

# The Molecular Organization of Endothelial Cell to Cell Junctions: Differential Association of Plakoglobin, $\beta$ -catenin, and $\alpha$ -catenin with Vascular Endothelial Cadherin (VE-cadherin)

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**Abstract.** In this paper we report that the assembly of interendothelial junctions containing the cell type-specific vascular endothelial cadherin (VE-cadherin or cadherin-5) is a dynamic process which is affected by the functional state of the cells.

Immunofluorescence double labeling of endothelial cells (EC) cultures indicated that VE-cadherin,  $\alpha$ -catenin, and  $\beta$ -catenin colocalized in areas of cell to cell contact both in sparse and confluent EC monolayers. In contrast, plakoglobin became associated with cell-cell junctions only in tightly confluent cells concomitantly with an increase in its protein and mRNA levels. Furthermore, the amount of plakoglobin coimmunoprecipitated with VE-cadherin increased in closely packed monolayers.

Artificial wounding of confluent EC monolayers resulted in a major reorganization of VE-cadherin,  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin. All these proteins decreased in intensity at the boundaries of EC migrating into the lesion. In contrast, EC located

immediately behind the migrating front retained junctional VE-cadherin,  $\alpha$ -catenin, and  $\beta$ -catenin while plakoglobin was absent from these sites. In line with this observation, the amount of plakoglobin coimmunoprecipitated with VE-cadherin decreased in migrating EC.

These data suggest that VE-cadherin,  $\alpha$ -catenin, and  $\beta$ -catenin are already associated with each other at early stages of intercellular adhesion and become readily organized at nascent cell contacts. Plakoglobin, on the other hand, associates with junctions only when cells approach confluence. When cells migrate, this order is reversed, namely, plakoglobin dissociates first and, then, VE-cadherin,  $\alpha$ -catenin, and  $\beta$ -catenin disassemble from the junctions. The late association of plakoglobin with junctions suggests that while VE-cadherin/ $\alpha$ -catenin/ $\beta$ -catenin complex can function as an early recognition mechanism between EC, the formation of mature, cytoskeleton-bound junctions requires plakoglobin synthesis and organization.

**T**HE endothelium forms a coherent lining of the inner surface of blood and lymphatic vessels and thus controls the passage of solutes and circulating cells from the lumen to tissues and vice versa (Simionescu and Simionescu, 1991; Haselton et al., 1992; Huang and Silverstein, 1992). This function requires highly effective intercellular junctions between endothelial cells (EC)<sup>1</sup>, the failure of which may lead to serious pathological manifestations. In-

tercellular junctions of various types were detected in EC (Franke et al., 1987, 1988; Schmelz and Franke, 1993). They are expected to play a major role not only in vessel permeability, as mentioned above, but also in endothelial surface polarity (Muller and Gimbrone, 1986), as shown in epithelial cells (McNeill et al., 1990). In addition, in most organs, the endothelium presents a low rate of turn over (Engermann et al., 1967; Folkman and Shing, 1992), probably due to contact-dependent growth inhibition. Indeed, when the continuity of the endothelial layer is interrupted, cells at the edge of the wound start to proliferate and migrate to the free area (Sholley et al., 1977; Schwartz et al., 1978). The molecular basis for contact-induced growth regulation and the involvement of specific cell junctions is still poorly characterized.

EC express at least two transmembrane molecules specific for inter-endothelial contacts: PECAM-1 (platelet endothe-

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1. *Abbreviations used in this paper:* EC, endothelial cells; PAF, paraformaldehyde; TX-100, Triton X-100; VE, vascular endothelial.

lial cell adhesion molecule-1 or CD31) that belongs to the immunoglobulin superfamily (Newman et al., 1990; Simmons et al., 1990; and which is present also in platelets and leukocytes) and vascular endothelial cadherin (VE-cadherin, Brevario, F., L. Caveda, M. Corada, I. Martin-Padura, P. Navarro, J. Golay, M. Inrona, M. G. Lampugnani, and E. Dejana, manuscript submitted for publication) or cadherin-5 (Suzuki et al., 1991; Lampugnani et al., 1992). VE-cadherin is a newly described member of the cadherin family that is selectively expressed by EC of all types of vessels both in culture and in situ (Lampugnani et al., 1992; Leach et al., 1993). VE-cadherin was shown to mediate homophilic intercellular adhesion in transfected cells (Brevario et al., manuscript submitted for publication) and to restrict endothelial permeability (Lampugnani et al., 1992). VE-cadherin is apparently unique in its junctional distribution in essentially all endothelia, unlike N-cadherin, the other major cadherin of EC, that is common also in non-endothelial tissues and is often diffuse on the cell membrane (Salomon et al., 1992; with some exception, Alexander et al., 1993). P-cadherin is expressed at low levels of EC (Liaw et al., 1990; Lampugnani, M. G., unpublished observations). The epithelial cadherin E-cadherin was found only in brain capillaries (Rubin et al., 1991).

It has been shown that the assembly of adherens type junctions is a complex process involving, besides the specific cadherins, also cytoplasmic junctional proteins (Geiger and Ginsberg, 1991). Classical cadherins (i.e., E-, N-, and P-cadherin) coimmunoprecipitate at least three cytoplasmic proteins,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -catenin (the latter is possibly identical with plakoglobin, Franke et al., 1989; Nagafuchi and Takeichi, 1989; Ozawa et al., 1989; McCrea et al., 1991a; Knudsen and Wheelock, 1992; Ozawa and Kemler, 1992; Piepenhagen and Nelson, 1993). It is believed that the catenins, together with other junction-associated proteins such as vinculin, are involved in the anchorage of cadherins to the cortical actin cytoskeleton (Hirano et al., 1987; Nagafuchi et al., 1988; Ozawa et al., 1989, 1990a; Tsukita et al., 1992; Kemler, 1993). These molecular interactions appear to be essential for E- and N-cadherin adhesive activity (Nagafuchi et al., 1988; Hirano et al., 1992; Kintner, 1992; Shimoyama et al., 1992; Fujimori et al., 1993).

In the present paper we described the interaction of VE-cadherin with catenins at different stages of endothelial junction assembly and disassembly. This process was studied using three different experimental conditions: (a) modulating extracellular  $\text{Ca}^{2+}$  concentrations; (b) following the progression of intercellular adhesion with increasing culture confluence and (c) wounding confluent EC monolayer and following the disassembly of intercellular junctions in the migrating cells.

We report here that VE-cadherin in EC exhibits a differential interaction with  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin, depending on culture condition and monolayer maturity. Thus, VE-cadherin,  $\alpha$ -catenin, and  $\beta$ -catenin formed complexes at relatively early stages even before acquisition of Triton X-100 insolubility by VE-cadherin. Plakoglobin binding, on the other hand, occurred at later stage of confluence. The formation of such complexes (VE-cadherin/ $\alpha$ -catenin/ $\beta$ -catenin/plakoglobin) was accompanied by a marked rise of the latter protein and by a moderate decrease of  $\beta$ -catenin. Upon disruption of intercellular junctions (due to cell migra-

tion into an in vitro wound) the reciprocal process occurred, namely, plakoglobin dissociated from VE-cadherin/ $\alpha$ -catenin complex while  $\beta$ -catenin accumulated into it. These findings suggest that the early formation of VE-cadherin/ $\alpha$ -catenin/ $\beta$ -catenin complexes and their association with inter-endothelial junctions does not require a strong connection with the cytoskeleton while plakoglobin accumulation at these sites depends upon the formation of organized and stable junctions.

## Materials and Methods

### Antibodies

The following antibodies were used: mouse monoclonal antibody (mAb) to human VE-cadherin (clone TEA 1.31 [Leach et al., 1993], clone BV9 and clone BV6); mouse mAb to human PECAM-1 (clone 9G11, British Biotechnology, Oxford, UK); mouse mAb to plakoglobin (clone PG5.1, Cowin et al., 1986; Franke et al., 1987) both kindly donated by Prof. W. Franke (German Cancer Research Center, Heidelberg, Germany) and purchased from American Research Products (Belmont, MA); rat mAb to  $\alpha$ -catenin (clone  $\alpha$ 18, kindly provided by Dr. A. Nagafuchi [National Institute for Physiological Sciences, Okazaki, Japan]; rabbit immunoglobulins to  $\alpha$ -catenin and to  $\beta$ -catenin (kindly gifted by Dr. D. Vestweber [Max Planck Institute for Immunobiology, Freiburg, Germany]); rabbit anti-pan-cadherin serum prepared as previously described (Geiger et al., 1990); mouse mAb to CD2 lymphocyte antigen (OKT11; Ortho Diagnostic System Inc., Raritan, NJ), that is not expressed by EC was also used as a negative control.

### Cell Culture

Human EC from umbilical vein were cultured and characterized as described in detail elsewhere (Lampugnani et al., 1992). Cells were routinely cultured on tissue culture vessels (Falcon; Becton Dickinson, Plymouth, England) coated with gelatin (1.5%; Difco, Detroit, MI). For immunofluorescence microscopy cells were cultured on glass coverslips (13-mm diam), precoated with human plasma fibronectin (7  $\mu\text{g}/\text{ml}$ ).

### EGTA Treatment

To decrease extracellular calcium concentration, as described by Volberg et al. (1986), 5 mM EGTA in serum-free 199 medium (culture medium; Gibco, Paisley, Scotland; pH of the medium readjusted to 7.4 with NaOH) was added to tightly confluent (see below) cell monolayers for the indicated time intervals. To recover from EGTA effect, cells exposed to EGTA for 30 min, were washed twice with serum-free culture medium and further incubated in serum-free culture medium as indicated.

### Effect of Cell Density on Junction Assembly

On the basis of preliminary experiments, EC were seeded at different initial densities to reach different stages of confluence at the same time after seeding in culture (72–96 h). Subconfluent cells (seeding density  $1.2 \times 10^3$  cells/cm<sup>2</sup>) presented sparse cell contacts at the time of experiment (when cell density reached  $5\text{--}6 \times 10^3$  cells/cm<sup>2</sup>). Recently confluent cells (seeding density  $4 \times 10^3$  cells/cm<sup>2</sup>) reached confluence no longer than 18 h before the experiment. These cells were characterized by absence of gaps between cells, yet spread morphology and relatively low cell density for confluent cells ( $25\text{--}28 \times 10^3$  cells/cm<sup>2</sup>). Long confluent cells (seeding density  $10 \times 10^3$  cells/cm<sup>2</sup>) reached confluence 48–72 h before the experiment. The cells showed limited spreading and reached high density ( $56\text{--}65 \times 10^3$  cells/cm<sup>2</sup>). The density of the EC used in these experiments was monitored by phase contrast microscopy several times a day by at least two independent observers. Care was taken to use cultures that had received the last change of medium no shorter than 48 h before the experiment.

### In Vitro Wounding Experiments

Highly confluent EC monolayers in 100-mm diam Petri dishes were wounded with a plastic tip (Pepper et al., 1992a). About 40 wounds (each 1 mm wide) parallel to each other were produced. The dish was then rotated through 90° and 40 further parallel wounds were produced. The cells were washed twice with complete culture medium (culture medium containing

20% newborn calf serum, 100  $\mu\text{g/ml}$  heparin and 50  $\mu\text{g/ml}$  endothelial cell growth factor, Lampugnani et al., 1992) and further incubated for 20 h. Cells were extracted for both Western and Northern blot analysis as described below.

For immunofluorescence, tightly confluent monolayers on glass coverslips were used. Culture medium was aspirated and the monolayer was wounded with a plastic tip (4 diam distanced 45° one from the other were removed). The wounded cell layer was washed twice with complete culture medium and either immediately fixed (see below) or supplemented with complete culture medium for the indicated time period. Control cells on glass coverslips were washed twice with culture medium and further incubated for the indicated length of time.

### **Immunoprecipitation and Western Blot Analysis**

EC were [ $^{35}\text{S}$ ]methionine labeled (see Lampugnani et al., 1992 for labeling details) for immunoprecipitation analysis.  $^{125}\text{I}$  labeling of VE-cadherin exposed at the cell surface was as described by Lampugnani et al. (1992). For immunoprecipitation coupled with immunoblotting experiments unlabeled cells were used. To separate the cellular extracts into Triton X-100 (TX-100)-soluble and insoluble fractions, extraction was performed as described below (Beer Stolz et al., 1992). The cell layer was washed twice with  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -containing PBS and twice with serum-free medium 199. Cells were extracted at 0°C with extraction buffer (10 mM Tris-HCl, 150 mM NaCl [TBS] with 2 mM  $\text{CaCl}_2$ , pH 7.5, 1 mM PMSF, 40 U/ml aprotinin, 15  $\mu\text{g/ml}$  leupeptin, 0.36 mM 1,10-phenanthroline [TBS with protease inhibitors], 1% NP-40 and 1% TX-100, 500  $\mu\text{l}$  for 25  $\text{cm}^2$  area) for 30 min on ice with occasional gentle agitation. The extraction buffer was collected and centrifuged at 14,000  $g$  for 5 min at 4°C. The supernatant was defined as the TX soluble fraction. After this extraction, at phase contrast microscopy the cells appeared homogeneously adherent to the culture vessel with well preserved nuclei and cytoskeletal fibers. They were then gently washed three times with TBS containing protease inhibitors and then extracted with 0.5% SDS and 1% NP-40 in TBS with protease inhibitors for 20 min on ice. The extract was collected, vigorously pipetted, and centrifuged at 14,000  $g$  for 5 min at 4°C. The supernatant was defined as the TX-insoluble fraction. When the amount of externally exposed  $^{125}\text{I}$ -labeled VE-cadherin present in this fraction was evaluated by immunoprecipitation SDS final concentration in the samples (both the TX-100-insoluble and the TX-100-soluble fractions analyzed in parallel) was adjusted to 0.2% with TBS with protease inhibitors and 1% NP-40 before immunoprecipitation.

Immunoprecipitation was performed as previously described (Lampugnani et al., 1992) with some modifications. Briefly, cell extracts were pretreated for 1 h at room temperature with protein G Sepharose (recombinant; Pharmacia, Uppsala, Sweden, 25–50  $\mu\text{l}$  for each sample of 500–1000  $\mu\text{l}$ ). The supernatant, separated by centrifugation (14,000  $g$ , 15 s at 4°C) was incubated with 25  $\mu\text{l}$  protein G Sepharose pretreated with 8–10  $\mu\text{g}$  mouse immunoglobulins (directed against VE-cadherin or PECAM-1, or CD2 antigen, as indicated) for 1 h at room temperature under continuous mixing. The resin pellet was then incubated for 30 min on ice in 1 ml TBS with 1 mM PMSF, 40 U/ml aprotinin, 2% TX-100 without or with 1% SDS. This was followed by three washes with 10 mM Tris-HCl, 0.5 M NaCl, pH 7.5, containing 2 mM  $\text{CaCl}_2$ , 0.05% NP-40, 1 mM PMSF, and 40 U/ml aprotinin. The resin pellet was boiled in sample buffer (Lampugnani et al., 1992) containing 2-mercaptoethanol (5% final concentration, reducing conditions).

For immunoblotting, after SDS-electrophoresis under reducing conditions, the polyacrylamide gel was incubated for 30 min (2  $\times$  15 min) in transfer buffer (Tris-HCl 50 mM, glycine 95 mM, 1 mM  $\text{CaCl}_2$ ). Separated proteins were then electrotransferred onto nitrocellulose (Bio Rad Labs, Richmond, CA), that was blocked with 10% low-fat milk in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -containing PBS (blocking buffer) and incubated overnight with the appropriate antibody (either TEA 1.31 or  $\alpha$ 18 hybridoma culture supernatants diluted one to two in blocking buffer or 5  $\mu\text{g/ml}$  PG5.1 or 5  $\mu\text{g/ml}$  immunoglobulins to  $\alpha$ -catenin or 5  $\mu\text{g/ml}$  immunoglobulins to  $\beta$ -catenin in blocking buffer). This was followed by a 60-min incubation with rabbit anti-mouse or rabbit anti-rat IgG, when required, (20  $\mu\text{g/ml}$ ; DAKO-PATTS, Glostrup, Denmark) and a 90-min incubation with  $^{125}\text{I}$ -labeled protein A ( $\sim 10^3$  cpm/ml; Amersham International, Buckingham, UK). Between the various incubation steps the nitrocellulose was washed several times with  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -containing PBS with 0.05% Tween 20. The immunoreactive bands were revealed by autoradiography. Autoradiography was also used to visualize the bands immunoprecipitated from  $^{125}\text{I}$ -labeled EC and from [ $^{35}\text{S}$ ]methionine metabolically labeled EC. In

this latter case gels were soaked in Enhance (Du Pont NEN, Dreieich, Germany) before drying.

Band intensity (mean optical density integrated for the band area) was quantified by a digital image analyzer (RAS 3000, Loats System; Amersham).

For immunoprecipitation of [ $^{35}\text{S}$ ]methionine metabolically labeled and  $^{125}\text{I}$ -labeled surface-labeled cell extracts, 10–15  $\times 10^6$  cpm and 1–2  $\times 10^5$  cpm (trichloroacetic acid insoluble) were used for each sample, respectively. For immunoblotting of unlabeled cell extracts, total cell extracts for each lane were from 1.5–3  $\times 10^5$  cells and immunoprecipitates for each lane were from 2–5  $\times 10^6$  cells. Cell number was determined from duplicate dishes.

### **VE-cadherin and Plakoglobin Probes and Northern Blot Analysis**

On the basis of the published cDNA sequences (Franke et al., 1989; Suzuki et al., 1991) we amplified from human EC RNA the coding region of VE-cadherin (from nucleotide 103 to nucleotide 2442) and a partial coding region of plakoglobin (from nucleotide 503 to nucleotide 2151). This was done according to the protocol of the RNA PCR kit (Perkin-Elmer Cetus Instrs., Norwalk, CT). The PCR products were subcloned into the pCRII plasmid using the TA cloning kit (Invitrogen Corporation, San Diego, CA). The cDNA-containing plasmids were analyzed by the dideoxynucleotide chain termination sequencing method (Tabor et al., 1987) and used as probes for subsequent Northern blot analysis. For Northern blot analysis total RNA was extracted and purified using the guanidium isothiocyanate/ $\text{CsCl}_2$  method as described (Golay et al., 1991). 10  $\mu\text{g}$  of total RNA were run in a standard formaldehyde/agarose gel, blotted onto nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany), and fixed at 80°C under vacuum for 2 h. Hybridization and washing conditions were as described (Golay et al., 1991).

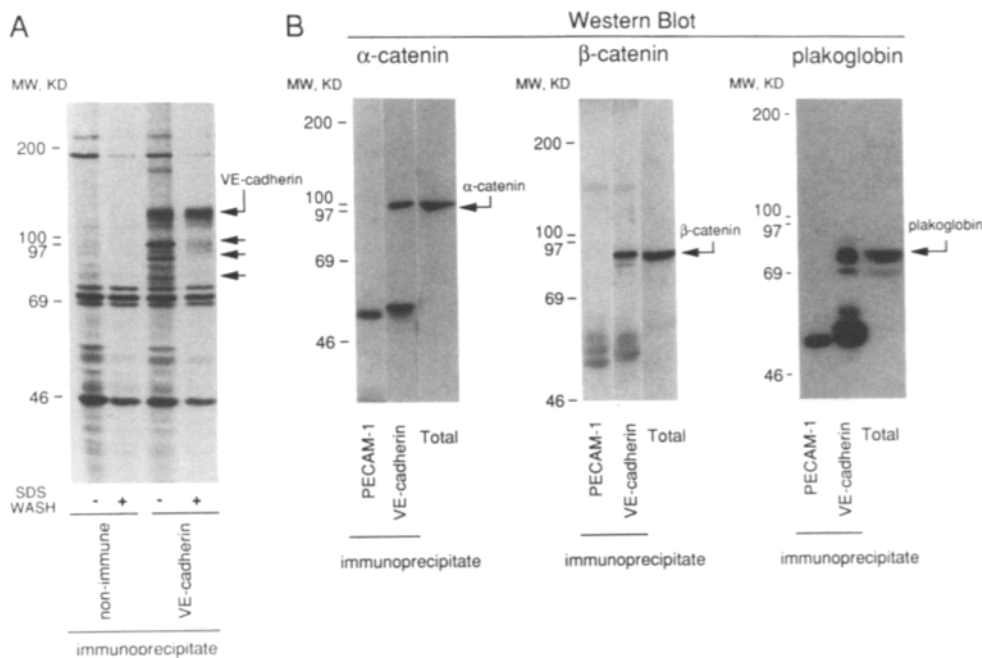
### **Immunofluorescence Microscopy**

Immunofluorescence was as described previously in detail (Lampugnani et al., 1992). Cells were fixed with 3% formaldehyde freshly prepared from paraformaldehyde (PAF) and permeabilized with 0.5% TX-100. In some experiments, to confirm with other fixation methods the results obtained, cells were fixed either with methanol (5 min at  $-20^\circ\text{C}$ ) or fixed and permeabilized at the same time with PAF (3%) with 0.5% TX-100 (3 min and an additional 15 min with PAF alone). Incubation with the primary antibody was followed by the rhodamine (TRITC)-conjugated secondary antibody, (DAKO-PATTS and Sigma Chem. Co., St. Louis, MO, to immunoglobulins of the appropriate species, depending on the primary antibody) in the presence of fluorescein (FITC)-labeled phalloidin (2  $\mu\text{g/ml}$ , Sigma Chem. Co.). In some experiments labeling was enhanced by an intermediate step of 20  $\mu\text{g/ml}$  of either rabbit anti-mouse or rabbit anti-rat immunoglobulins preceding the secondary antibody. For double staining the primary step was with mouse anti VE-cadherin in combination with rabbit anti  $\alpha$ -catenin or anti  $\beta$ -catenin and mouse anti plakoglobin in combination with rabbit anti  $\beta$ -catenin or pan-cadherin. This was followed by TRITC-coupled goat anti-mouse in combination with FITC-coupled donkey anti-rabbit (both from Jackson, West Grove, PA). Coverslips were then mounted in Mowiol 4-88 (Calbiochem-Novabiochem, La Jolla, CA) and examined with a Zeiss Axiophot microscope. Images were recorded on Kodak T MAX P3200 films with constant exposure of 40 s.

## **Results**

### **Association of VE-cadherin with Cytoplasmic Proteins**

Antibodies to VE-cadherin immunoprecipitated from [ $^{35}\text{S}$ ]methionine labeled confluent EC, besides VE-cadherin (130 kD), three major protein bands with apparent molecular mass 100, 93, and 83 kD (Fig. 1, *arrows*). These molecular masses are similar to those reported for catenins (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989; McCrea and Gumbiner, 1991b). In addition, the three bands disappeared after washing the immunocomplex with SDS as described for catenins associated to E-cadherin (Ozawa and Kemler, 1992). The three bands were not observed in VE-cadherin immunoprecipitates from  $^{125}\text{I}$ -labeled EC or in



**Figure 1.** VE-cadherin coimmunoprecipitates catenins. (A) Cell extracts from [<sup>35</sup>S]methionine-labeled confluent EC were immunoprecipitated with either mouse mAb to VE-cadherin or with irrelevant mouse IgG (non-immune, IgG to the T-lymphocyte antigen CD2, not expressed by EC). VE-cadherin (130 kD) coimmunoprecipitated bands of molecular mass 100, 93, and 80–83 kD, which were removed by SDS washing. These bands were not observed in the non-immune precipitates. (B) VE-cadherin immunoprecipitates were blotted with antibodies to either  $\alpha$ -catenin,  $\beta$ -catenin, or plakoglobin. As control of both specificity and blotting procedure, PECAM-1 immunoprecipitates and total cell ex-

tracts were analyzed in parallel. Molecular mass markers are shown on the left. The bands at  $\sim$ 50 kD position are the reduced immunoglobulins (to PECAM-1 and VE-cadherin, respectively) introduced in the samples at the immunoprecipitation step.

Western blots of VE-cadherin immunoprecipitates (not shown), indicating that they are distinct intracellular proteins. The residual faint band(s) of  $\sim$ 100 kD after SDS, most likely derives from degradation of VE-cadherin (as previously shown, Lampugnani et al., 1992) as it can be recognized in Western blotting by VE-cadherin antibodies (Lampugnani et al., 1992). Other minor bands of apparent molecular mass 190, 96, and 89 kD were also specifically and consistently observed in VE-cadherin immunoprecipitate, they disappeared by SDS washing suggesting that they could be either catenins degradation products or other unidentified molecules coimmunoprecipitated with VE-cadherin.

To directly determine whether VE-cadherin was associated with catenins, VE-cadherin immunoprecipitates were blotted with antibodies to either  $\alpha$ -catenin,  $\beta$ -catenin, or plakoglobin ( $\gamma$ -catenin). As reported in Fig. 1,  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin antibodies recognized bands of 100, 93, and 83–80 kD, respectively, in the VE-cadherin immunoprecipitates.

$\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin were not associated with PECAM-1 immunoprecipitates, confirming the specificity of their association to VE-cadherin. Plakoglobin appears in all experiments (compared also Figs. 2, 7, and 10) as a triplet. The bands at 83–80 kD represent 70–90% of total, with a slight prevalence of the 83-kD form. We found that these three bands recognized by plakoglobin antibodies were present both in the total cell extract and in the VE-cadherin coimmunoprecipitate. We do not have a direct explanation for this observation, we might be dealing with degradation products of the 83-kD form or with plakoglobin isoforms. Cowin et al. (1986) described a similar pattern of plakoglobin related bands after Western blotting analysis which they interpret as partial degradation.

#### **Effect of Low Extracellular Calcium Concentration on Junctional Components in EC**

Physiological concentrations of  $\text{Ca}^{2+}$  ( $>1$  mM) are required for maintaining EC restricted permeability properties (Shasby and Shasby, 1986; Lampugnani et al., 1992). We investigated whether low  $\text{Ca}^{2+}$  concentration induces redistribution of components at inter-endothelial junctions. It was found that reduced  $\text{Ca}^{2+}$  concentration had profound effect on the localization of plakoglobin,  $\alpha$ -catenin, and  $\beta$ -catenin to an extent and with a kinetics superimposable to VE-cadherin. Within 5 min after addition of EGTA, VE-cadherin, and catenins largely disappeared from cell junctions and become undetectable within 30 min of treatment (not shown). Restoration of physiological  $\text{Ca}^{2+}$  concentration resulted in a rapid and progressive recovery of VE-cadherin and catenins at junctional sites. This was already evident at 5 min and further progressed to almost normal appearance by 45 min of recovery. It should be mentioned that EGTA treatment of confluent cultures induced only very limited and sporadic cell retraction. The organization of actin microfilament was altered in EGTA-treated cells, but actin staining at contacts was preserved.

We then investigated whether the redistribution of VE-cadherin was associated with changes in its association with cytoskeletal proteins. Triton insolubility is commonly interpreted as an indicator for cytoskeletal association (McNeill et al., 1993 and references therein). Both control and EGTA-treated (30-min treatment) confluent EC monolayers were extracted with TX-100 and the soluble and insoluble fractions separated (Fig. 2). In control cells 20–26% of total VE-cadherin was recovered in the insoluble fraction, this amount was reduced to 6–10% after EGTA treatment.

The segregation of  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin

between the Triton-soluble and insoluble fractions was affected by EGTA in a manner very similar to VE-cadherin. Moreover low  $Ca^{2+}$  did not modify the composition of the complex between VE-cadherin and catenins as indicated by Western blotting of VE-cadherin immunoprecipitates (Fig. 2). As a control of the specificity of the VE-cadherin in the Triton-insoluble fraction, we analyzed the distribution of another transmembrane molecule of inter-endothelial contacts, PECAM-1. PECAM-1, however, could never be detected in the Triton-insoluble pellet even when the cells of origin were tightly confluent EC (Fig. 2).

### Effect of Confluence on VE-cadherin, $\alpha$ -catenin, $\beta$ -catenin, and Plakoglobin Organization at Inter-endothelial Junctions

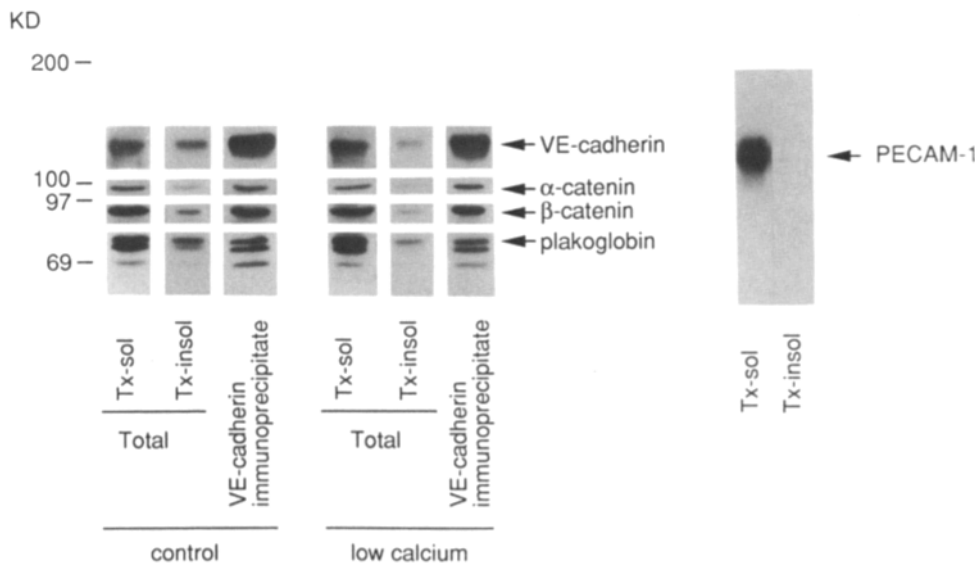
The organization and interaction of VE-cadherin,  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin at different stages following the establishment of inter-endothelial contacts was analyzed by immunofluorescence microscopy and immunoprecipitation. In subconfluent cultures, with EC touching each other discontinuously, VE-cadherin signal appeared only in the areas of cell to cell interaction (Fig. 3 a). In the absence of cell contact no localization of VE-cadherin at cell periphery was observed (Fig. 3 a, arrowhead).

In double staining experiments  $\alpha$ -catenin and  $\beta$ -catenin presented a very similar pattern (Figs. 4 and 5, a and b) while plakoglobin was hardly detectable in most regions of cell contacts (Fig. 6, b and h). When the cells were recently confluent (just enough to establish continuous contacts along their entire periphery; Fig. 3, c and d), VE-cadherin appeared as a fine, often segmented, line along the entire cell margins (Fig. 3, c).  $\alpha$ -catenin and  $\beta$ -catenin closely followed

VE-cadherin pattern (Figs. 4 and 5, c and d) while plakoglobin staining was still absent or very weak (Fig. 6, d and k).

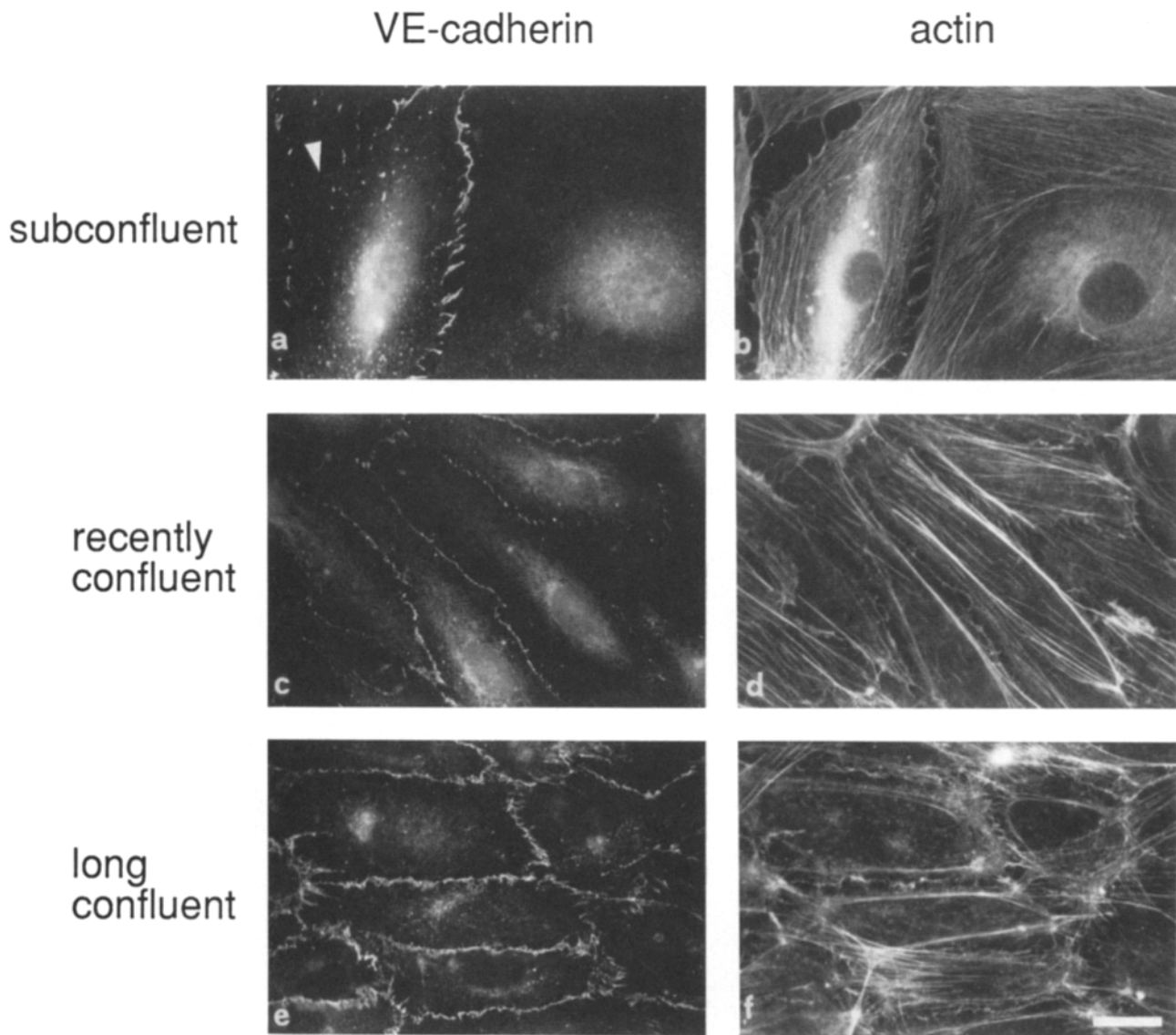
In long confluent EC monolayers (48–72 h after confluence) VE-cadherin appeared as a thick belt along the cell contact area (Fig. 3, e), presenting a much more complex pattern than that observed in loosely confluent EC (compare Fig. 3, c and e). Under the same condition,  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin were extensively labeled, showing a distribution comparable to that of VE-cadherin (Figs. 4–6, e and f, respectively). With confluence progressing, the area occupied by a single cell decreased and actin filaments reorganized from thick cables mostly oriented along the longest cell axis to shorter and more irregular fibers mostly at the cell periphery (Fig. 3, compare b, d, and f). Often, a circumferential actin ring was present (Fig. 3 f).

Double staining experiments on plakoglobin distribution had to be done with a rabbit polyclonal pan-cadherin antibody (Geiger et al., 1990) and a mouse monoclonal plakoglobin antibody since antibodies to VE-cadherin or plakoglobin of different species than mouse were not available. The pan-cadherin antibody has a wide recognition ability to all the cadherins possessing the specific cytoplasmic sequence against which it has been developed. In EC it recognizes, besides VE-cadherin, N-cadherin, and P-cadherin (Ayalon et al., 1994). Nevertheless up to now only VE-cadherin has been found to mark regularly the inter-endothelial contacts while the other cadherins have been observed at the junctions only occasionally (Salomon et al., 1992). However, as one cannot exclude that besides VE-cadherin other still unknown cadherins localized at interendothelial junctions can be recognized by the pan-cadherin antibody we also double stained EC for plakoglobin and



**Figure 2.** Effect of calcium ions on VE-cadherin partition between Triton X-100-soluble and insoluble fractions and coimmunoprecipitation of catenins. EC were incubated for 30 min in serum-free medium without (*control*) or with EGTA (5 mM, *low calcium*) before extraction and separation into Triton-soluble and -insoluble fractions (see Materials and Methods). Aliquots of these fractions (corresponding to  $1.5 \times 10^5$  cells for one lane) were analyzed in Western blot for the presence of VE-cadherin and catenins. The Triton-soluble fractions were also immunoprecipitated with antibodies to VE-cadherin to detect the presence of

catenins complexed with VE-cadherin by Western blotting. To save antibody and directly compare the level of VE-cadherin and catenins in the same sample, after blotting the nitrocellulose was cut perpendicularly to the direction of protein run, separating the areas of VE-cadherin and each catenin band. This was done with the reference of molecular weight standards. The sheets were then reacted with the appropriate antibody to reveal either VE-cadherin or  $\alpha$ -catenin or  $\beta$ -catenin or plakoglobin. The migration of molecular mass markers is shown on the left. PECAM-1, another transmembrane molecule of inter-endothelial contacts, used a control, could never be detected in the Triton-insoluble pellet.

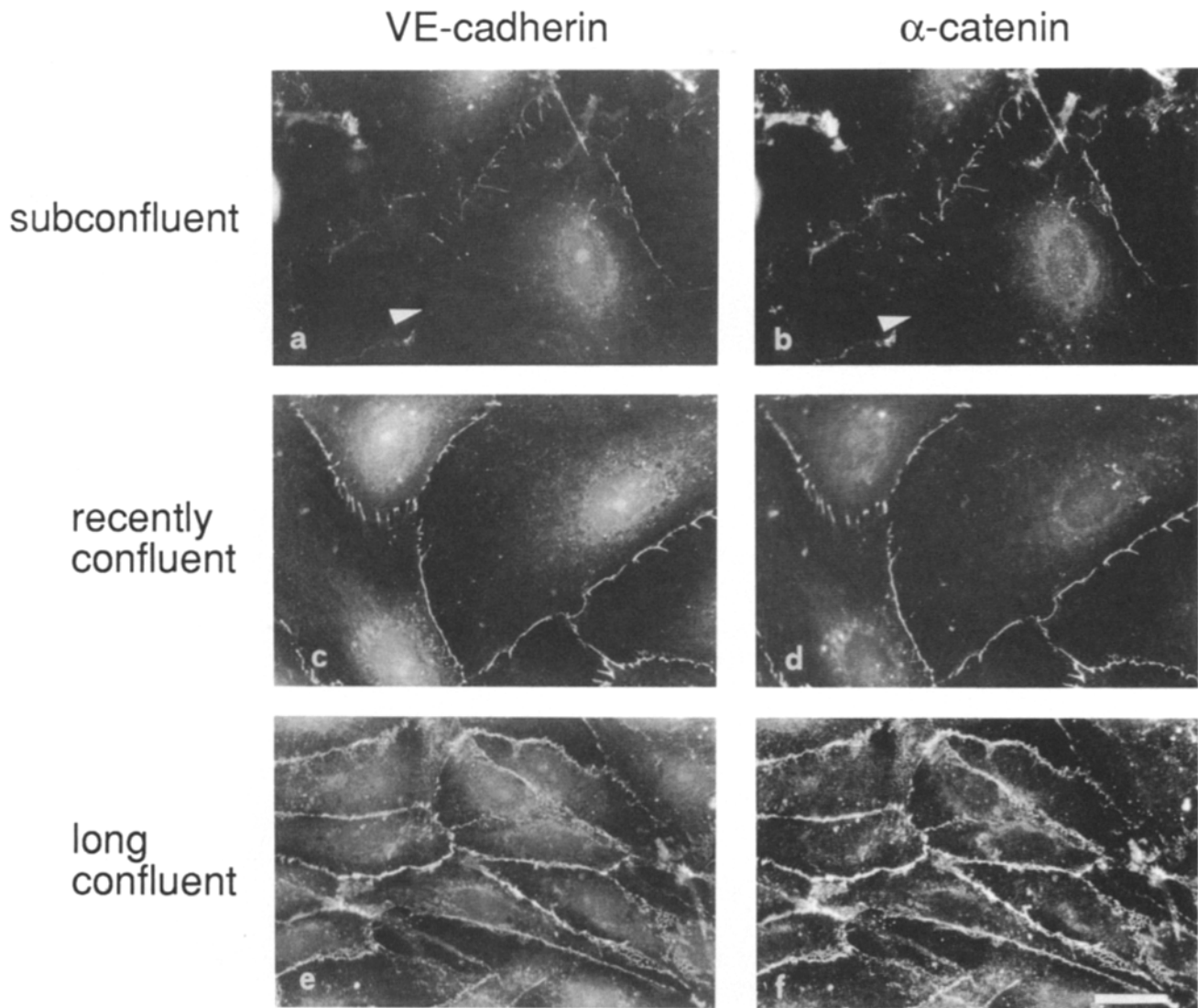


**Figure 3.** VE-cadherin localization in EC at different stages of confluence. Stages of confluence as defined in Materials and Methods. Cells were double stained for VE-cadherin (*a*, *c*, and *e*) and F-actin (*b*, *d*, and *f*). VE-cadherin staining was initially concentrated in the regions of cell to cell contact (*a* and *b*; arrowhead indicates a free cell margin, devoid of VE-cadherin staining). With the progression of intercellular contacts to the entire cell edge VE-cadherin signal extended at the entire cell margin: (*i*) as a fine, often segmented, line along the cell periphery at the beginning of the confluence (recently confluent: low density monolayer, confluence reached no longer than 18 h before staining, *c* and *d*); (*ii*) as a thick, complex line after prolonged confluence (long confluent: high density monolayer, confluence reached 48–72 h before staining, *e* and *f*). Bar, 20  $\mu\text{m}$ .

$\beta$ -catenin (which as shown in Fig. 5 codistributes with VE-cadherin) with results similar to the ones obtained for pan-cadherin and plakoglobin double labeling (Fig. 6).

To explore the quantitative significance of the apparent changes in junctional protein distribution, we coimmunoprecipitated  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin with VE-cadherin at different stages of confluence (Fig. 7 *A*). It was found that the association between VE-cadherin and  $\alpha$ -catenin did not vary significantly as a function of confluence. In contrast, the amount of plakoglobin immunoprecipitated with VE-cadherin, increased 2.2–4-fold (range of five experiments) in highly confluent cells. The total amount of plakoglobin changed in parallel to the amount immunoprecipitated. In contrast  $\beta$ -catenin associated to VE-cadherin

decreased 1.6–2-fold (range of three experiments) in tightly confluent monolayer. A similar effect was observed for the total amount of  $\beta$ -catenin. This biochemical modulation of  $\beta$ -catenin was not obvious in the immunofluorescence analysis of cell contacts (see above) possibly due to the relatively low level of sensitivity of immunofluorescence microscopy. VE-cadherin amount was not significantly affected by the degree of cell confluence (Fig. 7 *A*). Comparable results for VE-cadherin were obtained with extracts from  $^{125}\text{I}$ iodine surface-labeled EC, indicating that data obtained by Western blot reflected the behavior of the protein at the cell surface (Fig. 7 *B*). VE-cadherin was largely Triton-soluble ( $\sim 80\%$  of the total, see also Fig. 2) under any condition. The amount of Triton-insoluble cadherin increased by two- to fivefold



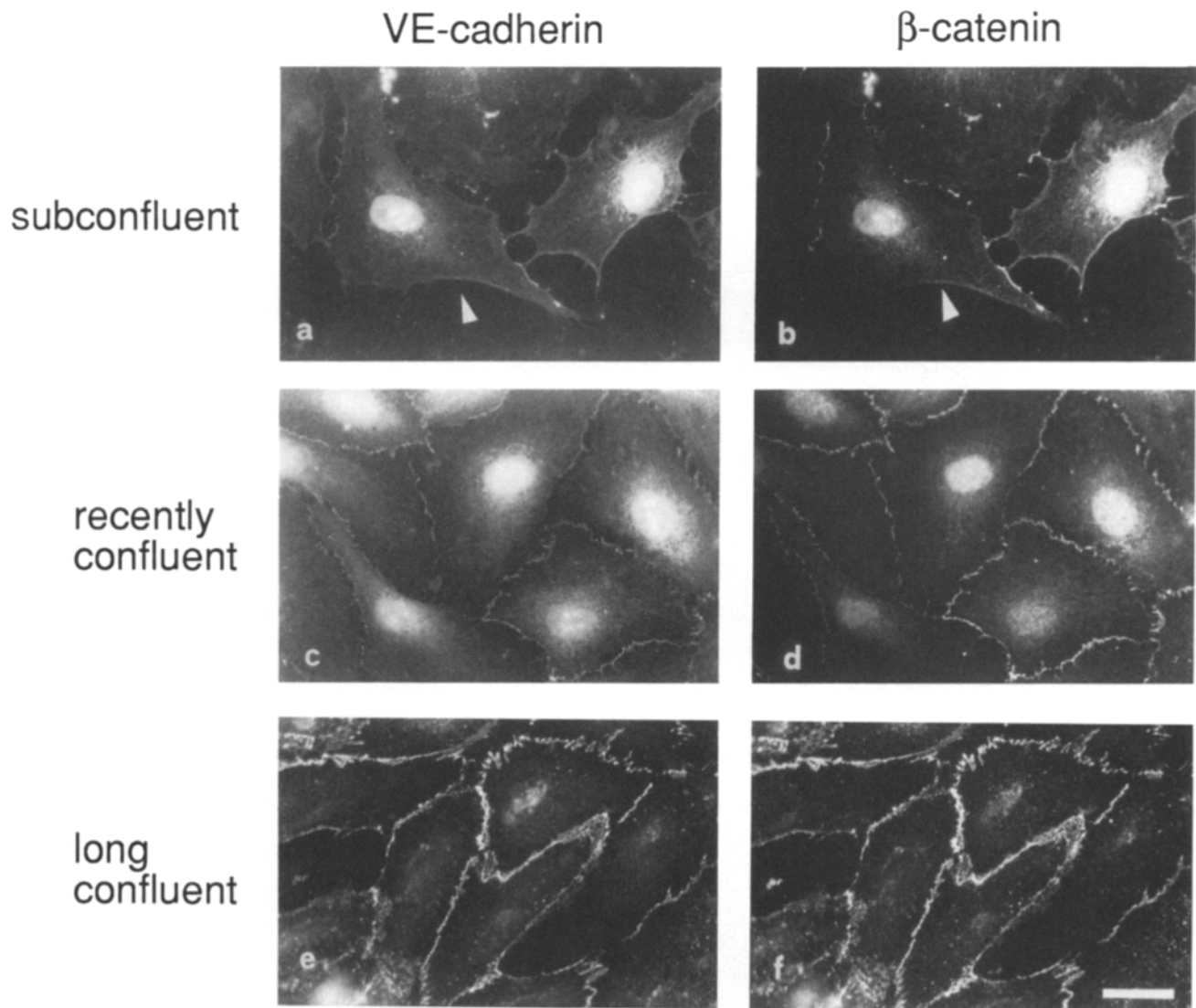
**Figure 4.** Comparison of  $\alpha$ -catenin and VE-cadherin distribution in EC at different stages of confluence. Cells were double stained for VE-cadherin (a, c, and e) and  $\alpha$ -catenin (b, d, and f).  $\alpha$ -catenin closely followed the pattern observed for VE-cadherin. It was present exclusively at cell contacts in subconfluent cells (a and b), discontinuously touching each other (arrowhead indicates a free cell margin, devoid of  $\alpha$ -catenin and VE-cadherin staining). It progressively concentrated at the cell periphery as the monolayer density increased (c and d, e and f). See also legend to Fig. 3. Bar, 20  $\mu$ m.

respectively in tightly confluent EC as compared to loosely confluent cells. We then analyzed whether level of mRNA for VE-cadherin and plakoglobin varied in parallel with the protein amount. As reported in Fig. 7 C, plakoglobin mRNA transcript increased of 64–83% in long confluent EC compared to recently confluent cells. In contrast, VE-cadherin mRNA (Fig. 7 C) and  $\beta$ -catenin mRNA (not shown) remained unchanged.

**Localization of VE-cadherin,  $\alpha$ -catenin,  $\beta$ -catenin, and Plakoglobin at Cell Junctions during Repair of a Wounded Endothelial Monolayer**

Tightly confluent (48–72 h confluent) EC monolayers were mechanically wounded. The wound releases EC at the front from close confluence and induces a complex response consisting both of early migration at the edge and later mitosis

in a more internal area (Sholley et al., 1977). Cells were examined immediately ( $t_0$ ), 3 and 20 h after wounding. Immediately after wound most of the cells at the edge did not show signs of damage and retained a continuous distribution of VE-cadherin (Fig. 8, a),  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin (not shown) at the cell contacts. Actin organization was also preserved in these cells (Fig. 8). 3–6 h after the introduction of the lesion, a marked modification of junctional organization was observed at the edge of the wound. The cells appeared to be stretching away from each other, forming small gaps along cell to cell contacts, thick stress fibers appeared, mostly oriented in the direction of apparent cell movement (Fig. 8 d). VE-cadherin staining was strong at the residual cell contacts, but redistributed in a zigzag pattern disappearing in correspondence of the intercellular gaps (Fig. 8 c). A very similar pattern was observed for  $\alpha$ -catenin,



**Figure 5.** Comparison of  $\beta$ -catenin and VE-cadherin distribution in EC at different stages of confluence. Cells were double stained for VE-cadherin (a, c, and e) and  $\beta$ -catenin (b, d, and f).  $\beta$ -catenin codistributed with VE-cadherin at intercellular junctions during each stage of formation of a densely packed monolayer from sparse cells. See also legend to Figs. 3 and 4. Arrowhead indicates a free cell margin devoid of  $\beta$ -catenin and VE-cadherin staining. Bar, 20  $\mu$ m.

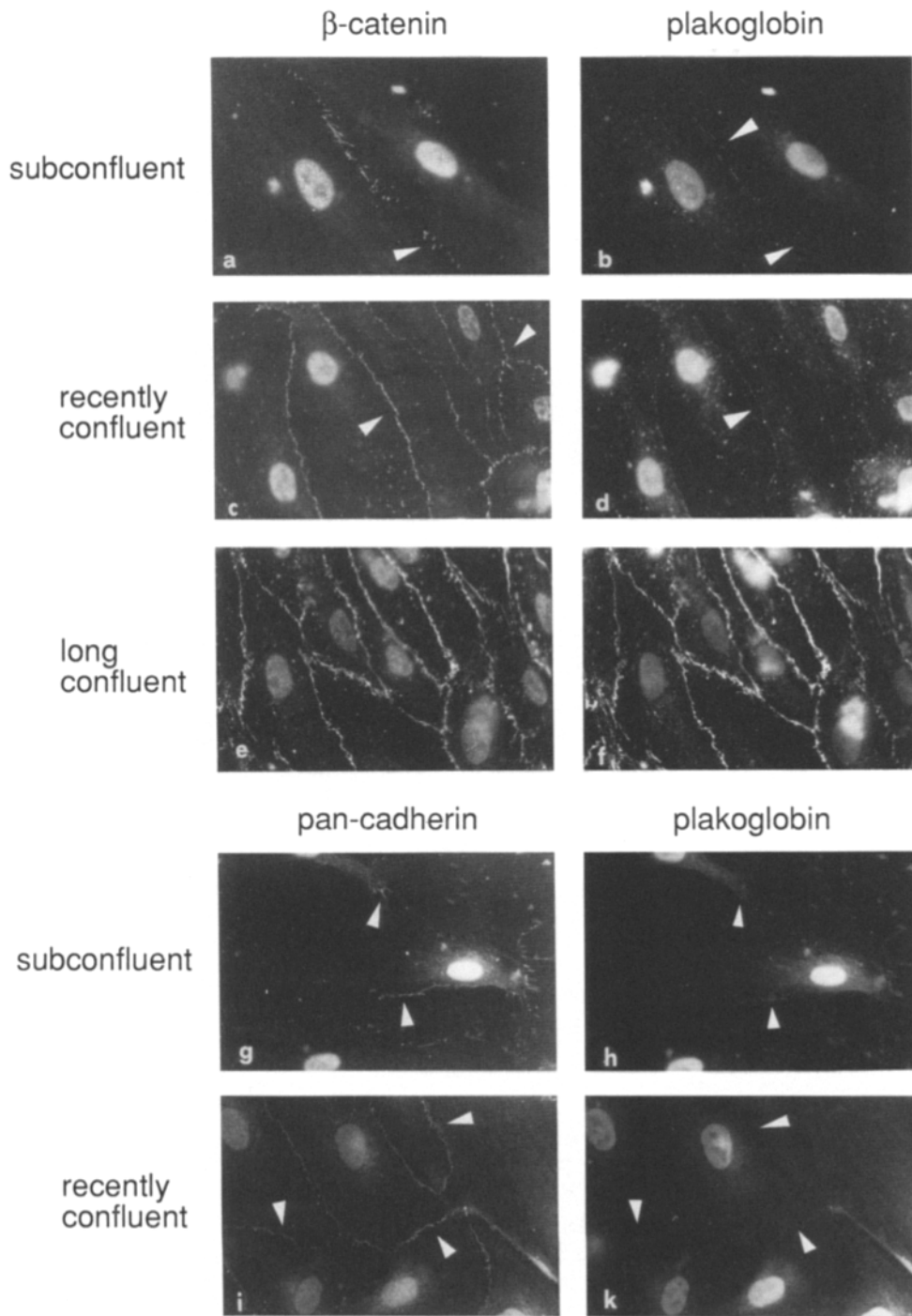
$\beta$ -catenin, and plakoglobin (not shown). This modification of the intercellular contacts was more pronounced at the wound border, but still detectable at a distance of 3–5 cells away from the front. At larger distances away from the wound no change in the organization of actin, VE-cadherin,  $\alpha$ -catenin,  $\beta$ -catenin, or plakoglobin distribution were noticed (not shown). 20 h after wounding a very different morphological pattern was observed when comparing the lesion front to internal areas. Actin distribution varied, as a function of distance from the wound. The first two lines of cells developed an extended actin-rich lamella in the apparent direction of migration (star; Fig. 8 f). The next 2–3 cell diameters away from the wound appeared as a loosely confluent monolayer, showing no intercellular gaps (compare with Fig. 3 d). Sporadic mitosis were present in this area. More distally, the layer appeared densely packed and quiescent (Fig. 8 k). Junctional VE-cadherin staining was relatively weak at the wound front (Fig. 8 e) while in adjacent

inner layers it appeared as a thin line at the periphery of the cells (Fig. 8 g) resembling the staining in loosely confluent EC, as described above (compare to Fig. 3 c). Far from the front, where the cells maintained the morphology of a densely packed monolayer, VE cadherin appeared as a thick continuous line following the cell borders (Fig. 8 i).  $\alpha$ -catenin and  $\beta$ -catenin strictly followed the pattern of VE-cadherin reorganization also in this experimental condition (Fig. 9).

Plakoglobin distribution was modified in a major way after cell wounding. 20 h after wounding plakoglobin was mostly either undetectable or barely detectable at contacts of both cells at the front (Fig. 9 f) and cells adjacent to them (Fig. 9 h), unlike  $\beta$ -catenin (Fig. 9, e and g). Plakoglobin labeling was still evident at cell to cell junctions in the distal, closely confluent areas (Fig. 9 k).

20 h after wounding we observed a significant decrease of plakoglobin level. Indeed, both the total amount of plako-



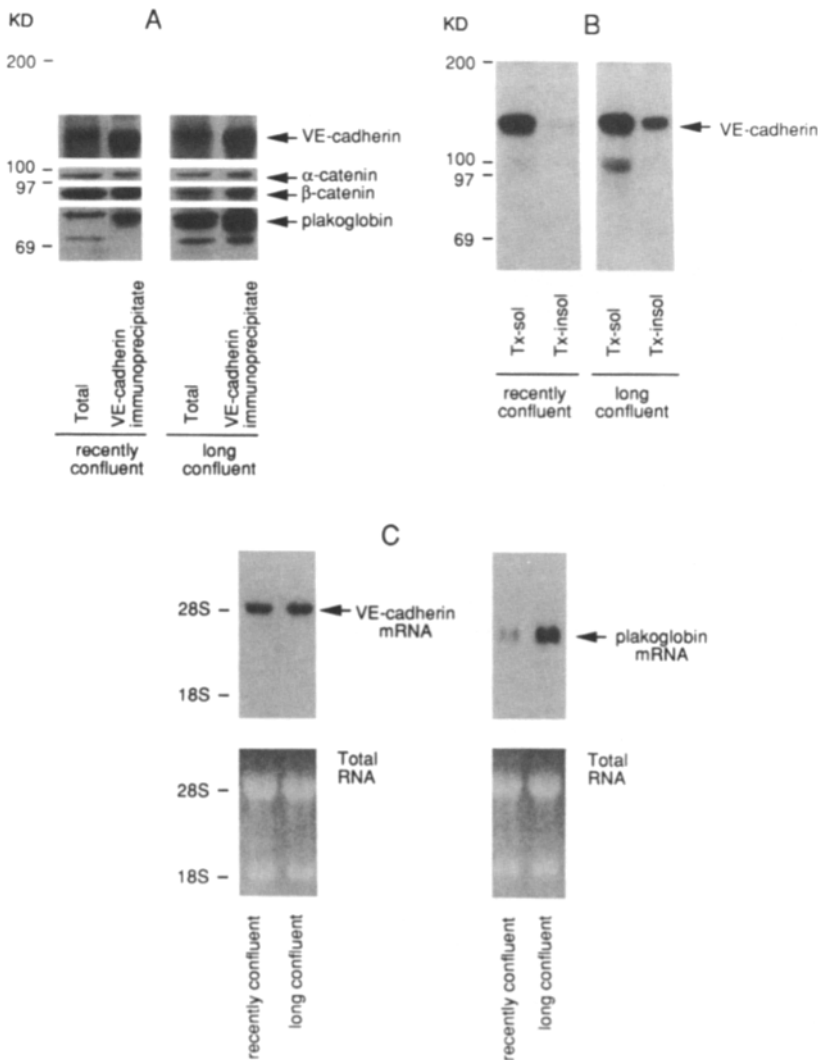


**Figure 6.** Comparison of plakoglobin and  $\beta$ -catenin and of plakoglobin and pan-cadherin localization in EC at different stages of confluence. Cells were double stained for  $\beta$ -catenin (a, c, and e) and plakoglobin (b, d, and f) and for pan-cadherin (g and i) and plakoglobin (h and k). Note that in subconfluent and loosely confluent EC plakoglobin staining was virtually absent or very weak (b, d, h, and k, arrowheads) also in areas of apparent cell contacts positive for  $\beta$ -catenin (a and c, arrowheads) and pan-cadherin (g and i, arrowheads). (Note that the arrowhead pointed cell margin positive for pan-cadherin in g is actually a region of intercellular contact. The nucleus of one of the adjoining cells lays out of the photographic field). Comparable areas were positive for VE-cadherin,  $\alpha$ -catenin and  $\beta$ -catenin (Figs. 3, 4, and 5, a–d). In long confluent monolayer (e and f) plakoglobin at the cell contacts presented an intensity and a pattern comparable to  $\beta$ -catenin. Bar, 20  $\mu$ m.

globin and the amount coimmunoprecipitated with VE-cadherin decreased 2.5–2.8-fold (range of five experiments) relative to tightly confluent EC (Fig. 10). Interestingly,  $\beta$ -catenin amount coimmunoprecipitated with VE-cadherin increased 2.1–2.6-fold (range of three experiments) 20 h after wounding (Fig. 10). The level of VE-cadherin did not significantly change in EC 20 h after wounding (Fig. 10). Both plakoglobin and VE-cadherin mRNA were not significantly modified in wounded EC (Fig. 10).

## Discussion

Adherens-type junctions are transmembrane, multimolecular complexes in which the actin-based cytoskeleton is linked to the plasma membrane through a specialized sub-membrane plaque (Geiger and Ginsberg, 1991; Tsukita et al., 1992). Major structural elements in this structure are the membrane receptors, namely members of the cadherin family, and a cytoplasmic anchoring system which binds these molecules to



**Figure 7.** Effect of confluence on VE-cadherin,  $\alpha$ -catenin,  $\beta$ -catenin and plakoglobin coimmunoprecipitation and messenger expression. (A) EC extracts from either recently confluent or long confluent monolayers were immunoprecipitated with VE-cadherin antibodies (extracts corresponding to  $5 \times 10^6$  cells were used for both recently and long confluent EC). This was followed by Western immunoblotting. Total: aliquots of cell extract corresponding to  $3 \times 10^5$  cells were run and blotted in parallel. To save antibodies and directly compare the level of VE-cadherin and catenins in the same immunoprecipitate, the nitrocellulose was cut perpendicularly to the direction of protein run. The cuts were chosen to separate the areas of VE-cadherin,  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin bands with the reference of molecular mass markers run in parallel (position on the left). The corresponding nitrocellulose sheets were then reacted with antibodies to either VE-cadherin,  $\alpha$ -catenin,  $\beta$ -catenin, or plakoglobin, respectively. (B) EC monolayers either recently confluent or long confluent were surface labeled with  $^{125}$ Iodine before extraction and separation into Triton-soluble and -insoluble fractions. This was followed by immunoprecipitation with VE-cadherin antibodies. The band at  $\sim 100$  kD is a degradation product of VE-cadherin (Lampugnani et al., 1992) which is produced during the manipulation of the cells in serum free medium as required by the labeling procedure. (C) Total RNAs ( $10 \mu\text{g}$  per lane) from recently confluent and long confluent EC were probed in Northern blot analysis for VE-cadherin and plakoglobin mRNA. Bands of the expected size ( $\sim 4,100$  bp for VE-cadherin and  $\sim 3,500$  bp for plakoglobin) were detected. Comparable amounts of total RNA were present in each lane, as indicated by the total RNAs observed after blotting (lower panels). Ribosomal RNA 28 S and 18 S were the main bands.

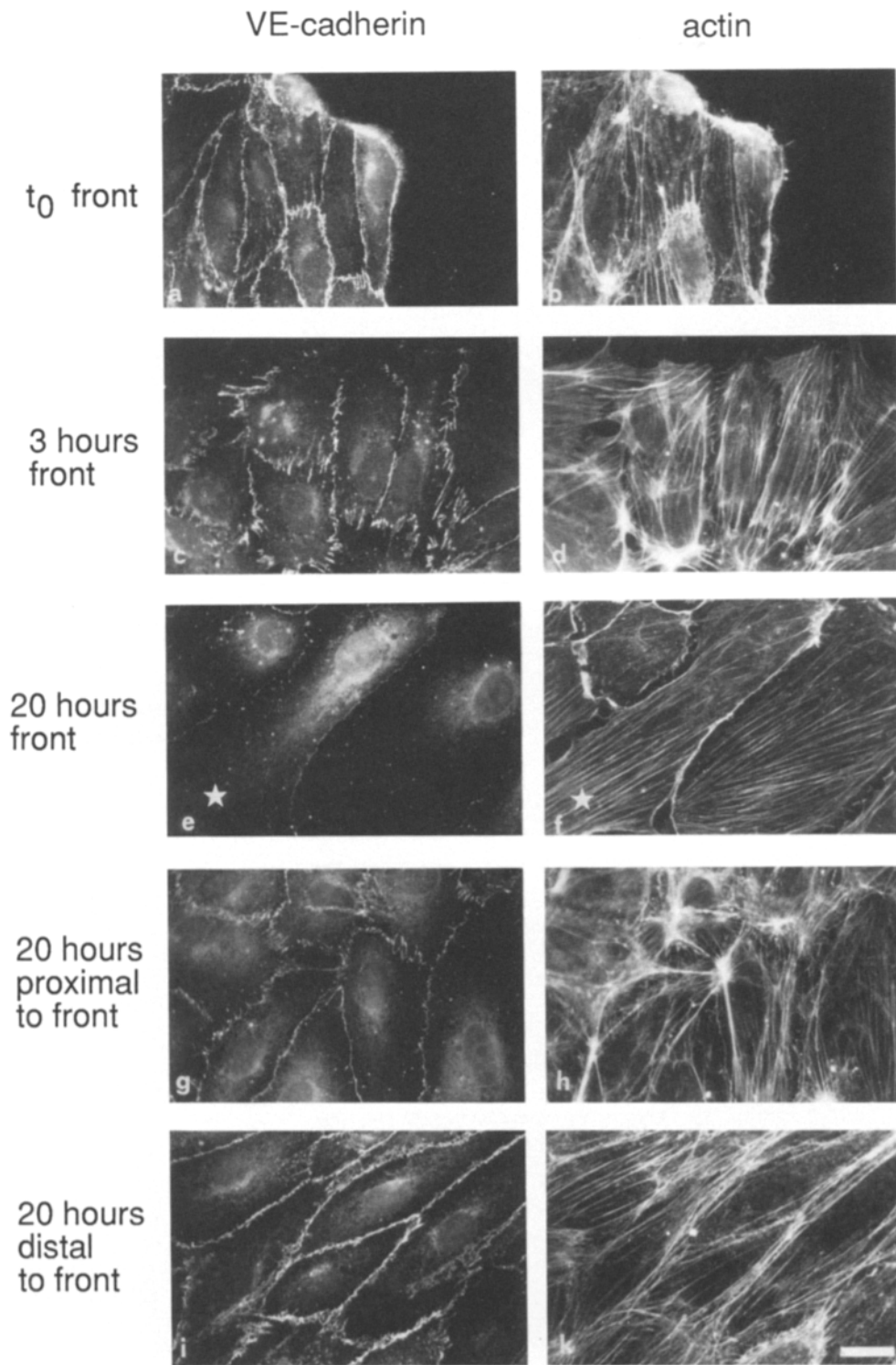
the microfilament network. Directly associated with the junctional cadherins are the anchor-proteins catenins,  $\alpha$ ,  $\beta$ , and  $\gamma$  (plakoglobin). In this study we have investigated the assembly of the cadherin-catenin complex in EC, showing that different members of the catenin triplet bind to the endothelium-specific VE-cadherin at vastly different stages of junction maturation.

#### ***VE-cadherin Organization Is Dynamically Regulated in EC in Response to Calcium Concentration***

The modulation of calcium ions in the culture medium has been successfully used to study the translocation of both calcium-dependent transmembrane proteins and cytoplasmic junctional proteins (Volberg et al., 1986; Kartenbeck et al., 1991; Citi, 1992). We report here that, similarly to the other cadherins (Grunwald, 1993), VE-cadherin organization at cell-cell contacts highly depends on extracellular  $\text{Ca}^{2+}$  concentration. This effect is rapid and reversible: VE-cadherin disappears from cell contacts within 5 min after EGTA addition and reorganizes at junctions within 5 min af-

ter restoration of physiological  $\text{Ca}^{2+}$  levels. The disappearance of VE-cadherin from cell margins is accompanied by a marked reduction in its Triton insolubility, reflecting on the association of the molecule with the actin cytoskeleton (Hirano et al., 1987; Nagafuchi and Takeichi, 1988; Ozawa et al., 1989; Shore and Nelson, 1991; McNeill et al., 1993 and references therein). The small expected increase in Triton-soluble VE-cadherin, could hardly be detected since the insoluble fraction obtained after the extensive extraction employed here, never exceeded 20–26% of total, even in tightly confluent EC. It should be noted that wide variations exist in the reported extractability of cadherins, using a variety of extraction conditions, ranging from  $\sim 60\%$  to less than 5% of the total (Hirano et al., 1987; Ozawa et al., 1989; McCrea and Gumbiner, 1991b and references therein; Shore and Nelson, 1991).

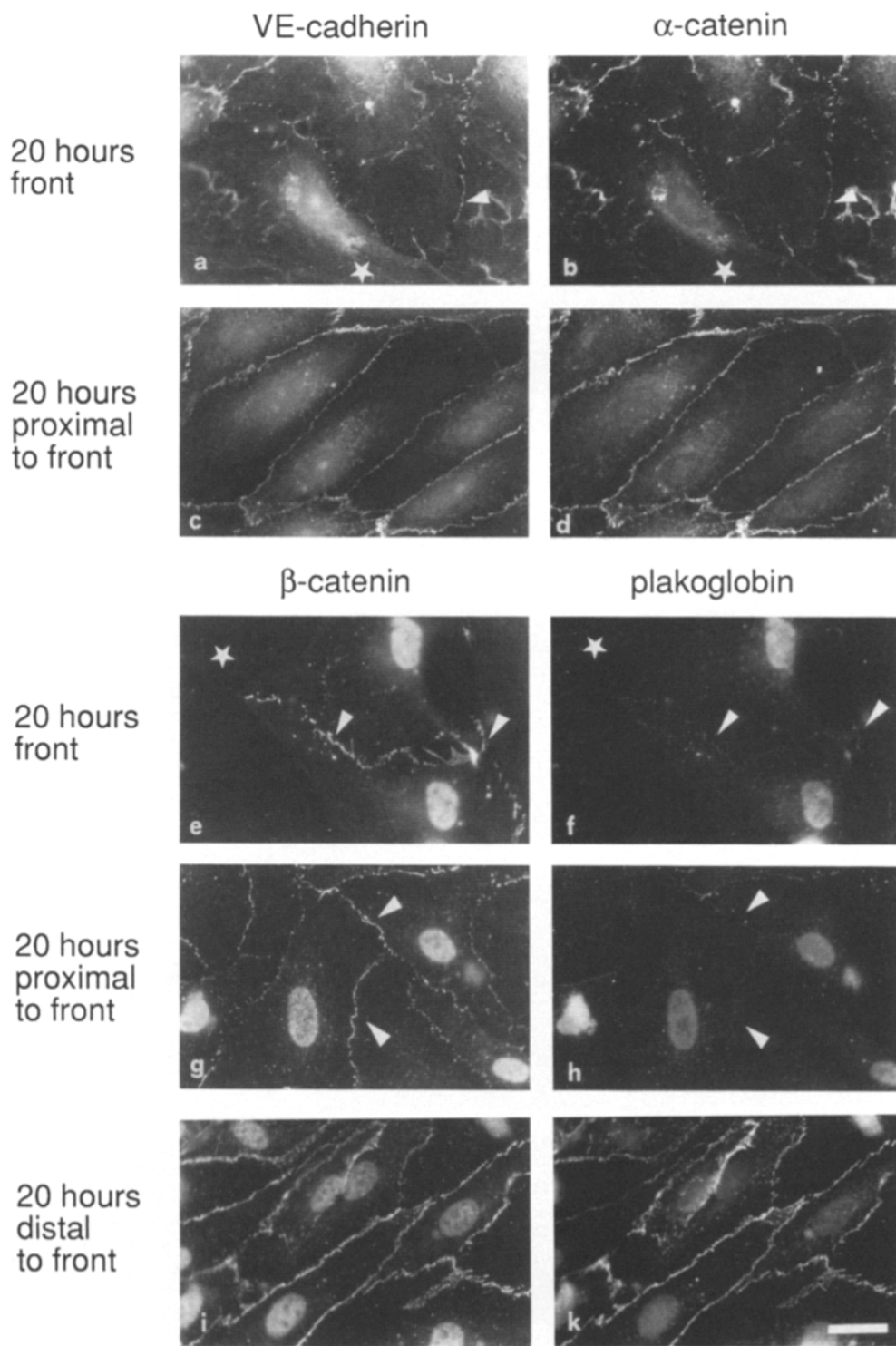
The mechanism of EGTA-mediated dispersal of VE-cadherin from endothelial junctions is not entirely clear.  $\text{Ca}^{2+}$  is believed to be required for maintenance of a functional conformation of cadherins which is essential for their



**Figure 8.** VE-cadherin distribution in EC during wound repair. Wounded edge: (a and b) immediately after the lesion, at  $t_0$ ; (c and d) 3 h after the lesion; (e and f) 20 h after the lesion. The stars in e and f indicate the direction of the front. The internal monolayer proximal to the front (2–3-cell diam away from the front, g and h) or distal to the front (i and k) 20 h after wounding is shown. A temporal and spatial gradient was observed. The wound induced morphological effects in the front and proximal to front monolayer. VE-cadherin staining in these areas progressively decreased, but was still clearly detectable in the regions of cell contacts (e and g). In areas distal to front VE-cadherin signal did not change during incubation (i; a comparable pattern was observed in equivalent areas also after shorter incubations). Cells were double stained for VE-cadherin (a, c, e, g, and i) and F-actin (b, d, f, h, and k). Bar, 20  $\mu\text{m}$ .

homotypic recognition and junctional interaction (Ozawa et al., 1990b). At low  $\text{Ca}^{2+}$  catenins ( $\alpha$ ,  $\beta$ , and plakoglobin) translocate from junctions concomitantly with VE-cadherin and apparently remain associated with it (see immunoprecipitation experiments, Fig. 2), suggesting that it is the entire complex of VE-cadherin/catenins that dissociates from the

junction. Interestingly, EGTA treatment of EC is accompanied by a marked increase in monolayer permeability, at the same time frame required for VE-cadherin redistribution (Lampugnani et al., 1992). Since this treatment is not associated to a major cell retraction or redistribution of other junctional adhesive proteins such as PECAM (Ayalon et al.,



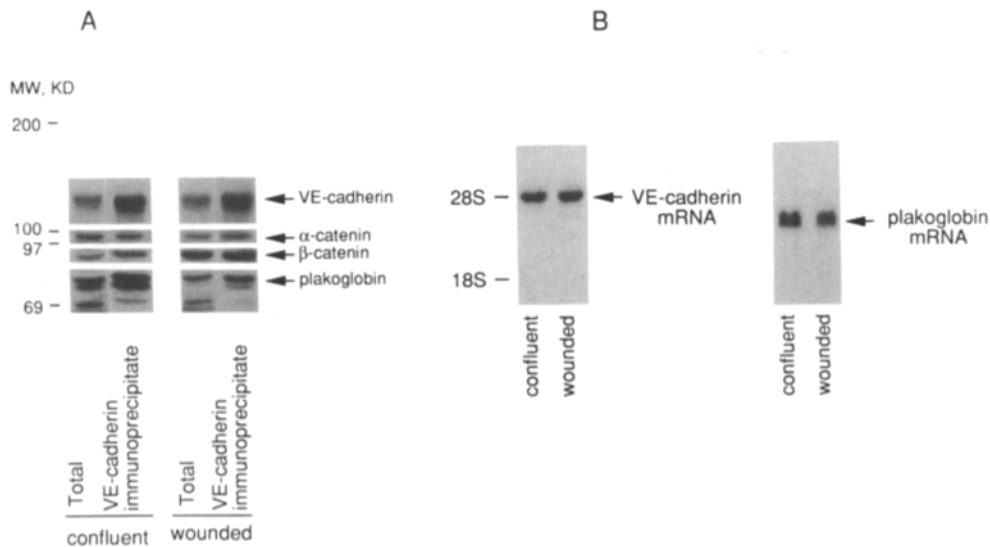
**Figure 9.**  $\alpha$ -catenin pattern in comparison to VE-cadherin and  $\beta$ -catenin pattern in comparison to plakoglobin in wound repairing EC. Cells were double stained for VE-cadherin (*a* and *c*) and  $\alpha$ -catenin (*b* and *d*) respectively and for  $\beta$ -catenin (*e*, *g*, and *i*) and plakoglobin (*f*, *h*, and *k*) respectively. Areas comparable to those in Fig. 8 are shown. Wounded edge: (*a*, *b*, *e*, and *f*) 20-h incubation. The stars in *a*, *b*, *e*, and *f* indicate the direction of the front. The monolayer internal, but proximal to the front after 20-h incubation is shown in *c*, *d*, *g*, and *h*. Wounding induced a progressive redistribution of  $\alpha$ -catenin and  $\beta$ -catenin similar to that observed for VE-cadherin. Compare Fig. 8 and relative legend. VE-cadherin,  $\alpha$ -catenin, and  $\beta$ -catenin signal at the intercellular contacts of EC was clearly present at the wound edge 20 h after lesion (arrowheads in *a*, *b*, and *e*). Up to 3 h after the wound plakoglobin staining at cell contacts was strong and comparable to that of VE-cadherin (Fig. 8 *c*). In contrast, 20 h after the wound plakoglobin staining at the front and proximal to the front was either virtually undetectable or barely detectable at most intercellular contacts (arrowheads, *f* and *h*). In areas distal to front plakoglobin signal was evident (*i*, *k*). Bar, 20  $\mu$ m.

1994), this argues for a specific role of VE-cadherin in the maintenance of a correct EC junctional organization and regulation of transendothelial permeability.

***The Effect of Junction Maturation in the Endothelium on VE-cadherin Organization and Its Association with  $\alpha$ -catenin,  $\beta$ -catenin, and Plakoglobin***

VE-cadherin was associated with inter-endothelial contacts

from early stages, in sparse cultures, to fully mature junctions in highly confluent monolayers. During all these stages VE-cadherin was, apparently, accompanied by  $\alpha$ -catenin and  $\beta$ -catenin which showed an essentially identical distribution. Plakoglobin, on the other hand, associated with the VE-cadherin-containing junctions only later, when junctions were tightly organized (usually within 48 h after reaching confluence). This was accompanied by a marked increase in



**Figure 10.** Effect of wound repair on VE-cadherin,  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin coimmunoprecipitation and messenger expression. (A) Cell extracts (corresponding to  $4 \times 10^6$  cells for one lane) from confluent and wounded (20 h after lesion) EC layers were immunoprecipitated with VE-cadherin antibodies. Total cell extracts (corresponding to  $1.5 \times 10^5$  cells, for one lane) were run in parallel (*Total*). This was followed by Western immunoblotting. To save antibody and directly compare the level of VE-cadherin and catenins in the same immunoprecipitate, after blotting the nitrocellu-

lose was cut perpendicularly to the direction of protein run separating the areas of VE-cadherin and each catenin band. This was done with the reference of molecular weight standards. The sheets were then reacted with the appropriate antibody to reveal either VE-cadherin or each of the catenins, respectively. (B) Total RNA (10  $\mu$ g) for confluent and wounded (20 h after lesion) EC layers were analyzed by Northern blot for the presence of mRNA transcripts for VE-cadherin and plakoglobin. Bands of the expected size ( $\sim 4,100$  bp for VE-cadherin and  $\sim 3,500$  bp for plakoglobin) were detected.

the quantity of plakoglobin coimmunoprecipitated with VE-cadherin. On the other hand  $\beta$ -catenin complexed with VE-cadherin decreased in the tightly confluent monolayer.

These data indicate that the establishment of VE-cadherin based inter-endothelial junctions consists of different temporally-regulated stages. First, VE-cadherin becomes organized at cell-cell contacts together with  $\alpha$ -catenin and  $\beta$ -catenin. This is followed, by a more elaborate junction assembly characterized by the binding of plakoglobin (and possibly other cytoplasmic proteins) to the junctional submembrane plaque which might partially substitute for  $\beta$ -catenin. At this stage, which we refer to as junction maturation, an overall increase in the Triton-insoluble fraction of VE-cadherin is observed.

These observations are in agreement with a work of McNeill et al. (1993), describing the early stages of cadherin-mediated adhesion in MDCK epithelial cells. In these cells, E-cadherin accumulates at nascent cell contacts, but becomes Triton-insoluble at a later stage.

Based on previous reports as well as on the results presented here we propose that the assembly of adherens-type intercellular junctions involves three major consecutive stages: (I) an initiation stage in which VE-cadherin,  $\alpha$ -catenin, and  $\beta$ -catenin are involved, yet the interaction of these complexes with the cytoskeleton is still very low; (II) an extension stage in which the junction expands and VE-cadherin,  $\alpha$ -catenin, and  $\beta$ -catenin (but not plakoglobin) associate with the microfilament system; (III) a maturation stage in which the junction reaches its final dimensions and plakoglobin associates with the submembrane plaque while part of  $\beta$ -catenin leaves it. Stage I, most likely, occurs concomitantly with the establishment of contact, the transition to stage II requires additional time (for example, 10 min in McNeill et al. [1993]). Interestingly, the maturation stage is not only accompanied by plakoglobin recruitment but also

by an increase in its synthesis (as demonstrated by the increase in total amount of the protein and of its related mRNA). On the other hand, under the same condition,  $\beta$ -catenin showed reduced association to VE-cadherin (in parallel to a decrease in its total steady state level). In this case  $\beta$ -catenin m-RNA level was not modified (not shown). These effects seem to be specific for plakoglobin and  $\beta$ -catenin since no change in  $\alpha$ -catenin or VE-cadherin amounts were observed. The signals which regulate plakoglobin gene transcription and translation are still unknown. It has been recently described that transfection with Wnt-1 gene increases steady state level of plakoglobin (Bradley et al., 1993), as well as the stability of the complex between plakoglobin and N-cadherin (Hinck et al., 1994a). In these cases the modulation was posttranscriptional as mRNA levels did not change. Concomitantly with these changes cells acquired an epithelioid morphology and increased intercellular adhesion (Bradley et al., 1993; Hinck et al., 1994a).

#### ***Exit of EC from a Confluent Monolayer Is Accompanied by VE-cadherin Redistribution, Decreased Association of Plakoglobin and Increased Association of $\beta$ -catenin with VE-cadherin***

When EC monolayers are wounded, EC migrate into the wound. During this process intercellular junctions dissociate, allowing cell detachment and movement. This process was accompanied by a concomitant decrease in junctional staining of VE-cadherin,  $\alpha$ -catenin, and  $\beta$ -catenin. Again, plakoglobin presented a different pattern of redistribution. At 20 h after wounding, when cells at or near the front still presented VE-cadherin,  $\alpha$ - and  $\beta$ -catenin at junctions, plakoglobin was essentially undetectable. The process of junction deterioration, in the migrating cells, was largely similar but reciprocally related to the one observed during the formation of stable contacts.

Interestingly, EC, (in contrast to smooth muscle cells or fibroblasts), tend to migrate from a wounded monolayer as a coordinated cell sheet, maintaining intercellular contacts during migration (Schwartz et al., 1978). Indeed EC show high levels of intercellular coupling and connexin 43 expression at early stages after wounding (Pepper et al., 1992b). The finding that cadherin organization is required for the establishment of intercellular communication through the connexin system (Jongen et al., 1991; Meyer et al., 1992) is in line with the observation reported here that VE-cadherin/ $\alpha$ -catenin/ $\beta$ -catenin complex is partially reduced but does not disappear from intercellular contacts of migrating EC. Overall this indicates that complete disruption of intercellular junction organization is not required for EC migration during the process of wound repair.

As observed in loosely confluent cells the total amount of plakoglobin, not only the amount associated with VE-cadherin, was lower in migrating cells compared to confluent monolayers. However, in these cells we did not observe a decrease in plakoglobin mRNA, suggesting that, at least within the time frame examined (20 h), the decrease in plakoglobin levels is related to translational down regulation or, more likely, increased breakdown. In addition, in parallel to loosely confluent cells, both the total amount of  $\beta$ -catenin and the amount associated to VE-cadherin increased in migrating EC at 20 h.

In summary, in this work we show that EC can differentially regulate the organization of VE-cadherin and associated cytoplasmic molecules at junctions in relation to different functional requirements. When VE-cadherin dislocation from junctions is artificially and rapidly induced by low  $\text{Ca}^{2+}$ ,  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin disappear in a concomitant way. In contrast, when the assembly and disassembly of endothelial junctions occurs slowly different temporally regulated steps can be distinguished. Loose junctions (as in early confluent cells or in cells migrating from the cell monolayer) are characterized by a weak VE-cadherin,  $\alpha$ -catenin, and  $\beta$ -catenin deposition at contacts and the absence of plakoglobin. Stable junctions (as in tightly confluent cells) are characterized by plakoglobin localization at intercellular contacts. These data suggest that while VE-cadherin/ $\alpha$ -catenin/ $\beta$ -catenin complexes can function as early recognition mechanisms between EC, the strength of the contact can be modulated by plakoglobin accumulation at junctions. Quantification through Western blotting of VE-cadherin coimmunoprecipitate confirms the immunofluorescence microscopy data for plakoglobin and suggests another possible regulatory system through  $\beta$ -catenin. Indeed the amount of  $\beta$ -catenin associated to VE-cadherin tends to vary in opposite direction to plakoglobin. Thus two highly homologous members of the Armadillo protein family might act as positive (plakoglobin) and negative ( $\beta$ -catenin) regulators to the strength of the junction. These molecules could act as a linker between cadherin/catenins complex and actin based cytoskeleton thus directly increasing or decreasing junction cohesion and strength. As an alternative  $\beta$ -catenin, in particular, might sequester the linked VE-cadherin away from the junction. Indeed strong modifications of VE-cadherin pattern at the junctions are not accompanied by variation in its total amount. It has to be noted that Hinck et al. (1994b) and Nathke et al. (1994) recently introduced the new concept of alternative complexes between E-cadherin and one or the

other of the catenins, modifying the previous view of a stable quaternary complex. Our data are in line with this model of flexible and possibly regulated relationships between cadherins and their cytoplasmic partners.

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