen at cell margins was essentially identical when EC were seeded on different substrata such as gelatin, fibronectin, or vitronectin. Further, staining pattern and intensity did not change up to 10 cell passages (not shown).

The presence of 7B4 antigen and, for comparison, that of PECAM-1/CD31 in other cell types including circulating cells (such as platelets, monocytes and granulocytes), other components of the vessel wall (such as fibroblasts and smooth muscle cells), or keratinocytes was examined by indirect immunofluorescence. mAb 7B4 recognized only EC while it did not bind to any other tested cell type (Table I and, e.g., Fig. 1, *c* and *d*). In contrast, as expected (Newman et al., 1990), PECAM-1 was detected in monocytes, polymorphonuclear cells and platelets (Table I). The reactivity of 7B4 was restricted to human EC; bovine EC from both large vessels and capillaries were negative (not shown). The enrichment of 7B4 at cell borders requires the establishment of intercellular contacts. In nonconfluent cultured EC examined by indirect immunofluorescence the antigen was detected only at contacting cell borders and never along free cell edges (Fig. 1, *e* and *f*). In nonconfluent EC the level of the antigen exposed at the cell surface was comparable to that of confluent cells. Indeed, mAb 7B4 immunoprecipi-

Table I. Cultured Cell Staining by 7B4 and PECAM-1 Antibodies

	7B4	PECAM-1
Endothelial cells	+	+
Platelets	−	+
Monocytes	−	+
Granulocytes	−	±
Keratinocytes	−	−
Fibroblast	−	−
Smooth muscle cells	−	−

Reactivity of human cultured cells and human blood cells to 7B4 and anti-PECAM-1 was tested by indirect immunofluorescence.

tated comparable amounts of ^{125}Iodine-labeled protein from the same number of either confluent or nonconfluent EC (not shown). The maintenance of 7B4 antigen at the cell–cell contacts depends on the presence of Ca^{2+}. EGTA (5 mM for 20 min) induced the disappearance of the antigen from the cell boundaries even in the areas of apparent residual intercellular contacts (Fig. 1, *g* and *h*). This effect was accompanied by increased permeability of the EC monolayer (see Table III). The amount of ^{125}Iodine-labeled protein immunoprecipitated by mAb 7B4 was comparable in EGTA-treated and in control cells (see also Fig. 4), thus indicating that the treatment induces a redistribution of the protein on the cell membrane but not its disappearance.

Expression of 7B4 Antigen In Situ

Expression of 7B4 antigen in situ was studied in a series of tissues, as listed in Table II. For comparison, the immunolocalization of PECAM-1/CD31 was also examined. The 7B4 antigen and PECAM-1 showed a different cellular distribution in tissue sections. Immunoreactivity to 7B4 was restricted to the vascular endothelial layer of the vessels in all the tissues examined, which comprised both normal and malignant tissues. The molecule was constitutively expressed in the endothelium of muscular arteries, arterioles, capillaries, venules, and veins and was mostly present at EC boundaries as shown in Fig. 2 (*a* and *c*). No significant staining was observed on the apical or basal aspect of EC membrane or in the cytoplasm. Cardiac and skeletal muscle, vascular smooth muscle, macrophages, fibroblasts, and any tested epithelial type did not contain detectable levels of this antigen. The immunostaining for PECAM-1/CD31 was different from that of 7B4 both in terms of tissue specificity and cellular localization. In fact, PECAM-1/CD31 was present also in nonendothelial cells, including platelets, megakaryocytes, and macrophages. In addition, the staining for PECAM-1/CD31 in EC lined intercellular contact rims and was also diffuse in the cytoplasm (Fig. 2 *b*). Interestingly, the pattern of cellular localization of 7B4 and PECAM-1/CD31 in tissue sections was similar to that observed in cultured EC (compare Fig. 1, *a* and *b*).

Biochemical Characterization of 7B4 Antigen

To identify the antigen recognized by mAb 7B4, extracts from [^{35}S]methionine-labeled EC were immunoprecipitated with the mAb. As shown in Fig. 3 (*A*), mAb 7B4 precipitated a major protein band of ~140 kD apparent molecular mass. As a control an isotype matched mAb to CD2 lymphocyte

Table II. Tissue Distribution of 7B4 and PECAM-1 Antibodies

Cell type	7B4	PECAM-1
Endothelial cells*	++	+++
Endothelial macrophages‡	++	+++
Megakaryocytes	−	++
Platelets	−	++
Epithelioid macrophages	−	++
Macrophages	−	+
Lymphocytes	−	−
Fibroblasts	−	−
Myocardium	−	−
Smooth muscle	−	−
Skeletal muscle	−	−
Peripheral nerve	−	−
Tissue examined		
Keratinizing epithelia		
Non-keratinizing epithelia		
Thymic		
Breast		
Thyroid		
Parathyroid		
Salivary gland		
Stomach		
Ileum		
Colon		
Lung		
Kidney		
Placenta		
Testis		
Squamous carcinoma		
Adenocarcinoma		

Cryostat sections of skin, kidney, heart, lung, thymus, lymph node, thyroid, parathyroid, salivary gland, stomach, ileum, colon, testis, placenta, bone marrow, lung squamous carcinoma, and colon adenocarcinoma were immunostained with mAb 7B4 and mAb 9G11 to PECAM-1 using avidin-biotin-peroxidase method.

* The antigen was constitutively expressed by EC in all tissues examined. The reactivity was present in muscular arteries, arterioles, capillaries, venules, and veins.

‡ The reactivity was expressed by macrophages lining lymph node sinuses. These cells represent a peculiar subset of macrophages sharing many features with EC.

antigen, which is not expressed by EC, was used in all the immunoprecipitation experiments (Fig. 3). These data indicate that 7B4 recognizes a protein synthesized by EC.

mAb 7B4 immunoprecipitated a major band of 140 kD also from ^{125}Iodine-surface labeled EC (Fig. 3 *B*), indicating that the antigen is externally exposed. After reduction with 2-mercaptoethanol, the apparent molecular mass increased slightly to 145 kD (Fig. 3 *B*), suggesting that intrachain disulfide bonds are present in the molecule.

Neuraminidase treatment (2 U/ml for 45 min) resulted in a decrease to 135 kD of the apparent molecular mass (Fig. 3 *C*), suggesting sialylation of the protein. Furthermore, the protein recognized by 7B4 bound to Con A, but not to WGA (Fig. 3 *D*) suggesting that it is a glycoprotein with mannose-containing oligosaccharide moieties.

EDTA (50 μM for 15 min at 37°C, Müller et al., 1989) did not strip 7B4 antigen from EC surface (data not shown), thus, indicating that it is an integral membrane protein. In apparent contrast, when ^{125}Iodine-labeled EC were extracted with TX-114 detergent the 7B4 antigen was mostly immunoprecipitated from the buffer phase (Fig. 4 *A*). This

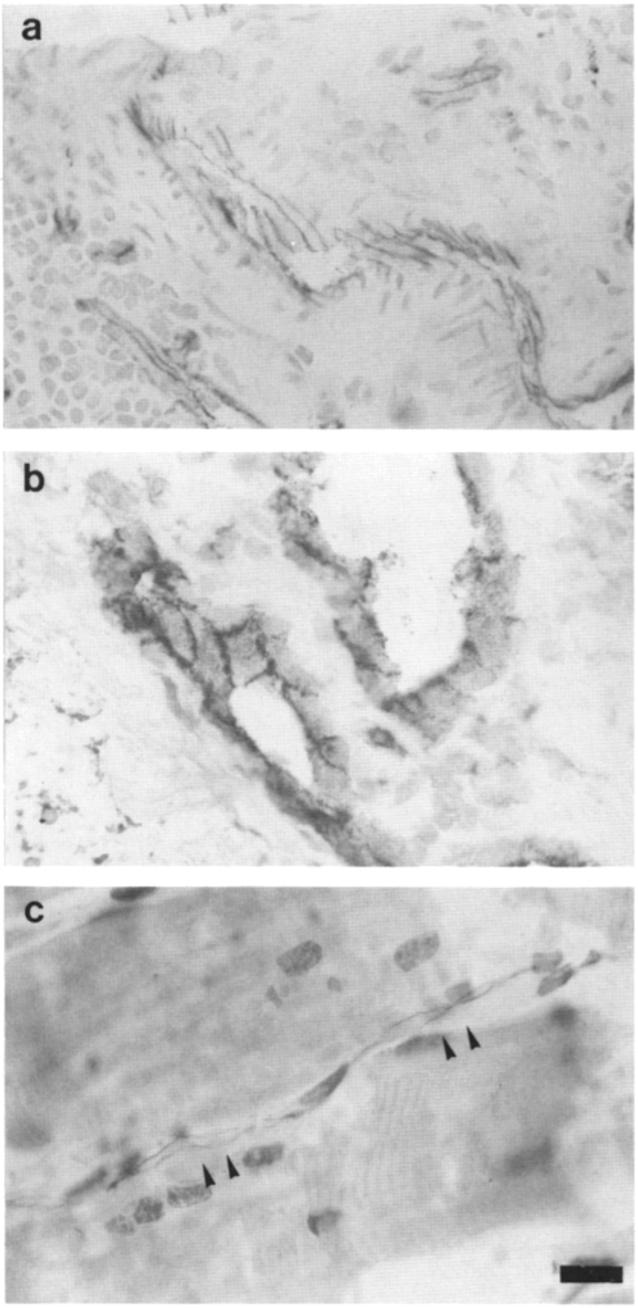

Figure 2. Immunoperoxidase localization of 7B4 antigen and PECAM-1. Cryostat sections of a human lymph node were stained with mAb 7B4 (*a*) and mAb to PECAM-1 (*b*). (*a*) Reactivity to mAb 7B4 is selectively expressed by EC lining an arteriole, a venule (*lower left*), and some cross-sectioned capillaries. The staining is present at lateral cell borders of contiguous EC. (*b*) PECAM-1 reactivity is expressed by EC lining two venules and some macrophages of the connective tissue (*upper left*). The staining of EC is weak in the cytoplasm and more pronounced at the intercellular borders. (*c*) Immunoperoxidase localization of 7B4 antigen in a cryostat section of a human skeletal muscle. An interstitial capillary vessel is highlighted by the linear staining of EC with mAb 7B4 (*arrows*). No reactivity is present on the striated muscle cells. Avidin-biotin-peroxidase complex, counterstained with hematoxylin. Bars: (*a*) 40 μm; (*b* and *c*) 25 μm.

unexpected distribution has been reported for other integral membrane proteins (Volk and Geiger, 1986 and references therein).

7B4 was sensitive to trypsin (0.01% for 20 min) both in the absence and presence of Ca^{2+} (Fig. 4 *A*).

PI-specific PLC did not detach 7B4 from the cell surface (Fig. 4 *A*). This makes unlikely a connection through PI-containing lipids as reported for other membrane proteins (Ferguson and Williams, 1988).

100-kD band(s) appeared constantly in the immunoprecipitate from ^{125}Iodine surface-labeled EC at variable intensity and was found occasionally in [^{35}S]methionine-labeled EC. Surface iodination requires longer cell manipulation before extraction than [^{35}S]methionine metabolic labeling (see Materials and Methods). In preliminary experiments, we found that the intensity of this band increased in parallel to a decrease of the 140-kD band when the cell extract was repeatedly frozen and thawed. We then suggest that the 100-kD band represents a degradation product of the higher 140-kD band. An unspecific band of ~180 kD appeared in the immunoprecipitates from ^{125}Iodine-surface labeled EC of both CD2 and 7B4. This protein had an immunoglobulin-like behaviour as it linked to protein G–Sepharose in the absence of CD2 or 7B4 (not shown) and its molecular mass decreased to ~55 kD upon reduction (Fig. 3 *B*). Immunoprecipitates of cell extracts precleared on protein G were depleted of this unspecific band (Fig. 4, *A* and *B*). It is not a metabolic product of the cells, as it never appeared in immunoprecipitates from [^{35}S]methionine-labeled EC. Overall, this strongly suggests that it represents serum derived IgG absorbed to EC from the culture medium.

Comparison of 7B4 to Other EC Adhesion Proteins

Intercellular location and size suggest that 7B4 antigen could be similar to other molecules previously found at intercellular EC contacts. By immunoprecipitation analysis, PECAM-1/CD31 migrates at a lower position than 7B4 antigen (Fig. 4 *B*). Furthermore, while 7B4 is sensitive to trypsin treatment (Fig. 4 *A*), PECAM-1/CD31 was detached in trypsinized cells (not shown). These data and the differences in cell recognition specificity (Tables I and II) indicate that 7B4 is a protein distinct from PECAM-1/CD31. Furthermore, transfected COS cells expressing a full-length PECAM-1 molecule are not recognized by mAb 7B4 (R. Pigott, unpublished results).

EC reportedly express mRNA for membrane proteins of the intercellular contacts belonging to the cadherin family: N and P (Liaw et al., 1990). Cadherins have a molecular weight in the range of that of 7B4 antigen. We therefore tested whether the 7B4 antigen could be recognized by cadherin antibodies. A polyclonal antibody directed to the COOH-terminal 24 amino acid domain of cadherins (Geiger et al., 1990) was used. The conserved nature of this domain allows this antibody to react with all known cadherins. This antibody detected a band at the expected 140 kD apparent molecular mass in the total EC extracts (Fig. 5 *A*), thus confirming the presence of cadherins in human EC. It also recognized a band of slightly lower molecular mass in the immunoprecipitate of mAb 7B4. As expected, no reactivity was found in the immunoprecipitate of anti-PECAM-1 (Fig. 5 *A*). When polyclonal antibodies to N- and P-cadherins

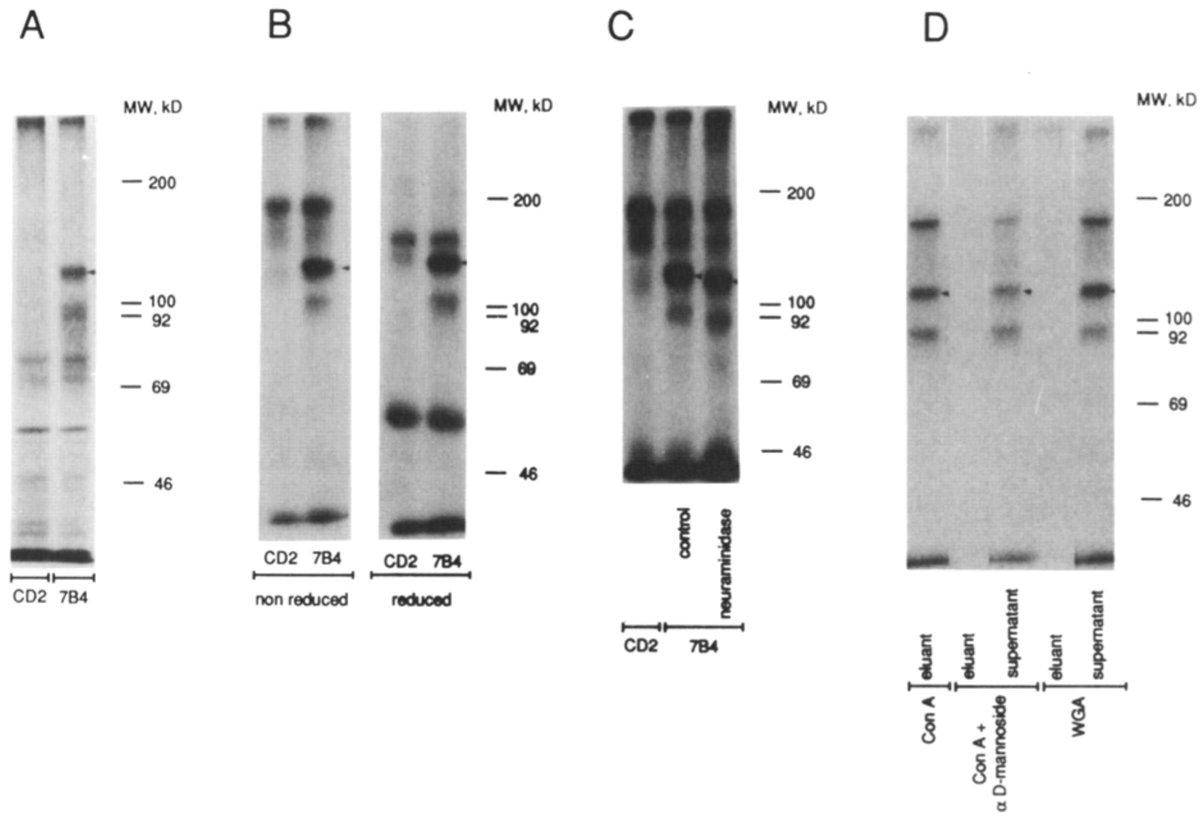

Figure 3. (*A*) Immunoprecipitation analysis of [^{35}S]methionine-labeled confluent EC. mAb 7B4 immunoprecipitated a band of ∼140 kD apparent MW (*arrow*). The isotype matched mAb to the lymphocytic CD2 antigen, not expressed by EC, was used as a negative control. (*B*) Immunoprecipitation analysis of ^{125}Iodine-surface labeled confluent EC. The effect of reduction on 7B4 antigen migration position (*arrows*) is shown. (*C*) The effect of treating the labeled EC monolayer with neuraminidase (2 U/ml for 45 min) before extraction and immunoprecipitation with mAb 7B4 (*arrows*) is shown. *D* illustrates the binding of 7B4 antigen to lectin-conjugated resins. 7B4 antigen (*arrows*) could be immunoprecipitated from the eluant of ConA-Sepharose obtained washing the resin with the competing sugar methyl α-D-mannopyranoside. When the cell extract was made to react with Con A in the presence of methyl α-D-mannopyranoside no 7B4 antigen could be immunoprecipitated from the eluant obtained washing the resin with the competing sugar methyl α-D-mannopiranoside. Under the same condition, as expected, the antigen could be immunoprecipitated from the unbound fraction (supernatant). 7B4 antigen (*arrows*) could not be immunoprecipitated from the eluant of WGA-agarose obtained washing the resin with the competing sugar *n*-acetylglucosamine. 7B4 antigen was present, as expected, in the unbound fraction supernatant. The migration of molecular weight standards run in parallel is shown on the right of each panel.

were used in the same kind of experiments they recognized a protein band of ∼140 kD in the total cell extract (Fig. 5 *B*). The band recognized by anti-P-cadherin was much fainter than that recognized by anti-N-cadherin, in apparent agreement with Liaw et al. (1990). None of the two antibodies detected any specific band in the immunoprecipitate of mAb 7B4 (Fig. 5 *B*) thus indicating that 7B4 antigen does not correspond to N- or P-cadherins. The high MW bands detected in both the 7B4 and PECAM-1 antibodies immunoprecipitates are the IgG recognized by iodinated protein A (Fig. 5, *A* and *B*). Both a monoclonal and a polyclonal antibody to bovine endothelial cell V-cadherin (Heimark et al., 1990) did not recognize any specific band in cultured human umbilical vein EC, possibly indicating a lack of crossreactivity.

Identification of 7B4 Antigen as a Novel Cadherin by Amino Acid Sequencing

Preparations of 7B4 antigen purified by immunoaffinity columns from placenta tissue were subjected to automated NH_2-terminal microsequencing yielding information up to residue 17 (Fig. 6). The 7B4 antigen NH_2-terminal sequence showed identity with the cDNA deduced NH_2-terminal sequence of a new member of the cadherin family recently characterized at the cDNA level and denoted cadherin-5 (Suzuki et al., 1991) (Fig. 6). Tryptic mapping of the protein and sequencing of the resulting peptides indicated identity also in some internal sequences (Fig. 6).

Effect of mAb 7B4 on Endothelial Cell Barrier Function

The intercellular location of 7B4 suggests that it can play a role in the functional organization of endothelial cell to cell junctions. When mAb 7B4 either in the form of intact IgG (up to 400 μg/ml) or Fab fragments (up to 400 μg/ml) were added to EC monolayers up to 12 h, they had no apparent effect on cell morphology as examined by phase-contrast microscopy and immunofluorescence by actin staining (not shown). To increase our sensitivity, we then used a permeability assay, which had been previously found to show varia-

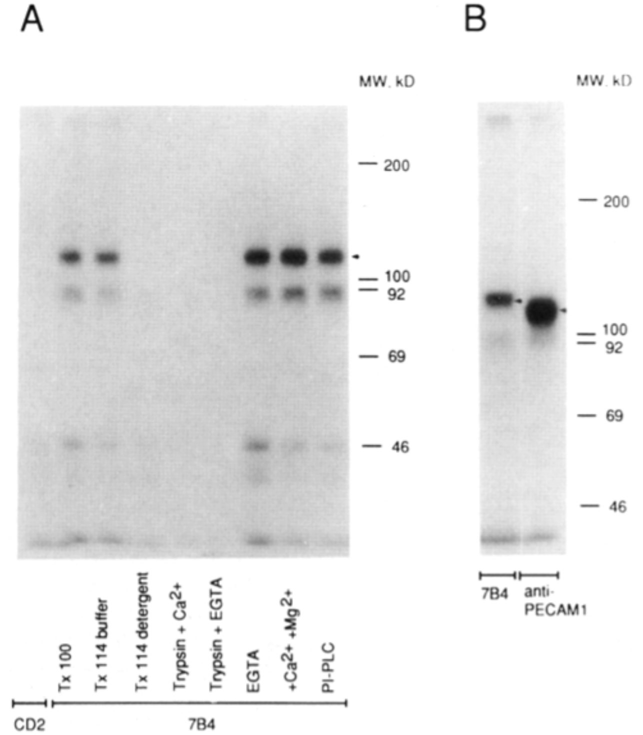

Figure 4. Immunoprecipitation analysis of [125]Iodine-surface labeled EC. (*A*) The effect of various treatments on 7B4 antigen is shown: partition of 7B4 antigen in TX-114 buffer and detergent phase; EC treatment with trypsin (0.01% for 20 min) in the presence of either 1 mM Ca^{2+} or 1 mM EGTA and for comparison 1 mM EGTA alone or 1 mM Ca^{2+} and Mg^{2+}; and EC treatment with PI-PLC (2 μg/ml for 20 min). (*B*) Comparison of the immunoprecipitation patterns obtained with mAb 7B4 and mAb to PECAM-1. In these experiments cell extracts were precleared by incubation for 1 h with uncoupled protein G Sepharose before immunoprecipitation. The migration of molecular weight markers run in parallel is shown on the right of each panel.

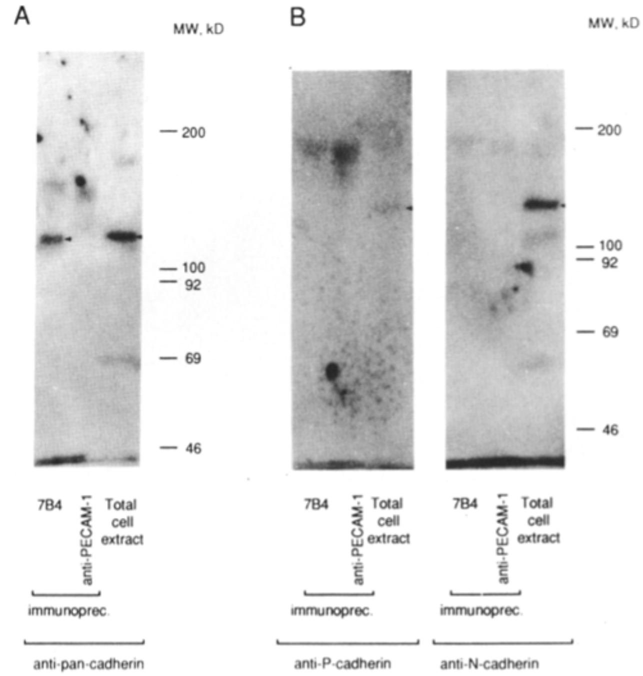

Figure 5. Immunoblot analysis with pan-cadherin, P-cadherin, and N-cadherin antibodies of mAb 7B4 and anti-PECAM-1 immunoprecipitates. (*A*) Cell extracts of confluent EC were immunoprecipitated with mAb 7B4 or anti-PECAM-1 (see Materials and Methods). The immunoprecipitates were blotted with anti-pan-cadherin. For comparison the starting cell extract (total cell extract) was blotted in parallel. (*B*) cell extracts of confluent EC were immunoprecipitated with mAb 7B4 or anti-PECAM-1. The immunoprecipitates and, for comparison, the starting cell extracts (total cell extract) were blotted with anti-P-cadherin and anti-N-cadherin. The migration of molecular weight markers is shown on the right of each panel.

tions of the barrier function of EC even in the absence of overt morphological alterations (Lampugnani et al., 1991). When Fab fragments were added to EC grown on Transwell filters a dose-response increase in the permeability of the EC monolayer was observed (Fig. 7). This increase was apparent at ~100 μg/ml mAb and at a minimal time of incubation of ~2 h reaching a plateau between 4 and 6 h (not shown). Comparable results were also obtained with intact IgGs.

7B4 Antigen Organization in Endothelial Cells Treated with Agents That Alter Monolayer Integrity

Several molecules have been reported to alter morphology and intercellular contacts of EC. As reported in Table III, treatment of EC with TNF and γIFN, with thrombin or elastase significantly increased monolayer permeability in Transwell experiments.

We checked first by indirect immunofluorescence the effect of these treatments on the distribution of 7B4 antigen. Thrombin added to the cells (5 U/ml for 30 min) induced retraction accompanied by small areas of detachment at intercellular boundaries (see actin, Fig. 8 *c*). 7B4 antigen disappeared from areas of cell retraction and the staining was restricted to residual cell contacts (Fig. 8 *d*, *arrows*). Changes in 7B4 antigen distribution was even more dramatic after elastase treatment of the cells (0.8 μM for 30 min) (Fig. 8 *f*). Cell retraction was observed after treatment (see actin, Fig. 8 *e*), yet 7B4 antigen did not show any discrete distribution even at residual intercellular contacts, but was diffusely distributed on EC membrane. Interestingly, the effect of thrombin on cell retraction and 7B4 localization was fully reversible in ~1 h, even in the continuous presence of the stimulus. In contrast the effect of elastase was not reversible up to 3–4 h (not shown).

When EC were incubated with γIFN and TNF (200 U/ml and 100 U/ml for 72 H) they acquired elongated morphology with relaxed intercellular contacts (Fig. 8 *g*) as described (Stolpen et al., 1986). 7B4 assumed a peculiar, punctate distribution all over the cell body and could be found at cell to cell contacts only in a few areas (Fig. 8 *h*).

We then investigated whether the morphological effects observed after EC treatment with thrombin, elastase or γIFN and TNF were paralleled by quantitative or qualitative modification of 7B4 antigen. As reported in Fig. 9, the amount of [125]Iodine-labeled antigen precipitable by mAb 7B4 was unmodified after γIFN and TNF (Fig. 9 *B* and par-

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                                                                                      D w I w N Q M H I D
M M L L A T S G A C L G L L A V A A V A A A G A N P A Q R D T H S L L P T H R R Q K R D W I W N Q M H I D
                                                                                     ↑
E e K - T - L                                                                                       L D r
E E K N T S L P H H V G K I K S S V S R K N A K Y L L K G E Y V G K V F R V D A E T G D V F A I E R L D R

E N I S E Y - L T A V I V
E N I S E Y H L T A V I V D K D T G E N L E T P S S F T I K V H D V N D N W P V F T H R L F N A S V P E S

S A V G T S V I S V T A V D A D D P T V G D H A S V M Y Q I L K G K E Y F A I D N S G R I I T I T K S L D

                                        G D - G - A T V L V T L Q d I N - N F P             Y T F V V P E
R E K Q A R Y E I V V E A R D A Q G L R G D S G T A T V L V T L Q D I N D N F P F F T Q T K Y T F V V P E

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D T R V G T S V G S L F V E D P D E P Q N R M T K Y S I L R G D Y Q D A F T I E T N P A H N E G I I K P M

                                                  y m s p p a g n
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N E K E L D R E V Y P W Y N L T V E A K E L D S T G T P T G K E S I V Q V H I E V L D E N D N A P E F A K

P Y Q P K V C E N A V H G Q L V L Q I S A I D K D I T P R N V K F K F I L N T E N N F T L T D N H D N T A

N I T V K Y G Q F D R E H T K V H F L P V V I S D N G M P S R T G T S T L T V A V C K C N E Q G E F T F C

E D M A A Q V G V S I Q A V V A I L L C I L T I T V I T L L I F L R R R L R L Q A R A H G K S V P E I H E

Q L V T Y D E E G G G E M D T T S Y D V S V L N S V R R G G A K P P R P A L D A R P S L Y A Q V Q K P P R

H A P G A H G G P G E M A A M I E V K K D E A D H D G D G P P Y D T L H I Y G Y E G S E S I A E S L S S L

G T D S S D S D V D Y D F L N D W G P R F K M L A E L Y G S D P R E E L L Y
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Figure 6. Comparison of the 7B4 NH_2-terminal sequence and sequences obtained from tryptic peptide mapping (*upper* lanes) with the cDNA derived sequence of human cadherin-5 (*lower* lanes; Suzuki et al., 1991). Sure residues are shown by capital letters and < 100% sure residues are indicated by lower letters. A "–" indicates that no residue was identified. The predicted proteolytic processing site of the precursor is indicated with an arrow. (*N*) Potential asparagine glycosylation sites. The putative transmembrane region is underlined.

tially reduced after thrombin or elastase (30 and 40%, respectively; Fig. 9 *A*).

Discussion

In this paper we describe several biological and biochemical characteristics of a novel, endothelial-specific cadherin.

This protein was identified adopting the indirect approach of developing mouse mAbs to human EC and selecting one mAb, denoted 7B4, on the basis of its ability to stain intercellular boundaries in indirect immunofluorescence.

mAb 7B4 stains EC both in vitro and in situ. In tissue sections it stains only the endothelium of blood vessels of different size and origin in a variety of normal and malignant tissues and it does not recognize circulating blood cells, such as monocytes, polymorphonuclear cells, and platelets. Furthermore, mAb 7B4 does not react with any other tested cultured cell types (including human fibroblasts, smooth muscle cells and keratinocytes).

The issue that 7B4 antigen is a component of intercellular junctions is supported by its exclusive immunocytochemical localization in situ and in vitro. The fluorescence pattern observed in cultured EC closely resembles that observed by immunohistochemistry in different vessels in situ. In either condition, mAb 7B4 identifies a fine, continuous line along

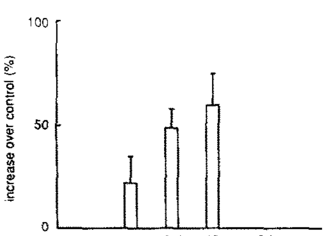

Figure 7. Effect of mAb 7B4 on the permeation through EC monolayer of HRP. EC were grown to confluency on Transwell filters. The EC monolayer was incubated for 6 h with the indicated doses of 7B4 Fab fragments and the passage of HRP through the endothelial layer was measured during the last hour. The ordinate is the percentage increase over control of the OD at 470 nm. Control EC were treated with Fab (400 μg/ml) from nonimmune mouse IgG. Control value was 235 ± 30 × 10^{-3} OD at 470 nm. The mean value of triplicate determinations ± SD in a representative experiment out of six performed are shown.

Table III. Permeation of HRP Across Transwell Filters

	OD × 10^{-3}
Endothelial cells	
Control	483 ± 6
γIFN and TNF	1,167 ± 130
Thrombin	
20 U/ml	1,165 ± 191
5 U/ml	874 ± 63
Elastase	
0.6 μM	1,415 ± 291
0.3 μM	722 ± 38
–EGTA	856 ± 36
Empty filter	1,334 ± 103

EC were grown to confluency on Transwell filters and treated with the indicated doses of thrombin, elastase or EGTA (5 mM) for 30 min. γIFN and TNF (200 and 100 U/ml, respectively) were given to the cells for 72 h. Permeation of HRP, measured as described in Materials and Methods, was quantified as OD at 470 nM. Mean of triplicate determinations ± SD.

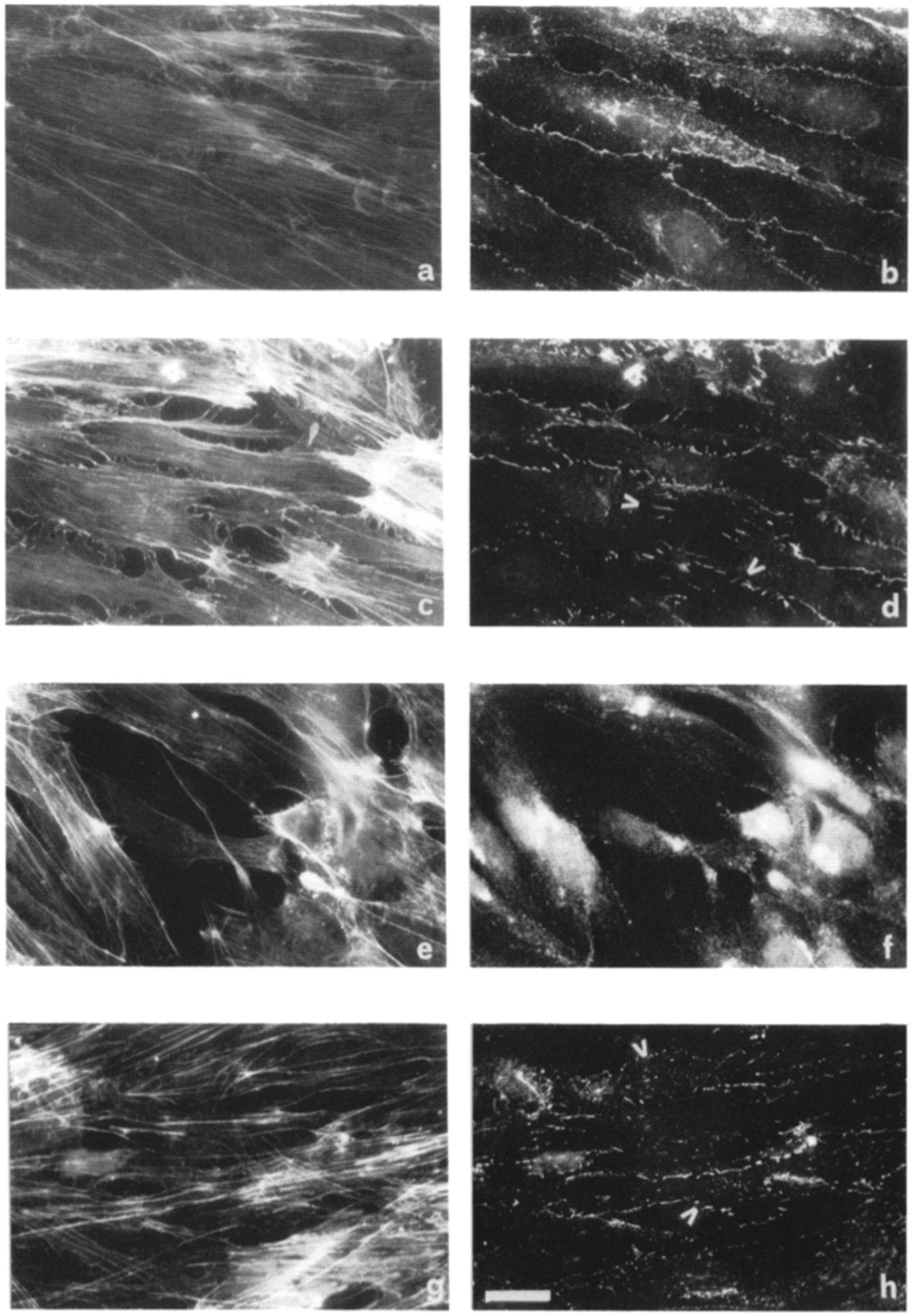

Figure 8. Immunofluorescence of cultured EC after treatments affecting cell to cell contacts. The cells were double stained with mAb 7B4 followed by rhodaminated secondary antibody to mouse immunoglobulins (*b, d, f, h*) and for F-actin with F-PHD (*a, c, e, g*). (*a* and *b*) Control. (*c* and *d*) Thrombin (5 U/ml for 30 min). (*e* and *f*) Elastase (0.8 μM for 30 min). (*g* and *h*) γIFN and TNF (200 U/ml and 100 U/ml, respectively, for 72 h). Arrows in *d* indicate residual areas of cell to cell contacts stained by mAb 7B4. Arrows in *h* point to 7B4 positive dots scattered within the cell body. Bar, 5 μm.

the rim of intercellular boundaries. In cultured EC, peripheral staining is observed exclusively at contact rims, while contact-free segments of the cell membrane are negative in subconfluent cultures. A constant feature of 7B4 antigen is its abundant intracellular storage in vitro, presumably in a Golgi-like vesicular compartment.

Data of 7B4 distribution are consistent with a role of 7B4 antigen in regulating EC cell–cell adhesion structures. Additional functional data supports this hypothesis. First, mAb 7B4 increases the permeability of EC cultured or Transwell filters by ~40% over the control value. Second, comparing confluent vs subconfluent cells, the antigen is localized at cell boundaries only where cells come in touch. Third, 7B4 topography is markedly modified by treatments such as γIFN and TNF, thrombin and elastase that increase EC permeability.

Interestingly, in cells treated with thrombin and elastase, 7B4 disappearance at cell boundaries is accompanied by a moderate decrease in the amount of antigen expressed by the cells. In contrast, in sparse cells or in cells treated with γIFN and TNF the changes in 7B4 distribution do not correspond to detectable changes in 7B4 qualitative or quantitative expression. This suggests that 7B4 organization in intercellular junctions could be regulated not only by direct digestion/de

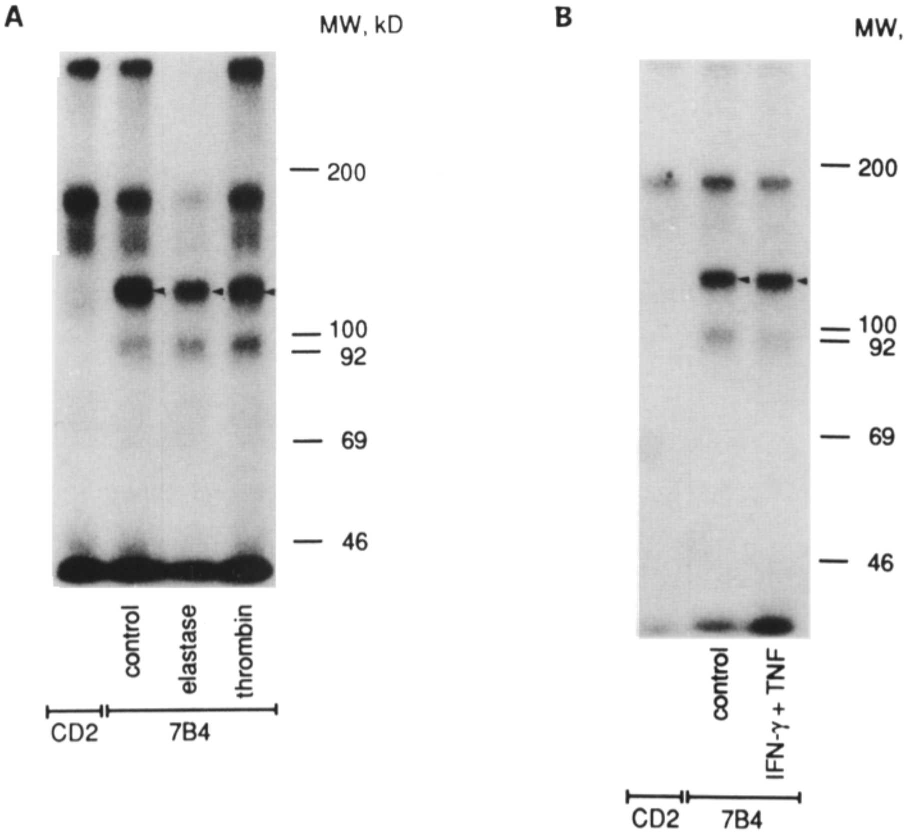

Figure 9. Effect of treatments affecting EC cell to cell contacts on the level of the externally exposed 7B4 antigen. (*A*) Confluent EC were surface labeled with ^{125}Iodine then treated with elastase (0.8 μM for 30 min) or thrombin (5 U/ml for 30 min) before extraction and immunoprecipitation with mAb 7B4. (*B*) EC were grown for 72 h in the presence of γIFN and TNF (200 and 100 U/ml, respectively) before surface labeling with ^{125}Iodine and immunoprecipitation. Molecular weight markers are shown on the right of each panel.

novo exposure of the antigen but also by antigen disassembling and relocation within the cell membrane.

The 7B4 antigen has been characterized for several biochemical properties. First, by lactoperoxidase-catalyzed iodination it appears to be externally exposed and EDTA treatment supports its firm association with the phospholipid bilayer (Müller et al., 1989). Further, it is produced by EC since it can be immunoprecipitated from metabolically labeled cell extracts. A second prominent biochemical property is its content of mannose and sialic acid oligosaccharide moieties witnessing its glycoprotein nature. The apparent molecular mass of the 7B4 antigen is 140 kD. An additional band or group of bands of ~100 kD is occasionally precipitated from metabolically labeled and constantly precipitated from surface-labeled EC extracts. Since its intensity increases in parallel to a decrease of the 140-kD band in several experimental tests, including repeated freezing and thawing cycles, it is likely that it represents a degradation product of the higher MW protein.

Many characteristics of 7B4 are in common with some members of the cadherin family. These include (*a*) sensitivity to trypsin digestion (Albelda, 1990); (*b*) partition in TX-114 buffer phase (Volk and Geiger, 1986); (*c*) binding to ConA (Damsky et al., 1983; Volk and Geiger, 1986); (*d*) requirement of Ca^{2+} for the localization of the antigen at the cell contacts (Volk and Geiger, 1986; Vestweber et al., 1985; Takeichi, 1990). In addition, 7B4 antigen is recognized by a polyclonal antibody directed to the conserved COOH-terminal cytoplasmic domain of all known cadherins.

The NH_2-terminal sequence data and sequence data from tryptic peptide mapping confirmed that 7B4 is indeed a member of the cadherin family. The sequences essentially showed identity with the cDNA deduced sequence of a new member of the cadherin family recently characterized and denoted as cadherin-5 (Suzuki et al., 1991). This molecule was identified in human placenta cDNA by the aid of polymerase chain reaction using degenerated primers corresponding to highly conserved amino acid sequences from the cadherin cytoplasmic domain. The entire putative coding sequence of cadherin-5 has been reported (Suzuki et al., 1991) and exhibits significant homology with those of the previously described cadherin sequences and the overall molecular structure is essentially the same as that of the other cadherins. Data reported here confirm the existence of a cadherin-5 protein and characterize its biochemical and biological behaviour. The specific localization of this protein in endothelial cells distinguishes this cadherin from the other members of the family that present a quite widespread distribution. This molecule appears to be a candidate not only as a specific marker for endothelial cell junctions but also as a potential regulator of endothelial continuity required for the maintenance of permeability control and the antithrombotic properties of the endothelium.

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