A HUMAN ENDOTHELIAL CELL-RESTRICTED, EXTERNALLY DISPOSED PLASMALEMMAL PROTEIN ENRICHED IN INTERCELLULAR JUNCTIONS

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Cultured vascular endothelial cells (EC)[1] actively maintain a polarized distribution of integral membrane proteins between their apical and basal surfaces (1). The intercellular contact region of EC is an additional physiologically distinct zone that contains specialized junctional complexes through which leukocytes emigrate during inflammation and atherogenesis. We are currently studying the intercellular zone and its role in EC function.

For this purpose, we have generated mAbs against human EC (HEC) membrane proteins restricted to the intercellular junctional domain by immunizing mice with human umbilical vein EC cultured on collagen. Under these conditions the EC junctions resemble those of the vascular intima in both structure and function. Such cultures produce tightly apposed, growth-arrested monolayers whose cell borders stain with $AgNO_3$ and have well-developed adherens and tight junctions. Monocytes that settle on these monolayers migrate rapidly to the cell borders where they tightly adhere to and quickly transmigrate the EC monolayer to the abluminal surface (2).

We report here the characterization of one plasmalemmal protein that is not only restricted in its distribution to the intercellular domain of the EC surface in vitro, but is also restricted in its cellular distribution to vascular EC in situ.

Materials and Methods

Materials. The following materials were routinely procured from the indicated sources for tissue culture: Collagenase, CLS II, Worthington Biochemical Corp. (Freehold, NJ); clotted human blood and human plasma fibronectin, The New York Blood Center (New York, NY); medium 199 with Earle's salts, HBSS, trypsin/EDTA mixture, penicillin/streptomycin, RPMI 1640 medium, and FCS, Grand Island Biological Company (Grand Island, NY); Vitrogen, Collagen Corporation (Palo Alto, CA); 10× medium 199, specialty media (Lavalette, NJ). Glutaraldehyde and osmium tetroxide were purchased from Electron Microscope Sciences (Fort Washington, PA); Epon, Ernest Fullum (Latham, NY); protein A-Sepharose, Pharmacia Fine Chemicals (Piscataway, NJ), Autofluor, National Diagnostics (Manville, NJ); molecular weight markers and OVA, Sigma Chemical Co. (St. Louis, MO).

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[1] *Abbreviations used in this paper:* DPBS, Dulbecco's PBS; EC, endothelial cells; EC-col, HEC cultured on dehydrated collagen gels; EC-fn, HEC cultured on fibronectin-coated tissue culture dishes; HEC, human endothelial cells; LPO, lactoperoxidase; PD/OVA, PBS without divalent cations plus 0.2% OVA.

LPO-latex was synthesized as described (1) using lactoperoxidase from Calbiochem-Behring Corp. (San Diego, CA), carboxylate-modified polystyrene latex from Polysciences, Inc. (Warrington, PA), and coupling reagents from Aldrich Chemical Co. (Milwaukee, WI). Carrier-free $Na^{125}I$ (NEZ-033) was purchased from NEN Products (North Billerica, MA). For metabolic labeling a ^{35}S *Escherichia coli* hydrolysate containing 70% ^{35}S-methionine (Tran^{35}S label) was purchased from ICN Radiochemicals (Irvine, CA).

All polyclonal antisera were purchased from Dako Corp. (Santa Barbara, CA). Monoclonal hybridomas W6/32 (directed against MHC class I H chains) and 9.3C9 (directed against MHC class II) were obtained from the American Type Culture Collection (Rockville, MD). Protein A-colloidal gold was the generous gift of Dr. Patricia Detmers (The Rockefeller University).

$CD2F_1$ mice were obtained from the Trudeau Institute (Saranac Lake, NY). All other materials were reagent grade or better.

Isolation of Human Umbilical Vein Endothelial Cells (HEC). Umbilical cords from uncomplicated deliveries of healthy women were collected within 2 d of birth in sterile glucose-containing PBS. HEC were isolated by standard techniques (3) from cannulated umbilical veins using collagenese at 75 U/ml. Harvested cells from each cord were plated as separate primary cultures in 25-cm^2 tissue culture flasks coated with human plasma fibronectin at >1 $\mu g/cm^2$. Cultures were washed free of nonadherent cells 4 h after initial plating and returned to culture in complete medium. Cultures isolated from cords >20 cm long generally reached confluence in 3–5 d.

Culture of HEC. Culture medium consisted of 20% normal human serum in medium 199. No exogenous growth factors were added. Medium was supplemented with penicillin and streptomycin (100 U/ml and 100 μg/ml, respectively). Medium was replaced every 3 d. Cultures were passaged at confluence by harvesting in trypsin/EDTA. Healthy primary isolates were pooled for passaging, and split generally at ratios of 1:2 to 1:4. Subcultures were grown on tissue culture plastic coated with human fibronectin (EC-fn) or on polymerized dehydrated collagen gels (EC-col) coated with fibronectin.

Collagen Gels. Vitrogen was mixed with 10× medium 199 and 0.1 N NaOH and pH adjusted to neutrality according to the manufacturer's directions. Tissue culture vessels were coated with appropriate volumes of the chilled, sterile solution (1.0 ml for a 35-mm dish, 8.0 ml for a 100-mm dish). The collagen was allowed to gel in the tissue culture incubator for up to 1 h; then sterile M199 was added to the dishes.

Hydrated collagen gels absorbed soluble reagents and eluted them slowly, making the analyses to be performed in this study uninterpretable. We found that dehydrated collagen gels supported the growth of HEC monolayers that were essentially identical biochemically and phenotypically, yet allowed such experiments to be performed. Dehydrated collagen gels were made by aspirating the M199 and allowing the gels to dry overnight in a laminar flow hood. The dehydrated gels were covered with salt crystals, which were removed by repeated washes with sterile, pyrogen-free water. These gels did not rehydrate to any appreciable extent. Dehydrated gels were used as culture substrata for HEC in all immunochemical and radiolabeling procedures.

Silver Nitrate Staining. Silver nitrate staining of live or paraformaldehyde-fixed EC monolayers was performed as described (4).

Production of mAbs. HEC were grown to confluence on hydrated collagen gels. A test culture demonstrated strong $AgNO_3$ staining of intercellular junctions. HEC were nonenzymatically resuspended by rocking the culture flasks in 10 mM EDTA in HBSS. The recovered cells were washed and resuspended in Dulbecco's PBS (DPBS). Female $CD2F_1$ mice were immunized by injecting 6×10^5 live HEC in 0.25 ml DPBS via tail vein. The mice were subsequently boosted by an intravenous injection of 4×10^5 similarly prepared cells 1 and 4 mo later. 4 d after the last boost, the spleen was removed from one mouse. A sterile suspension of spleen cells was fused with NS-1 myeloma cells at a 10:1 ratio using polyethylene glycol by standard methods (5). 3×10^4 cells were plated in each well of a 96-well tray and grown in HAT medium with 20% FCS.

Supernatants from growing hybridoma cultures were screened by immunoperoxidase simultaneously on confluent EC-col and EC-fn monolayers derived from the same parent culture

and otherwise treated identically. Those supernatants that stained EC-col intensely in the region of intercellular junctions, but stained EC-fn with a diffuse surface pattern, were selected for cloning. The hybridomas were cloned on $CD2F_1$ thymocyte feeder layers and rescreened as above.

Clone hec7 was adapted to growth in 10% iron-supplemented calf serum (HyClone Laboratories, Logan, UT) in RPMI medium.

Isotyping was performed on culture supernatants by ELISA using a mouse monoclonal sub-isotyping kit (HyClone Laboratories). The mAb reported here is an IgG2a.

Immunocytochemistry (Light Microscopy). The method of Lane and Lane (6) was adapted for our cultures. EC-col or EC-fn monolayers were fixed in 2% paraformaldehyde for 10 min at 4°C or a 1:1 (vol/vol) mixture of acetone and methanol for 10 min at room temperature. The monolayers were allowed to air dry. Small (2–5 μl) aliquots of primary antibody diluted in PBS without divalent cations plus 0.2% OVA (PD/OVA) or mAb culture supernatant were carefully placed on the monolayer surface. The culture dish was incubated in a moist chamber for 30 min at room temperature. The dish was washed extensively with PD/OVA. Subsequent incubations consisted of covering the dish (generally, 35-mm diameter) sequentially with rabbit anti-mouse IgG (1:400 in PD/OVA), swine anti-rabbit IgG (1:40), and rabbit antiperoxidase–horseradish peroxidase soluble complexes (1:50) each 0.5 ml for 30 min. Antibody was localized with DAB-H_2O_2 (7). Cells were counterstained in Gill's Hematoxylin.

Immunofluorescence was performed in an identical manner except that the secondary antibody was a fluorescein-labeled rabbit anti-mouse IgG, $F(ab')_2$ fragment. After this step, staining was visualized by epi-illumination in a Nikon fluorescence photomicroscope equipped with fluorescein filters.

Immunohistochemistry. Frozen sections 6 μm thick were cut from OCT-embedded human tissue obtained at surgery or autopsy under proper Institutional Review Board protocols. Sections were picked up on gelatin-coated slides and stored dessicated at −20°C. Tissue sections were rehydrated in PD for 30 min then incubated for 30 min at room temperature in 0.3% hydrogen peroxide to quench any endogenous peroxidase activity. Subsequent immunoperoxidase staining was performed as for the monolayer cultures above.

Immunoelectron Microscopy. In this experiment, the antibody was applied to the apical surface of the culture. Since the iodination experiments revealed that the vast majority of the antigen was not present on the apical surface, we tested monolayers that were incubated for 2 min at room temperature in HBSS without divalent cations before fixation. This incubation caused the cells to retract slightly, as assessed by phase microscopy; such cultures no longer stained with silver nitrate, even though cells were still flat and in a monolayer conformation. We thought that this would allow better penetration of the antibody and protein A–colloidal gold to the lateral and basal surfaces of the cells.

Confluent EC-col monolayers were fixed in 2% paraformaldehyde in PBS for 10 min on ice. After extensive washing of the 35-mm culture dishes, 1 ml of the primary antibodies (all as culture supernatants) hec7, W6/32 (anti-class I MHC), and 9.3C9 (anti-class II MHC) were added to separate dishes for 1 h on ice. The dishes were washed extensively with PD/OVA and incubated for 1 h on ice with a 1:100 dilution of protein A–colloidal gold (8-nm diameter) in PD/OVA. After further extensive washing, the cells were post-fixed in "mixed fix" (0.67% osium tetroxide and 0.83% glutaraldehyde), washed in saline, dehydrated overnight in graded ethanols, removed from the dish with propylene oxide, and embedded in Epon.

Thin sections (700 Å) were cut with a diamond knife and picked up on collodion-coated 100-mesh grids. The sections on the grids were stained with 1% uranyl acetate and Reynold's lead citrate. They were examined in a JEOL CX-100 electron microscope at 80 kV.

Analysis of Immunoelectron Micrographs. To determine whether the junctional enrichment of hec7 seen by light microscopy was present at the ultrastructural level, we compared the number of protein A–colloidal gold particles associated with intercellular junctions of HEC incubated with either hec7 or W6/32. W6/32 stained EC-col diffusely at the light microscope level.

EC-col were fixed as intact monolayers in this experiment so that we could identify apical, basal, and junctional surfaces. 50 random photographs taken at 20,000× were made for each specimen. The brief wash in HBSS had loosened most cell junctions, but the surfaces of ap-

position were still readily apparent by EM. Gold particles were counted directly from the negatives and assigned uniquely to the apical, basal, or junctional compartments. A particle near a junction at the apical or basal surface was arbitrarily assigned to the junctional zone. Because this pre-embedding method most likely does not allow all surfaces of the EC equal access to reagents and washes, absolute antigen density cannot be determined accurately. Instead, we report the number of gold particles per junction for comparison relative to W6/32 and an isotype-matched negative control mAb.

Selective Cell Surface Iodination. Selective radioiodination of the apical or total cell surfaces of HEC monolayers was performed as previously described (1).

Metabolic Labeling. HEC cultures were washed three times in DMEM without methionine (DMEM −) and incubated for 30 min in DMEM −. Cells were then labeled in DMEM − supplemented with ^{35}S-methionine and ^{35}S-cysteine (Tran^{35}S label) at 200–400 μCi/ml followed by a "chase" in complete culture medium for the times indicated in Results.

Immune Precipitation. Radiolabeled HEC were lysed in 0.05% NP-40 or 1% Triton X-114 and lysates clarified as described (1). Clarified lysates were preadsorbed by shaking (300 rpm, 30 min, 4°C) with Protein A-Sepharose. Equal volumes of adsorbed lysate were added to microfuge tubes containing 100 μl of primary antibody or culture supernatant. The mixture was incubated for 1 h at 4°C and immune complexes were retrieved on protein A-Sepharose and washed as described (8).

SDS-PAGE and Autoradiography. Samples were prepared for electrophoresis as previously described (9) and analyzed on 4–11% acrylamide gradient gels 19.5 cm long using a discontinuous buffer system (10). Samples were run at 20-mA constant current. Gels containing ^{125}I-labeled samples were dried and exposed for autoradiography on Kodak XAR 5 film with the aid of Cronex Lightening Plus enhancing screens (DuPont Co., Wilmington, DE) (9). Gels containing ^{35}S-labeled samples were equilibrated with Autofluor after staining. All dried gels were exposed at −80°C.

Results

When HEC are cultured on amnionic stroma (4) or hydrated collagen gels (2), they form cobblestone monolayers of polygonal cells with a low mitotic index. The cell borders stain discretely with silver nitrate and the monolayer resembles the vascular intima in situ. In contrast, HEC cultured on conventional tissue culture plastic coated with geletin or fibronectin (EC-fn) are more elongated in shape, have a relatively high mitotic index at confluence, and do not make close, silver-staining junctions.

Hydrated matricies absorb soluble molecules and make standard biochemical and immunochemical analyses difficult. We therefore developed a technique in which HEC were cultured on dehydrated collagen gels (EC-col) and were phenotypically and biochemically identical to those grown on hydrated collagen gels. The dehydrated gels allowed the use of procedures such as surface iodination and immunoperoxidase, since the thin substratum of collagen did not expand upon rehydration.

mAbs Selective for the Junctional Zone. To identify proteins enriched in intercellular junctional zones, mAbs against EC-col were produced in mice and screened, as described in Materials and Methods, for their ability to bind to the intercellular region. Those hybridomas producing antibodies that selectively stained EC-col at the cell junctional zone, but stained EC-fn diffusely, were subsequently cloned. From 314 hybridomas of one fusion, five such cultures were selected; two yielded stable clones that produced high titer antibody in culture supernatants. This report concerns one of these, mAb 7 (hec7).

Cellular Localization of hec7. The immunoperoxidase staining pattern of hec7 on HEC is shown in Fig. 1. Four subclones of hec7 gave identical results. When applied to live, confluent monolayers, staining with hec7 was weak. However, on monolayers

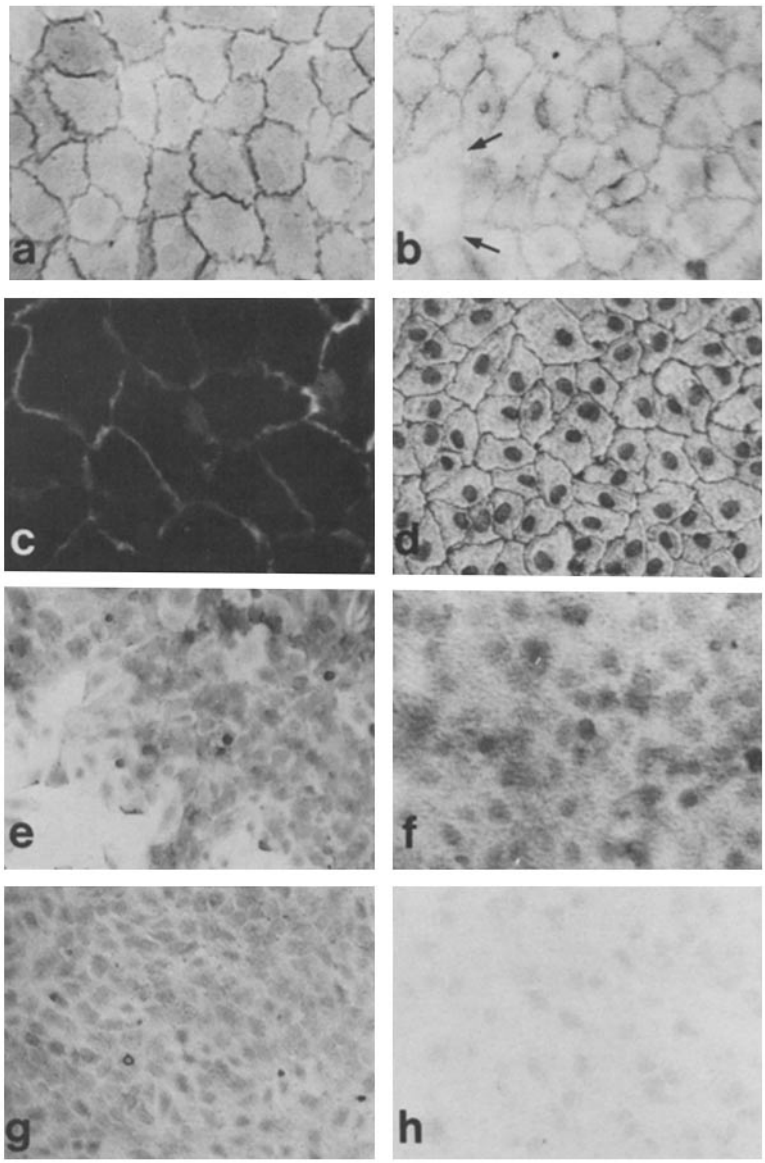

FIGURE 1. mAb 7 detects an antigen enriched in the intercellular borders of confluent EC-col monolayers. EC-col (*a–d*, *f–h*) and EC-fn (*e*) were stained with silver nitrate (*d*) or fixed in acetone/methanol (*a*) or paraformaldehyde (*b*, *c*, *e–h*). Fixed cell monolayers were air dried and subjected to immunoperoxidase (*a*, *b*, *e–h*) or immunofluorescence (*c*) with hec7 or control antibodies as described below. Monolayers were counterstained with hematoxylin after immunoperoxidase and Wright-Giemsa after silver nitrate.

Staining by hec7 was restricted predominantly to the intercellular borders of confluent EC-col (*a–c*). Occasional senescent cells were seen whose borders stained less intensely (*arrows in b*). The staining by hec7 very closely resembled the silver nitrate staining pattern of the same confluent EC-col monolayers (*d*). In contrast, on EC-fn monolayers, which do not make silver-staining junctions, hec7 produced a diffuse cell-surface staining pattern (*e*). A portion of the EC-fn monolayer in *e* was inadvertantly scraped away during processing. Diffuse surface staining on EC-col monolayers was produced by W6/32, an isotype-matched mAb against HLA-A and B H chains (*f*) and by rabbit anti-human GPIIIa (β_3 integrin family) antisera (*g*). An isotype-matched negative control, mAb 9.3C9, produced no staining (*h*). Magnifications: *e* and *g*, × 90; *a*, *b*, *d*, *f*, and *h*, × 180; *c*, × 250.

rendered permeable by fixation in acetone/methanol (Fig. 1 *a*) or by air drying after aldehyde fixation (Fig. 1, *b* and *c*), hec7 detected an antigen that was heavily enriched in the intercellular junctional zones of EC-col. The immunostaining pattern closely resembled the silver nitrate staining pattern of EC-col junctions (Fig. 1 *d*). In contrast, staining of EC-fn, which do not make silver-staining junctions, was much more diffuse (Fig. 1 *e*). This staining pattern was not an artifact of the collagen culture system, since mAb W6/32, directed against HLA-A and B H chain framework determinants, produced diffuse cell surface staining in the same EC-col cultures (Fig. 1 *f*). Similar results were obtained using antibodies against angiotensin I converting enzyme, intercellular adhesion molecule I, and the common β chains of class III (Fig. 1 *g*) and class I integrins (the glycoprotein IIb/IIIa-related protein/vitronectin receptor family and the VLA family, respectively). An isotype-matched mAb against class II MHC framework determinants, which are not expressed on nonactivated HEC in culture (11), served as the negative control (Fig. 1 *h*).

Subconfluent EC-col stained lightly and diffusely with hec7. However, when growing cells made contact and began to flatten out along their appositional borders, the antigen was strongly concentrated in the zones of cell-cell contact (Fig. 2). Free edges of these same cells did not stain.

Postconfluent EC-col retained the hec7 staining pattern for at least 10 d, the longest time point examined. As confluent cultures aged, the staining became heterogeneous. The majority of the cells staining less intensely were the larger senescent cells, and their junctions also stained poorly with silver nitrate (Fig. 1 *b* and reference 2). Thus, the antigen is truly enriched on the junctional surfaces of the cells under conditions in which the EC make junctional complexes adequate to be stained by $AgNO_3$, but is more homogeneously expressed over the plasma membrane under conditions in which they do not (EC-fn; Fig. 1 *e*, subconfluent cells; Fig. 2 *a*).

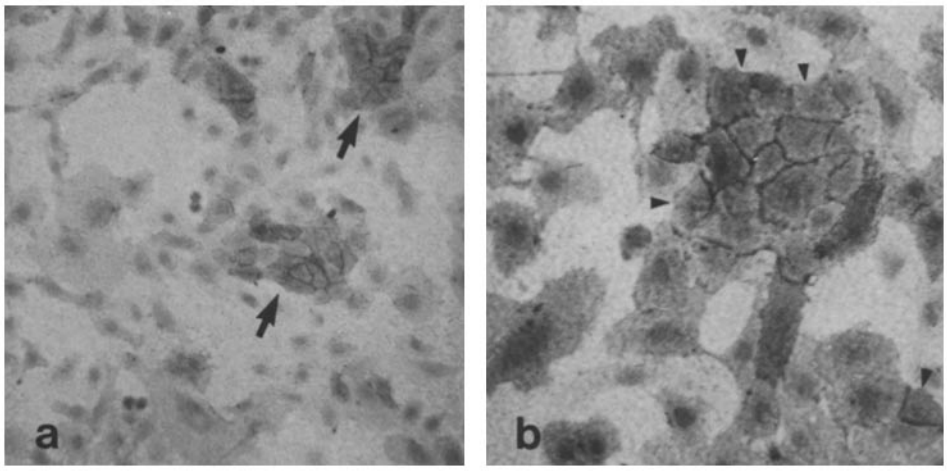

FIGURE 2. mAb hec7 selectively stains endothelium at intercellular borders. Immunoperoxidase cytochemistry was performed on EC-col split 1:10 and cultured for 5 d. Staining by hec7 was increased on islands of cells in contact and staining was particularly intense along appositional borders (*arrows in a*). In contrast, the free borders of the same cells did not stain with hec7 (*arrowheads in b*). *a*, × 90; *b*, × 180. Counterstained with hematoxylin.

Identification of the Plasmalemmal Protein Recognized by hec7. mAb hec7 immunoprecipitated a 135-kD protein from metabolically labeled HEC cultures (Fig. 3). The molecular mass was not changed by running the SDS-PAGE gels under nonreducing conditions (not shown). A faint 110-kD band was always identified. Pulse-chase experiments (Fig. 3 *B*) indicated that this was a metabolic precursor of the 135-kD protein. However, even when the "chase" period continued for 2.5 h, the band persisted.

EC-fn expressed the same antigen as EC-col in roughly similar amounts per cell, as judged by metabolic labeling. Only the cell surface distribution of the protein appears to be different in the two culture systems.

The protein recognized by hec7 is an integral membrane protein. It was not stripped from the surface of HEC by EDTA (Fig. 4) or by extensive washing with chaotropic agents, including carbonate buffer at pH 11 and 2 M NaCl (data not shown).

The hec7 antigen is an externally disposed plasmalemmal protein. It could be labeled on the apical surface of EC-col monolayers by solid phase lactoperoxidase under conditions in which the cells remained >99% viable (Fig. 4). The 110-kD band was iodinated to the same relative extent on the surface of viable HEC using solid phase lactoperoxidase (Fig. 4) as it was present in the immunoprecipitates of metabolically labeled and chased cells (Fig. 3). These data suggest that a trace of the smaller polypeptide was transported to the cell surface.

The lactoperoxidase in this experiment was covalently attached to 0.7-μm polystyrene latex spheres, and iodination was restricted to the apical surface of endothelial cells (1). When the same solid phase LPO was used to iodinate the total cell surface of nonenzymatically resuspended EC-col, much more radiolabeled antigen was immunoprecipitated from the same number of cells. The radioactive bands were cut from the gel in Fig. 4 and incorporated radioactivity quantitated directly in a gamma scintillation counter. When normalized for the radioactivity incorporated into the

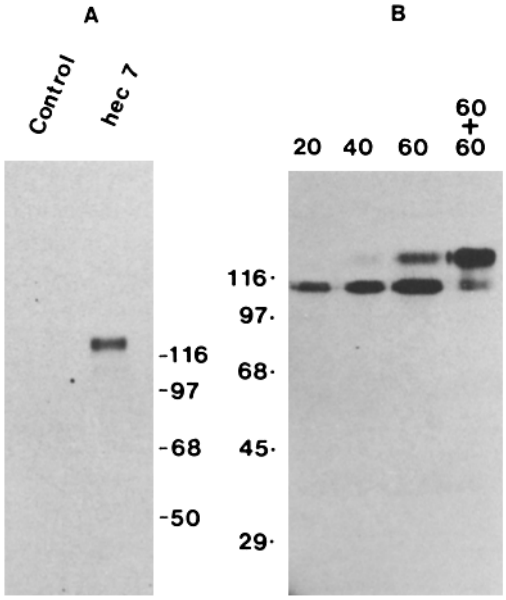

FIGURE 3. The antigen recognized by hec7 is a protein synthesized by HEC. (*A*) Confluent EC-col monolayers were labeled with ^{35}S-methionine and cysteine for 4 h then cultured for 1 h in nonradioactive complete medium. Equal volumes of cell lysate were immunoprecipitated with hec7 or an isotype-matched negative control. A major band at 135 kD and a minor one at 110 kD are specifically recognized by hec7. (*B*) Pulse-chase experiment. Replicate cultures of EC-col were metabolically labeled for 20, 40, or 60 min and lysed immediately, or labeled for 60 min and "chased" for 60 min in complete medium (60 + 60). Immunoprecipitation using hec7 showed that most, but not all, of the label in the 110-kD band was "chased" into the 135-kD polypeptide. SDS-PAGE and fluorography were performed as described in Materials and Methods. Numbers at the margins of the autoradiographs indicate positions of molecular mass markers in kilodaltons. (*A*) 1-d exposure; (*B*) 2-d exposure.

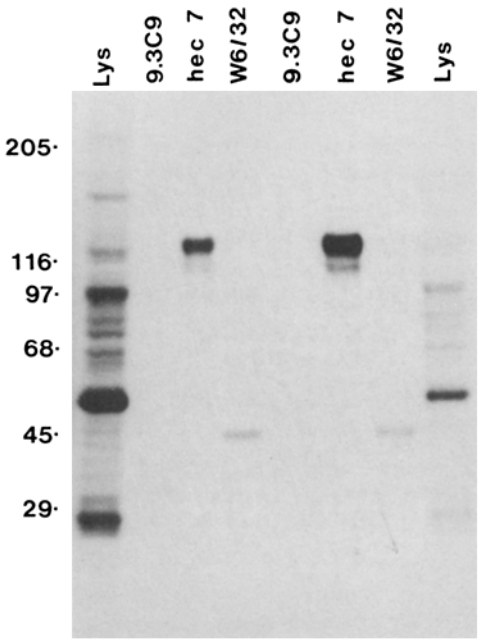

FIGURE 4. The hec7 antigen is an externally disposed plasmalemmal protein relatively inaccessible to iodination at the apical cell surface. Confluent HEC were radioiodinated by solid phase lactoperoxidase under conditions in which labeling was restricted to the apical surface (four lanes on left) or evenly distributed over the entire cell surface (four lanes on right) (reference 1). Equal volumes (200 μl) of cell lysates from each sample were subjected to immune precipitation using hec7 or isotype-matched positive (W6/32) and negative (9.3C9) controls. A 20-μl aliquot of the initial lysate of both samples, representing 10% of the volume subjected to immune precipitation, was run on the same gel to serve as a denominator for total radioiodine incorporated into all membrane proteins in the apical and total cell surface samples. Thus, the relatively intense labeling of the hec7 antigen in the total surface sample compared with the apical surface sample is even more impressive considering that the overall specific activity of the total surface sample was much lower (compare "lysate" lanes). Positions of molecular mass markers (in kilodaltons) are shown on the left.

all apical or total cell surface proteins, 85% of the antigen was determined to be inaccessible to the solid phase LPO. This is consistent with enrichment of this antigen in the intercellular domain of EC-col.

Ultrastructural Localization of the hec7 Antigen. In an initial attempt to localize the antigen recognized by hec7 at the ultrastructural level, immunoelectronmicroscopy was performed on EC-col using pre-embedding techniques (see Materials and Methods for experimental rationale). Analysis of 50 randomly photographed fields of cell monolayers revealed that protein A–colloidal gold particles were enriched in intercellular junctional zones of EC-col incubated with hec7 (Table I).

Glutaraldehyde fixation destroyed the antigenicity of the 135-kD protein on cells; therefore, EC-col were fixed in paraformaldehyde. This resulted in suboptimal ultrastructural preservation. However, the overall cellular architecture and particularly plasma membrane structure at cell borders was clearly discernable. A wide

TABLE I

Analysis of Immunoelectron Micrographs

Antibody	Total junctions	Particles on junctions	Particles/junction	
			mean ± SD	*median*
9.3C9*	47	45	0.96 ± 2.22	0
W6/32‡	20	33	1.65 ± 2.37	1
hec7	30	236	7.87 ± 8.48	6

* IgG2a negative control mAb directed against class II MHC antigens.
‡ IgG2a positive control mAb directed against class I MHC antigens.

range of values was obtained when the number of gold particles per junction was calculated for each sample. This is reflected in the large standard deviations of the mean values (Table I). The overall pattern of binding is better reflected in the median value for each specimen (Table I). A control antibody, 9.3C9, directed against MHC class II framework determinants, did not produce detectable staining of EC-col by immunoperoxidase at the light microscope level (Fig. 1 *h*) and displayed low background binding to EC-col in these specimens (Table I). mAb W6/32, directed against MHC class I framework determinants, produced a diffuse cell surface staining by immunoperoxidase (Fig. 1 *f*) and produced a low density of junctional gold particles at the EM level (Table I). W6/32 gave the qualitative impression of having the same density on the junctional and apical surfaces. hec7 produced a distinct pattern of gold particles under these conditions. Immunogold particles were enriched in the remaining cell-cell junctions and the edges of adjacent endothelial cells (median, six particles/junction) compared with W6/32 (median, one particle/junction). Selected profiles of cells whose junctions remained particularly well preserved are shown in Fig. 5.

Expression of the hec7 Antigen In Situ. The antigen recognized by hec7 is present on HEC in situ (Fig. 6). It was present constitutively on endothelium of all vessel types tested (see Table II), including muscular arteries, arterioles, capillaries, venules, and veins. Continuous endothelia of all capillary types stained, including fenestrated endothelium of the adrenal cortex (Fig. 6 *e*) and intestine, glomerular capillaries (Fig. 6 *h*), and capillaries of the central nervous system that constitute the blood-brain barrier. The one interesting exception may be discontinuous sinusoidal en-

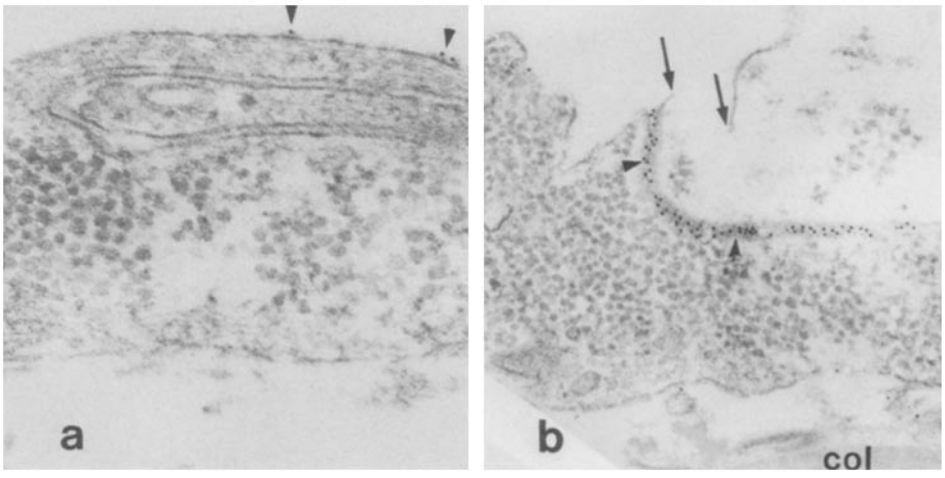

FIGURE 5. Ultrastructural localization of hec7 antigen to intercellular junctions. Immunoelectron microscopy was performed on paraformaldehyde-fixed EC-col monolayers. Selected profiles of cells stained with (*a*) mAb W6/32 (anti-class I MHC H chain) and (*b*) hec7 are shown. The poor preservation of ultrastructural detail in paraformaldehyde resulted in occasional profiles, such as in *b*, where the apical plasma membrane is clearly disrupted (*between arrows*). Nevertheless, the plasmalemma at the cell border is plainly visible. Arrowheads mark protei . A-colloidal gold particles on the apical cell surface in *a*, but predominantly in intercellular junctions in *b*. Note that the cell in *b* was not among the 50 random fields chosen for quantitation and was therefore not included in the data of Table I. Col, collagen matrix. Both micrographs, × 50,000.

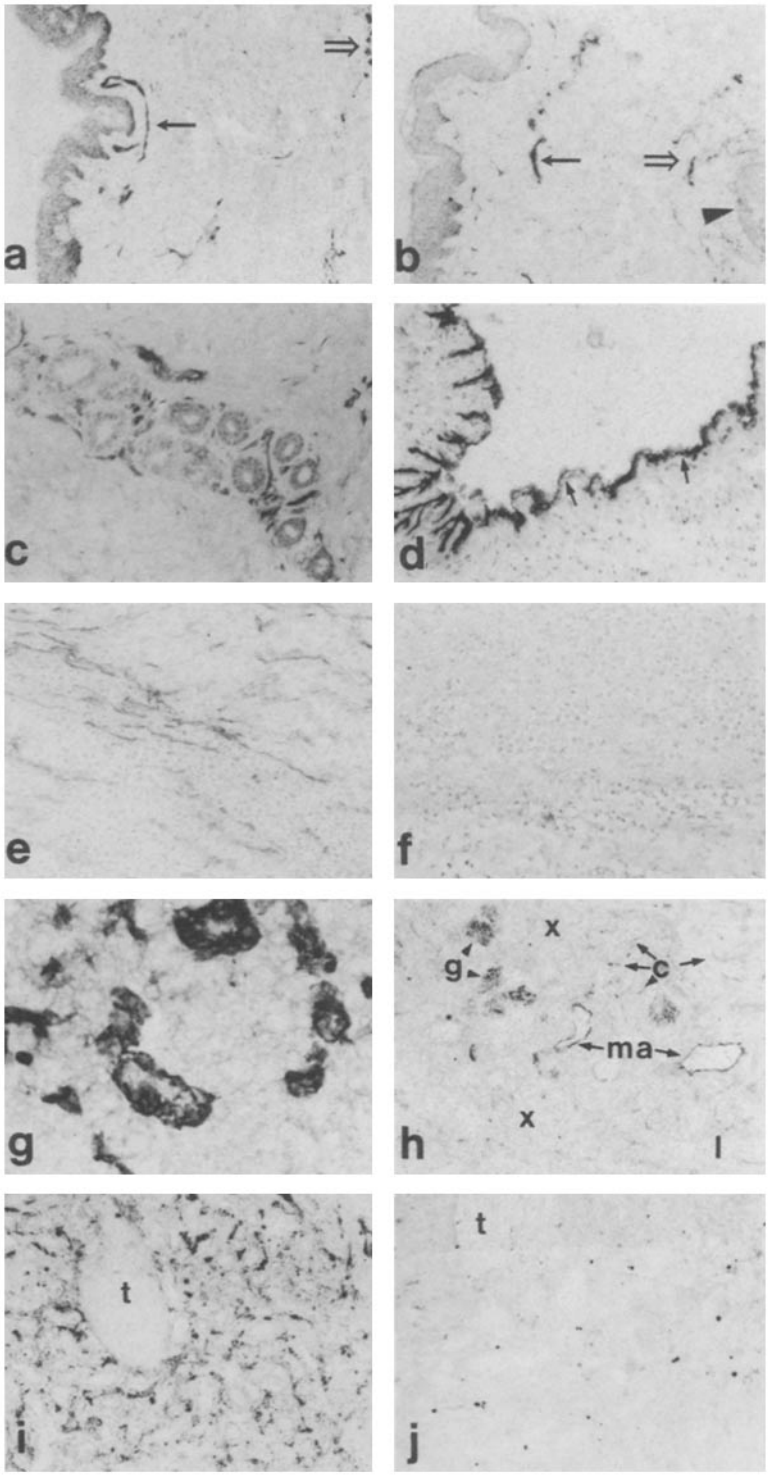
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TABLE II
Blood Vessels Stained by hec7

Vessel type	Tissue source (examples)*
Elastic artery	Aorta
Muscular artery	Kidney, lung, colon
Arteriole	Skin, kidney
Capillary	
Continuous	Skin, skeletal muscle
Fennestrated	Adrenal gland, colon lamina propria
Glomerular	Kidney
Alveolar	Lung
(Discontinuous sinus)‡	Liver (gradient)
Blood-brain-barrier	Cerebral cortex
Post-capillary venule	Lymph node, inflamed skin
Venule	Colon, skin, thymus, trachea
Vein	Lung, skin, colon

* Tissue source lists some examples of human tissue examined. Endothelium of a particular vessel type stained positively for hec7 in every organ in which that vessel type was examined.

‡ Not detectable in splenic red pulp vascular sinuses. Focally detectable on hepatic sinusoidal endothelium in periportal zone (see text).

dothelium. Staining of sinusoidal endothelium in the red pulp of the spleen was not detected (Fig. 6 *j*), although endothelium of larger splenic vessels clearly expressed the antigen. hec7 stained sinusoidal endothelium of the liver in periportal zones, but expression decreased dramatically along the sinusoids as they radiated toward the central vein. The liver tissue was an autopsy specimen; however, other antigens, such as von Willebrand Factor (vWF) and class II MHC antigens, were clearly expressed by endothelium in the centrilobular zone.

FIGURE 6. Expression of hec7 Ag is restricted to HEC in situ. Representative samples are shown from immunoperoxidase studies performed on 6-μm frozen sections of normal tissue obtained at surgery. (*a*) Skin stained with rabbit antisera to vWF shows immunoreactivity in the superficial (*single arrow*) and deep (*double arrow*) venular plexuses as well as in dermal capillaries. (*b*) An adjacent section stained with hec7 demonstrates immunoreactivity on endothelium of all vessels producing a staining pattern identical to that of vWF. Note that stratified squamous epithelium, dermal fibroblasts, and epithelium of a hair follicle (*arrowhead*) are not stained by hec7. (*c*) Eccrine gland in the deep dermis does not stain with hec7 while the surrounding capillaries do. (*d*) Umbilical vein endothelial cells stain intensely and discretely with hec7, while underlying vascular smooth muscle cells are negative. In favorable views (*arrows*) staining is seen to be predominantly on the basolateral side of the endothelium. (*e*) Fenestrated capillaries of the adrenal cortex stain with hec7. (*f*) Adjacent section of adrenal cortex stained with irrelevant control antibody shows no immunoreactivity. (*g*) High endothelial venules of the lymph node stain with hec7 while lymphocytes, macrophages, and dendritic cells are negative. (*h*) hec7 stains endothelium of all vessel types in the kidney. Shown here is staining of glomerular capillaries (g), peritubular capillaries (c), and muscular arteries (ma). Mesangial cells of the glomerulus and epithelium of tubules shown cut in cross-section (x) or longitudinally (l) do not stain. (*i*) Red pulp of the spleen stained with vWF delineates the discontinuous endothelium of the vascular sinuses. t, trabecula. (*j*) In contrast, the red pulp is not stained by hec7. Adjacent fields of this specimen demonstrated obvious staining of endothelium of splenic arteries. t, trabecula. Magnifications: *a*, *b*, *h*, *i*, and *j*, × 20; *c*, *d*, *e*, and *f*, × 40; *g* × 80.

Endothelium of at least some larger and more structurally defined lymphatic channels appeared to stain with hec7. These included subcapsular sinus in the lymph node and several capacious, erythrocyte-free, thin-walled vessels in the serosa of the colon and the adventitia of the ureter: structures that we interpret as lymphatics.

The antigen was present on normal endothelium in all tissues tested, and it was specific for endothelial cells in all of these tissues (Table III, Fig. 6). Vascular smooth muscle, macrophages, fibroblasts, and every type of epithelium tested did not contain detectable levels of the antigen by this technique. The number of vascular profiles we encountered in which we thought we could distinguish vascular pericytes from endothelial cells was small; yet these cells did not stain with hec7.

TABLE III

Cells and Tissues Testing Negative for hec7 Staining

Tissue	Organ (source)
Epithelia	
Stratified squamous	Skin, tonsil
Eccrine duct	Skin
Mucus gland	Trachea
Salivary gland	Peritonsilar soft tissue
Bile duct	Liver
Hepatocytes	Liver
Respiratory epithelium	Lung (bronchus)
Simple squamous	Lung (pneumocytes)
	Mesothelium (colonic serosa)
Simple columnar	Colonic mucosa
Transitional	Urinary bladder
Renal tubular	Kidney
Thymic epithelium	Thymus
Mesodermal mesenchymal derivatives	
Skeletal muscle	Diaphragm
Smooth muscle	Colon, vascular media
Perineurium	Skin, colon
Fibroblasts	Skin, umbilical cord
Cartilage	Trachea
Mesangial cells	Renal glomerulus
Bone marrow-derived cells	
Erythrocytes	Intravascular
Leukocytes	Intravascular
Tissue macrophages	Skin, spleen, liver (Kupfer cells)
Lymphoid follicles	Lymph node (peripheral and mesenteric), spleen, tonsil, thymus
Other	
Endocrine glands	Adrenal cortex, parathyroid
Neuroendocrine glands	Adrenal medulla
Peripheral neurons	Skin, myenteric plexus
Central nervous system	Cortical neurons and glia
Trophoblast	Placenta

A partial list of human cell and tissue types tested for hec7 staining. A representative organ for each class is mentioned. Nonendothelial cell types did not stain with hec7 in any organ examined.

Discussion

We report on a previously undescribed 135-kD plasmalemmal protein of HEC. Immunohistochemistry shows that: (*a*) this protein is enriched in the intercellular junctions of cultured HEC monolayers; (*b*) this protein is expressed by continuous endothelium of all vessel types in situ; and (*c*) expression of this protein or, at least the epitope recognized by hec7, is restricted to vascular endothelial cells in situ.

mAb hec7 stains cultured HEC preferentially along borders between tightly confluent cells. Staining of monolayers grown under conditions where cell-cell apposition is looser (EC-fn; Fig. 1 *e*) show diffuse surface staining with hec7. Staining in older cultures is heterogeneous, and older senescent cells stain less intensely. This correlates with the loss of $AgNO_3$ staining of their junctions. Subconfluent HEC grown on collagen (EC-col) stain diffusely and relatively weakly with hec7. As the cells appose their neighbors and form small islands of polygonal cells, staining is concentrated at the borders of apposition, while the free edges do not stain (Fig. 2). The antigen is largely inaccessible to iodination on the apical surface by solid phase lactoperoxidase, but labels readily on resuspended cells. At the electron microscope level, antigen density is greatest at adjacent edges of cultured HEC.

The above findings raise some interesting biological questions. Is the concentration of this molecule in the junctions of EC-col due to a redistribution of extant surface protein or selective insertion of nascent molecules directly into junctional zones? Does the small percentage of the 135-kD protein present on the apical surface represent imperfect cell polarity in our in vitro system, or does it serve a physiologic purpose in that location? The answers to these questions may shed light on the natural function of this molecule.

To assess the true ultrastructural distribution of the 135-kD antigen in vitro and in situ will require immunoelectronmicroscopic localization using post-embedding techniques or ultra-thin frozen sections where the antibody has equal access to the entire cell profile. Such techniques generally require polyclonal antisera to detect membrane proteins. hec7 will be used to affinity purify its antigen for the purpose of making a polyclonal rabbit antiserum.

The antigen recognized by hec7 does not correspond to any other reported HEC protein. Angiotensin converting enzyme and a GPIIb-related protein have a similar M_r (130–140 kD) (12–14), but antibodies against angiotensin converting enzyme and the common β chains of both class I and class III integrins all have a diffuse distribution over the apical surface of EC-col by immunoperoxidase (Fig. 1 *g*; additional data not shown). Furthermore, 88% of the total cell surface ACE can be iodinated on the apical surface (Muller, W. A., unpublished data).

The antigen recognized by hec7 is present constitutively on the surface of HEC at all levels of the vascular tree. Specialized capillary types (glomerular, central nervous system) also express the antigen. We did not appreciate any consistent difference in the staining intensity of endothelium from different vessel types. Endothelium in inflamed tissue retains the hec7 antigen and generally stains somewhat more intensely (Table II). No other cell or tissue type of the dozens tested in frozen human tissue sections stain appreciably by our sensitive immunoperoxidase procedure that uses four layers of antibody in the "sandwich."

hec7 may therefore be a useful histologic marker for endothelium. Conventional histochemical endothelial-specific markers are vWF and *Ulex europaeus* I lectin. vWF

is an intracellular protein and is also present on platelets and megakaryocytes (15). *Ulex europaeus* I lectin is also present on some epithelial cell types (16).

There are no established surface markers unique to HEC. Scattered reports of endothelial cell-specific mAbs have appeared in the literature (17–19). Kaplan et al. (18) reported an mAb that recognized a 92-kD antigen on the surface of HEC both in culture and in situ. However, the antigen was also on the surface of some fibroblasts, mononuclear cells, and placental trophoblasts. Schlingemann et al. (19) derived an mAb, PAL-E, that stained EC of medium sized veins, venules, and capillaries in frozen tissue sections. Arteriolar and small artery EC stained weakly; large vessel EC did not stain. The antigen recognized by PAL-E was not identified.

One likely use of hec7, and a clear advantage over the intercellular marker vWF, may be the ability to purify live surface-stained EC from any tissue or organ (save, perhaps splenic and hepatic sinuses) by FACS to establish pure cultures. While hec7 will not select among EC from the different vessel sizes, this could be performed by sieving methods currently in use followed by FACS to separate EC from enzymatically digested tissue. Several trypsin cleavage products of the 135-kD antigen remain in the cell membrane and are recognized by hec7 (data not shown).

The function of the 135-kD antigen is not known. It is tempting to speculate that its enrichment in the intercellular junctional domains of confluent EC bespeaks a cellular function localized in that region. Its intense enrichment in the intercellular domain as cells come to confluence and its molecular weight are suggestive of cadherins, a class of integral membrane proteins mediating calcium-dependent adhesion between similar cell types (20, 21). In this regard it is interesting that the only type of endothelium in situ that stained weakly, inconsistently, or not at all with hec7 was the discontinuous endothelium of hepatic and splenic sinuses.

Cadherins have not been described on endothelial cells to date. However, in preliminary experiments, when hec7 supernatant (but not control) was allowed transient access to the junctions of live EC-col, the cell borders lost their ability to stain with silver nitrate. Prolonged incubation led to retraction of the cells from each other without loss of cell viability, similar to the effects reported with anti-A-CAM (N-cadherin) antibody (22).

There is at least one other externally disposed HEC membrane protein enriched in intercellular contact zones. We have recently cloned another hybridoma that recognizes an antigen in this location that is distinct from the one recognized by hec7. These proteins may serve endothelial-specific, junction-restricted functions such as cadherin-like homotypic cell-cell adhesion, or heterotypic cell-cell adhesion such as occurs during transendothelial migration by monocytes. The physiologic role of these proteins is currently under investigation.

Summary

We have raised an mAb to a previously undescribed 135-kD externally disposed integral membrane protein that is enriched in the intercellular junctional domain of cultured human umbilical vein endothelial cells. This protein localizes at the appositional surfaces of cells as they become confluent and is stably expressed in the junctional zones of confluent monolayers. This protein is expressed in situ on continuous endothelia of all blood vessels in all human tissues examined. Moreover,

this protein, as determined by mAb immunocytochemistry, is not expressed by any other cell type. This protein may mediate endothelial-specific functions restricted to the intercellular domain. It may also serve as a unique cell surface marker for the identification and purification of human endothelial cells.

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Note added in proof: Several other investigators, working with bovine aortic endothelial cells, have independently generated mAbs that recognize a protein(s) enriched in the intercellular junctions of bovine EC that is of similar molecular weight to the hec7 antigen. (R. Heimark, manuscript submitted; S. Albelda and C. Buck, personal communication.) A rabbit antiserum against one of these proteins (provided by S. Albelda, The Wistar Institute) immunoprecipitates the hec7 antigen (W. A. Muller, unpublished data).

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