

## Catenin-dependent and -independent Functions of Vascular Endothelial Cadherin\*

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**Vascular endothelial cadherin (VE-cadherin, cadherin-5, or 7B4) is an endothelial specific cadherin that regulates cell to cell junction organization in this cell type. Cadherin linkage to intracellular catenins was found to be required for their adhesive properties and for localization at cell to cell junctions. We constructed a mutant form of VE-cadherin lacking the last 82 amino acids of the cytoplasmic domain. Surprisingly, despite any detectable association of this truncated VE-cadherin to catenin-cytoskeletal complex, the molecule was able to cluster at cell-cell contacts in a manner similar to wild type VE-cadherin. Truncated VE-cadherin was also able to promote calcium-dependent cell to cell aggregation and to partially inhibit cell detachment and migration from a confluent monolayer. In contrast, intercellular junction permeability to high molecular weight molecules was severely impaired by truncation of VE-cadherin cytoplasmic domain. These results suggest that the VE-cadherin extracellular domain is enough for early steps of cell adhesion and recognition. However, interaction of VE-cadherin with the cytoskeleton is necessary to provide strength and cohesion to the junction. The data also suggest that cadherin functional regulation might not be identical among the members of the family.**

Cadherins are a family of integral membrane glycoproteins responsible for homotypic calcium-dependent cell-cell adhesion (1–3). All members of the family present strong homology at the amino acidic level (4–9), but the degree of conservation differs with the different domains of the molecules. The cytoplasmic domain presents the highest conservation level and is involved in the binding to cytoplasmic proteins called catenins, that contribute to the anchorage of cadherins to the cytoskeletal network (10–15).

Previous studies have shown that deletions or substitutions of the cytoplasmic domain of cadherins results in loss of association to catenins and in inhibition of cell aggregation (12, 16). Furthermore, recent studies indicate that tyrosine phosphorylation of cadherins and/or catenins decreases the amount of

actin-bound complexes, and this was related to impairment of cadherin-mediated cell to cell aggregation and increased cell mobility (17–19). Most of these observations were made taking E-cadherin (or uvomorulin) as a model. However, considering other cadherins, the requirement of cytoskeletal interaction for the maintenance of adhesive properties does not seem to be absolute. For instance, T-cadherin, which naturally lacks the cytoplasmic and transmembrane domains, is still able to promote homotypic cell to cell aggregation (20).

A recently discovered member of the cadherin family, vascular endothelial cadherin (VE-cadherin),<sup>1</sup> also known as cadherin-5 or 7B4 (8, 21), was found to be selectively expressed in endothelial cells of about all types of vessels (21). This molecule plays a role in the organization of lateral endothelial junctions (22) and in the control of permeability properties of vascular endothelium (21). Transfection of the VE-cadherin cDNA confers adhesive properties to the cells (23). The VE-cadherin amino acidic sequence showed considerable modifications with respect to the other members of the family (23% identity when compared with classical cloned human cadherins: E-, N-, and P-cadherin). In addition, in the extracellular amino-terminal domain, VE-cadherin lacks the HAV motif (single-letter code for amino acids) that in classical cadherins (24) seems to play a relevant role in cadherin homotypic recognition (25–27). These sequence modifications suggest that the molecule might present different functional and structural properties with respect to the other members of the family.

We have recently found that the VE-cadherin association with cytoskeleton (as measured by Triton X-100 insolubility) was not required for its localization at cell to cell contacts during early steps of endothelial cell junction organization (28). This suggests that the extracellular domain of the molecule could be enough for initial homotypic recognition.

To get further insights into this aspect, a mutated form of VE-cadherin, lacking the cytoplasmic domain responsible for its interaction with catenins, was constructed and transfected in Chinese hamster ovary (CHO) cells. Truncated VE-cadherin could localize at cell to cell contacts, promote homotypic aggregation, and partially inhibit cell detachment from a confluent monolayer. However, in contrast to the wild type form, truncated VE-cadherin was unable to reduce cell junction permeability.

These results suggest that functional differences may exist between cadherins and that cell junction might present different degrees of complexity. VE-cadherin does not require catenin and cytoskeletal binding for initial cell recognition and

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<sup>1</sup> The abbreviations used are: VE-cadherin, vascular endothelial cadherin; CHO, Chinese hamster ovary; mAb, monoclonal antibody; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; kb, kilobase pair(s).

adhesion; however, this step seems necessary for strengthening junction cohesion and limiting cell permeability.

#### MATERIALS AND METHODS

All reagents were purchased from Sigma unless indicated otherwise.

**Antibodies and Recombinant Fragments**—Mouse monoclonal antibodies (mAb) to human VE-cadherin were: clone TEA 1.31 (22), clone BV9 (Hemeris, Grenoble, France) (22, 28), and clone BV6 (Bioline Diag., Torino, Italy) (28). These antibodies were characterized by immunodepletion analysis and by positive staining of VE-cadherin transfectants. All three reacted with epitopes on the extracellular domain of the molecule as indicated by positive staining of intact living cells (fluorescence-activated cell sorting analysis) and by binding to a recombinant VE-cadherin amino-terminal fragment (Cad1, see below) in enzyme-linked immunosorbent assays (29, 30).

Rabbit pan-cadherin antiserum, against the conserved cytoplasmic sequence of all cadherins (31), was a kind gift of Dr. B. Geiger (Weizmann Institute, Rehovot, Israel). Rabbit polyclonal antibodies against  $\alpha$ - and  $\beta$ -catenins were kindly provided by Dr. D. Vestweber (Max Planck Institute for Immunobiology, Freiburg, Germany). The following peptides were used to immunize the rabbits: CKKHVNPVQALSEFK (COOH terminus of  $\alpha$ -catenin), CMATQADLMELDMAMEPDRK (NH<sub>2</sub> terminus of  $\beta$ -catenin). The anti-catenin antibodies were used and characterized in previous studies (28).

The mAb BV7 recognizes the human  $\beta$ 1 integrin chain and has been previously characterized (32).

*Escherichia coli* produced recombinant fragments spanning the extracellular domains either of VE-cadherin (Cad 1) or of the GpIIIa subunit were a kind gift of Dr. D. Gulino (CEA, INSERM U217, CEN-G, Grenoble, France). cDNA fragments containing the sequences from nucleotide 166 to 1482 for VE-cadherin (8) and from nucleotide 658 to 1200 for GpIIIa subunit (33) were generated. The fragments were then cloned into the expression vector pTG1924. The resulting pTG-derived vectors expressed VE-cadherin (Asp<sup>48</sup>-Lys<sup>486</sup>) and GpIIIa (Phe<sup>214</sup>-Leu<sup>394</sup>). Production and purification of these two recombinant proteins were made as described previously (34).

**Cells**—Human endothelial cells were isolated from umbilical cord vein and cultured as described previously (21). CHO cells were grown as previously described (23). CHO cells transfected with the full-length cDNA of human VE-cadherin, and control cells transfected with the empty pECE plasmid were obtained as described previously (23). CHO cells transfected with integrin  $\beta$ 1 subunit cDNA were a kind gift of Dr. G. Tarone (University of Torino, Torino, Italy). These cells were characterized previously in detail (35).

**Construction of VE-cadherin Cytoplasmic Deletion Mutant cDNA**—The pORFcad plasmid containing the human endothelial VE-cadherin coding sequence (23) was cut with *EcoRI* enzyme and the insert subcloned into the pBluescript vector. To generate the truncated form of VE-cadherin, the successive construct was cut with the restriction enzyme *SmaI*. Two *SmaI* sites are present in the open reading frame of the VE-cadherin cDNA sequence (positions 2130 and 2359), and a unique restriction site in the pBluescript polylinker (see Fig. 1, Panel A). This digestion eliminates the coding region for the last 82 amino acids of the cytoplasmic domain (Fig. 1, Panel B). A synthetic oligonucleotide containing stop codons sequences was then ligated to the previously *SmaI*-digested vector. The resultant plasmid was then cut with *KpnI* and *SacI* enzymes, and the insert was subcloned into the pECE eucaryotic expression vector (36) to give the pECE-truncated VE-cadherin construct. The construct was subsequently checked for correct orientation by sequence analysis using the dideoxynucleotide chain termination method (37).

**Transfection Procedure**—CHO cells were plated at 3–4 × 10<sup>6</sup> cells/100-mm Petri dish in Dulbecco's modified Eagle's medium with 10% fetal calf serum. 24 h later they were cotransfected by calcium phosphate precipitation method (38) using 20  $\mu$ g of pECE-truncated VE-cadherin and 2  $\mu$ g of pSV<sub>neo</sub> plasmids. G418 (Life Technologies, Inc.)-resistant colonies were isolated and tested for VE-cadherin expression by enzyme-linked immunosorbent assays. Positive cells were subcloned and used for further studies.

**RNA Extraction and Northern Blot Analysis**—Total RNA was isolated by the guanidium isothiocyanate method as described previously (39). Denatured RNA (10  $\mu$ g) from each sample was fractionated in a standard 1% formaldehyde/agarose gels, transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany), and fixed at 80 °C under vacuum for 2 h. Hybridization and washing conditions were as described elsewhere (38).

**Western Blot and Immunoprecipitation**—Whole cell extracts were

obtained from confluent cells as described previously (21). Detergent solubilization was carried out essentially as reported previously in detail (28) but using Nonidet P-40 (Nonidet P-40-soluble and -insoluble fractions) instead of Triton X-100. Different cell extracts were adjusted to 1 × Laemmli sample buffer and fractionated under reducing conditions on 7.5% SDS-polyacrylamide gels (40).

Western blot analyses of the various cell extracts were carried out essentially as previously described (28). After blocking with 10% nonfat milk the proteins of interest were detected by specific monoclonal or polyclonal antibodies at the optimal dilution in blocking buffer (1:2 dilution for TEA 1.31, BV9, or BV6 hybridoma culture supernatants against VE-cadherin and 10  $\mu$ g/ml for rabbit polyclonal serum against  $\alpha$ - or  $\beta$ -catenin). This was sequentially followed by incubation with goat anti-mouse IgG peroxidase conjugated (1  $\mu$ g/ml) for monoclonal antibodies or protein A peroxidase conjugated (1  $\mu$ g/ml; Pierce) for polyclonal antibodies and further development of peroxidase activity using an enhanced chemiluminescence kit (ECL; Amersham International, Buckingham, UK) and autoradiography.

Immunoprecipitation of the cadherin-catenin complex was performed as previously reported (28) with some modifications. Briefly, cell extracts were precleared by incubation with uncoupled protein G or protein A-Sepharose CL-4B (Pharmacia Biotech Inc.) for 2 h. After centrifugation, the precleared supernatants were incubated with protein G or protein A-Sepharose coupled to mAb TEA 1.31 or polyclonal antibody against  $\alpha$ -catenin, respectively, for 2 h. Immunocomplexes were collected by centrifugation, washed 5 times in a buffer containing 0.05% Nonidet P40, 0.1% bovine serum albumin, 50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, and finally resuspended in 30  $\mu$ l of 1 × Laemmli sample buffer and boiled for 5 min. Samples were analyzed by electrophoresis, transferred to nitrocellulose membranes, and immunoblotted sequentially with mAb TEA 1.31 to VE-cadherin and polyclonal antibodies to  $\alpha$ - and  $\beta$ -catenin as described above.

**Cell Surface Biotinylation**—Biotinylation of cell surface proteins was performed as described elsewhere (41) using sulfo-nitrohydroxysuccinimido-biotin (Pierce). Samples were analyzed by electrophoresis on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked with 10% low fat milk and then incubated in fresh blocking solution with horseradish peroxidase-conjugated streptavidin (Biospa Division, SPA, Milano, Italy) for 1 h at room temperature. After three washes with PBS (1 mM calcium chloride and 0.5 mM magnesium chloride) containing 0.1% Tween 20, peroxidase-conjugated streptavidin was visualized using the ECL kit as described in the previous section.

**Immunofluorescence Microscopy**—CHO cells were seeded on glass coverslips and grown to confluence before immunofluorescence staining. For endothelial cell culturing, glass coverslips were coated with human plasma fibronectin (7  $\mu$ g/ml). Cells were fixed with 3% paraformaldehyde, permeabilized with 0.5% Triton X-100 for 2 min, and processed for indirect immunofluorescence microscopy as described elsewhere (21). Briefly, incubation with the primary antibody was followed by rhodamine-conjugated secondary antibody (Dakopatts, Glostrup, Denmark) in the presence of fluorescein-labeled phalloidin (1  $\mu$ g/ml) with several washes with 0.1% bovine serum albumin in TBS between the various steps. Coverslips were then mounted in Mowiol 4–88 (Calbiochem). A Zeiss Axiophot microscope was used for observation and image recording on Kodak TMax P3200 films.

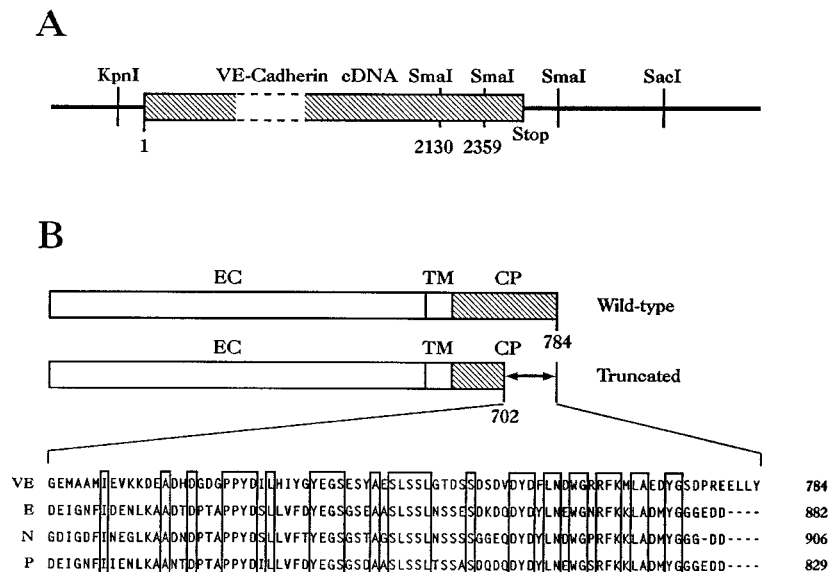
**EGTA Treatment**—EGTA was used for chelating calcium ions in the culture medium as previously described (42). A buffered stock solution of 100 mM EGTA was used to obtain a final concentration of 5 mM. Cells grown to confluence on 13-mm glass coverslips were incubated with 5 mM EGTA at 37 °C for 30 min, fixed, and processed for indirect immunofluorescence as described above.

**Cytochalasin D Treatment**—To study the effect of cytochalasin D on the localization of wild type and truncated VE-cadherin, cells were cultured to confluence on glass coverslips and treated with 1  $\mu$ g/ml cytochalasin D in culture medium. 60 min later cells were fixed with 3% paraformaldehyde, permeabilized with 0.5% Triton X-100, and processed for indirect immunofluorescence as described above.

**Cell Aggregation**—To determine the calcium-dependent cell-aggregating activity, the cell monolayer of control and transfected with wild type or truncated VE-cadherin CHO cells were washed several times with PBS and dissociated by treatment with 0.01% trypsin (from bovine pancreas, type III) in Hank's balanced salt solution containing 25 mM Hepes, 10 mM calcium chloride, and 5 mM magnesium chloride. This solution was maintained on the cells for the shortest interval after which initial intercellular retraction appeared (usually 5–7 min). Trypsin action was stopped by addition of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 0.1% soybean trypsin

FIG. 1. Construction of VE-cadherin cytoplasmic domain deletion mutant.

**A**, schematic representation of the cDNA sequence of VE-cadherin in the pBlue-script vector. The truncated form of VE-cadherin was constructed by digesting the wild type VE-cadherin cDNA with *SmaI* restriction enzyme, followed by the ligation of a synthetic oligonucleotide to insert the stop codon in frame (see "Materials and Methods"). Truncated VE-cadherin was further subcloned into pECE expression vector after *SacI* and *KpnI* restriction enzyme digestion. **B**, schematic representation of wild type and cytoplasmic deletion mutant VE-cadherin. The deleted cytoplasmic fragment corresponds to the most conserved amino acid sequence among the four cloned human cadherins. EC, extracellular domain; TM, transmembrane domain; CP, cytoplasmic domain; VE, VE-cadherin; E, E-cadherin; N, N-cadherin; P, P-cadherin.



inhibitor (Sigma). VE-cadherin is particularly sensitive to trypsin even in the presence of calcium (21), but the treatment here described preserves VE-cadherin expression on the cell surface at least by 80% as measured by flow cytometry. After trypsin neutralization, cells were sedimented, washed twice with 1% bovine serum albumin in Hanks' balanced salt solution and resuspended at a density of  $4 \times 10^5$  cells/ml in washing solution. The cell suspension (0.5 ml/well) was seeded in a 24-well plate previously coated with 1% bovine serum albumin and allowed to aggregate in the presence of 5 mM calcium chloride or 5 mM EGTA for 90 min at 37 °C on a rotating platform (80 rpm).

For those experiments where actin cytoskeleton disruption agent was used, cytochalasin D was added at 1  $\mu$ g/ml after the first centrifugation, and the cells were incubated at 37 °C for 30 min. In some experiments, recombinant fragments of VE-cadherin (Cad1) or of the GpIIIa subunit (see above) were added (100  $\mu$ g/ml) to the cell suspension. The aggregation assay was then performed as described before.

Cell aggregates were observed by phase contrast microscopy or fluorescence microscopy as described below. The reaction was stopped by addition of 0.5 ml/well 5% glutaraldehyde (electron microscopy grade) in PBS. The initial number of particles ( $N_{t_0}$ ) and the number of particles at 90 min ( $N_{t_{90}}$ ) were counted using a ZM Coulter Counter (window set 6–100  $\mu$ m). Quantification of aggregation was estimated by the index  $(N_{t_0} - N_{t_{90}})/N_{t_0} \times 100$  as described elsewhere (43).

For photography, in order to better visualize the aggregates, cells were labeled with 2  $\mu$ M 2',5'-bis [2-carboxyethyl]-5[and-6] carboxyfluorescein acetoxyethyl ester (Molecular Probes, Eugene, OR) in Hanks' balanced salt solution for 10 min at 37 °C, immediately after the first centrifugation and processed as described above.

**Cell Migration**—The migration assays were performed as described (23). The distance migrated by the cells was measured using a micrograduated scale (Nikon) adapted in the ocular of a Nikon inverted microscope under phase contrast (100 $\times$  magnification). Twelve values, obtained in three different wells, were recorded for each cell type at each time interval.

**Permeability Assay**—Cells ( $1.5 \times 10^4$  at seeding) were cultured for 5 days in Transwell units (with polycarbonate filter, 0.4- $\mu$ m pore, Costar, Cambridge, MA). For endothelial cells the Transwell units were previously coated with fibronectin (2  $\mu$ g/well) before seeding. Horseradish peroxidase conjugated to goat immunoglobulins (8  $\mu$ g/ml initial concentration in the upper chamber, minimal calculated molecular mass 200 kDa, specific activity 28 units/mg) were added to the upper compartment. At different time points of incubation, 100- $\mu$ l samples were withdrawn from the lower compartment and assayed photometrically for the presence of enzymatic activity (23). In some experiments, 5 mM EGTA or 1  $\mu$ g/ml cytochalasin D were added both in lower and upper compartments during incubation with immunoglobulins (2 h for EGTA and 1 h for cytochalasin D). Samples of three different wells from at least three independent experiments were grouped for statistical analysis.

## RESULTS

### Construction and Characterization of a Mutated Form of VE-cadherin Lacking the Region of the Cytoplasmic Domain

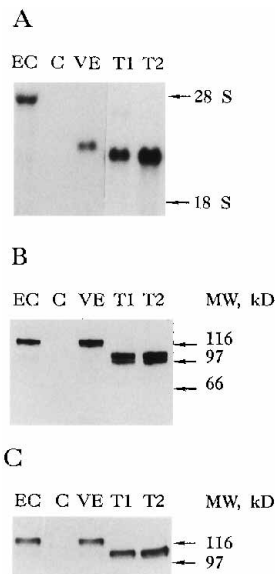
**Involved in Catenin Association**—The coding VE-cadherin cDNA sequence in the pBluescript vector was digested with *SmaI* restriction enzyme, ligated to a synthetic oligonucleotide containing stop codon sequences, and further subcloned into the expression vector pECE, as described under "Materials and Methods" (Fig. 1, Panel A). The constructed cDNA encodes a deletion mutant of VE-cadherin lacking the last 82 amino acids of the cytoplasmic domain. This deleted fragment corresponds to the most conserved region of the four cloned human cadherins (Fig. 1, Panel B) which was found to be necessary for the anchorage of these molecules to catenins (11–16, 44).

This mutant cDNA was stably transfected into CHO cells, which have no endogenous cadherin activity (23). G418-resistant clones were selected by enzyme-linked immunosorbent assay method, and two clones (clones T1 and T2) were chosen for further analysis.

As shown in Fig. 2, Panel A, in a Northern blot analysis, a band of approximately 2.2-kb size was detected in the truncated VE-cadherin clones (*lanes T1* and *T2*) as expected from deletion. Wild type VE-cadherin transfectants (*lane VE*) showed a band of 2.5 kb corresponding to the coding cDNA sequence, while endothelial cell (*lane EC*) endogenous VE-cadherin mRNA was 4.1 kb as described previously (28). No VE-cadherin mRNA was detected in empty vector transfected cells (*lane C*).

Expression of truncated VE-cadherin protein was analyzed by Western blot using TEA 1.31 mAb. As reported in Fig. 2, Panel B, two intense bands were detected in the mutant VE-cadherin transfectants: one of 110-kDa apparent molecular mass corresponding to the expected size, and a faster migrating one of 98 kDa (*lanes T1* and *T2*). No detectable VE-cadherin expression was found in control transfectants, while wild type VE-cadherin transfectants and endothelial cells expressed comparable amounts of the molecule at the 135-kDa apparent molecular mass (*lanes C*, *VE*, and *EC*, respectively). Identical results were obtained using two different VE-cadherin mAbs (BV9 and BV6) (data not shown).

To evaluate the amount of the truncated molecule exposed on the cell surface, confluent cell monolayers were incubated with sulfo-nitrohydroxysuccinimido-biotin and immunoprecipitated with TEA 1.31 mAb. By avidin-horseradish peroxidase blotting we detected only one band in clones T1 and T2 with the expected apparent molecular mass of 110 kDa (Fig. 2, Panel C, *lanes T1* and *T2*). This strongly suggests that the smaller band



**FIG. 2. mRNA and protein expression of wild type and cytoplasmic deletion mutant of VE-cadherin.** *A*, Northern blot analysis of VE-cadherin mRNA expression. mRNA of endothelial cells (*EC*), wild type (*VE*) and truncated (*T1* and *T2*) VE-cadherin transfectant cells run to positions 4.1, 2.5, and 2.2 kb, respectively. 10  $\mu$ g of total RNA from the different cell lines were loaded in each lane. Control transfectants (*C*) did not express detectable amounts of VE-cadherin mRNA. *B*, expression of VE-cadherin protein by Western immunoblot. The estimated molecular mass of the antigens detected was 135 kDa for endothelial cells (*EC*) and wild type (*VE*) VE-cadherin transfectants, and 110 and 98 kDa for the truncated VE-cadherin transfectants (*T1* and *T2*). Control transfectants (*C*) did not show detectable amounts of VE-cadherin. Whole cell extracts of the different cell lines were obtained in the presence of 2 mM calcium chloride and protease inhibitors. Cell lysates from the same number of cells ( $5 \times 10^4$ ) were loaded on 7.5% SDS-polyacrylamide gels, transferred to nitrocellulose membranes and detected with mAb TEA 1.31. Migration of the molecular mass markers is indicated on the right. *C*, expression of surface associated VE-cadherin. Biotin-labeled whole cell membranes were immunoprecipitated with mAb TEA 1.31, subjected to gel electrophoresis and blotted with avidin-horseradish peroxidase. One band of an estimated molecular mass of 110 kDa was detected in truncated VE-cadherin transfectants (*T1* and *T2*). *C*, control transfectants; *VE*, wild type transfectants; *EC*, endothelial cells. Migration of the molecular mass markers is indicated on the right.

detected by Western blot of the whole cell extracts (Fig. 2, *Panel B*, lanes *T1* and *T2*) corresponds to an immature, intracellular form of truncated VE-cadherin. Endothelial cells and wild type VE-cadherin transfectants expressed comparable amounts of VE-cadherin on the cell surface (Fig. 2, *Panel C*, lanes *EC* and *VE*, respectively).

**Truncated VE-cadherin Association to Catenins and Actin Cytoskeleton**—To verify whether cytoplasmic truncation disrupts VE-cadherin linkage to catenins, cell extracts of VE-cadherin transfectants were immunoprecipitated with mAb TEA 1.31 against VE-cadherin (Fig. 3, *Panel A*) or polyclonal antibody against  $\alpha$ -catenin (Fig. 3, *Panel B*), and sequentially blotted with mAb TEA 1.31 and with antibodies directed to  $\alpha$  and  $\beta$ -catenins. As observed in Fig. 3, *Panel A*, endogenous and wild type transfected VE-cadherin was associated with two major polypeptides with apparent molecular masses of 105 and 95 kDa recognized by  $\alpha$ - and  $\beta$ -catenin antibodies, respectively, (lanes *EC* and *VE*, respectively). In contrast, these polypeptides were absent in immunoprecipitates from truncated VE-cadherin transfectants (lanes *T1* and *T2*). As expected, no specific bands could be detected in control transfectants (lane *C*). Fig. 3, *Panel B*, shows that after  $\alpha$ -catenin immunoprecipitation association with VE-cadherin and  $\beta$ -catenin can be only detected in endothelial cells and wild type VE-cadherin transfectants (Fig.

3, *Panel B*, lanes *EC* and *VE*, respectively) but not in the truncated mutants and control cells, despite the presence of  $\alpha$ -catenin (Fig. 3, *Panel B*, lanes *T* and *C*, respectively). The higher expression of  $\alpha$ -catenin in endothelial cell, and wild type VE-cadherin transfectants (shown in Fig. 3, *Panel B*) can be explained by a stabilization of the whole cadherin-catenin complex as described previously (15).

Analysis of the solubilization of cadherins in Nonidet P40 detergent has been described as an indirect indication of their association with the cytoskeleton (11, 16, 45). Western immunoblotting of the Nonidet P40-soluble and -insoluble fractions was carried out with the mAb TEA 1.31. Whereas a significant amount of VE-cadherin was found in the Nonidet P40-insoluble and -soluble fractions of endothelial cell, and wild type VE-cadherin transfectants (Fig. 3, *Panel C*, lanes *EC* and *VE*, respectively), the truncated mutant was found only in the Nonidet P40-soluble fraction (Fig. 3, *Panel C*, lanes *T1* and *T2*).

**Immunofluorescence Analysis of VE-cadherin Distribution**—By immunofluorescence staining, mAbs directed to VE-cadherin (TEA 1.31, BV6, and BV9) decorated cell to cell contacts of wild type and truncated VE-cadherin transfectants (Fig. 4, *b* and *c*). In contrast,  $\alpha$ -catenin localized at cell junctions only in wild type (Fig. 4*f*) but not in truncated VE-cadherin transfectants (Fig. 4*g*). The same pattern was observed using an anti- $\beta$ -catenin antibody (data not shown). None of these antibodies stained cell contacts of control cells (Fig. 4, *a* and *e*).

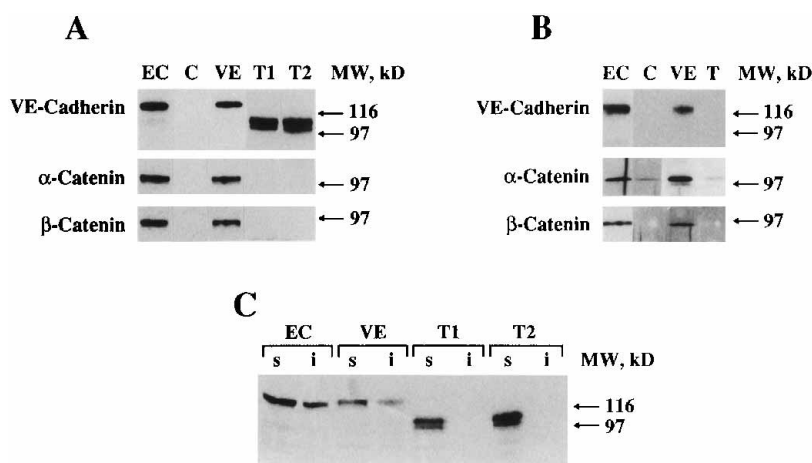
To exclude that the increase in staining of truncated VE-cadherin at junctions was simply due to membrane overlapping, we analyzed CHO cells transfected with another membrane protein ( $\beta$ 1 integrin) (35). As reported in Fig. 4*d*, no apparent enrichment of this molecule at cell junctions was observed by staining with an anti- $\beta$ 1 mAb. Similarly,  $\alpha$ -catenin antibodies did not stain cell to cell contacts in these cells.

To further confirm the specificity of the truncated VE-cadherin distribution at intercellular contacts (46) we treated the cells for 30 min with 5 mM EGTA (Fig. 5). In these conditions even if no substantial cell retraction was observed by actin staining (Fig. 5, *e* and *f*), both wild type and truncated VE-cadherin lost intercellular contact localization and showed a wide punctate pattern over all of the cell surface (Fig. 5, *a* and *b*).

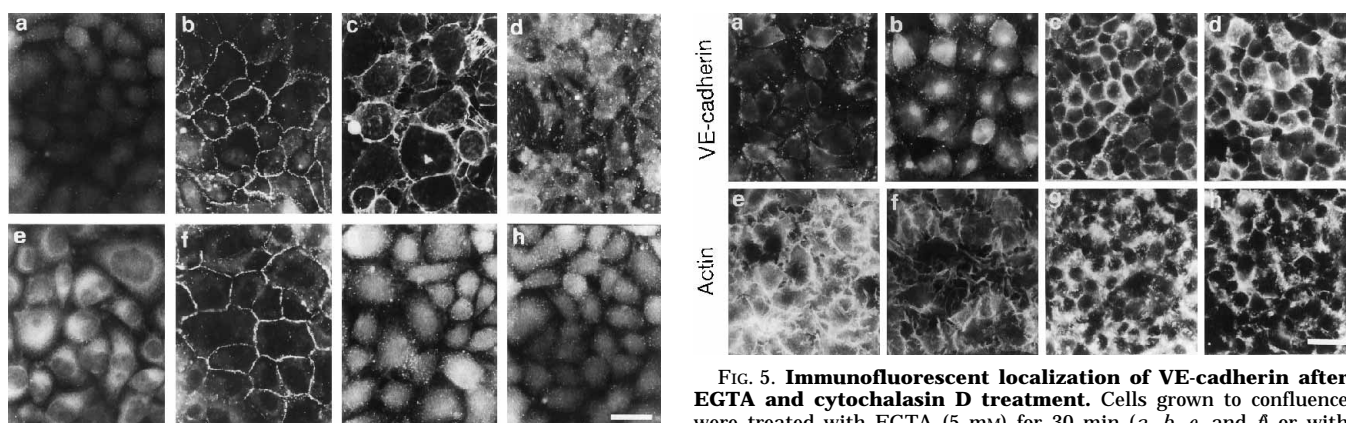
Furthermore, when confluent cells were treated with cytochalasin D in order to disrupt the actin microfilament network (Fig. 5, *g* and *h*) both wild type and truncated VE-cadherin were still localized at cell-cell contacts (Fig. 5, *c* and *d*, respectively) indicating that an intact actin cytoskeleton is not required for maintaining VE-cadherin at cell junctions.

Finally, as displayed in Fig. 6, truncated VE-cadherin can be driven at junctions through the interaction with wild type transfectants, showing that it retains the capacity to interact with the native molecule (Fig. 6, *a* and *b*). Additionally, truncated VE-cadherin, similar to the wild type molecule, was not significantly enriched at heterogeneous cell junctions (*i.e.* when cells were adhering to control transfectants) (Fig. 6*c*), indicating that homotypic binding is required for junction localization.

**Aggregating Properties of Truncated VE-cadherin**—We studied whether the lack of association to catenins could modify VE-cadherin adhesive properties. CHO parental cells show little endogenous calcium-dependent aggregation. Transfection of these cells with wild type VE-cadherin confers calcium-dependent aggregating activity (Fig. 7). Surprisingly, truncated VE-cadherin was even more efficient in promoting calcium-dependent aggregation of transfectants than the wild type molecule. Since none of the three available VE-cadherin mAbs presented a significant blocking activity, the specificity of cell aggregation was analyzed by adding a soluble recombinant fragment of



**FIG. 3. VE-cadherin association to catenins and to detergent soluble/insoluble fractions.** *A*, detection of VE-cadherin associated proteins in endothelial cells (EC), wild type (VE), and truncated (T1 and T2) VE-cadherin transfectants. Equivalent samples of VE-cadherin immunocomplexes from the different cells were sequentially immunoblotted with antibodies to VE-cadherin (TEA 1.31),  $\alpha$ - and  $\beta$ -catenin. VE-cadherin expressed by endothelial cells (EC) and wild type VE-cadherin transfectants (VE) was associated with  $\alpha$ - and  $\beta$ -catenins. No signal for catenins was detected in truncated VE-cadherin transfectants (T1 and T2) and control cells (C). Migration of the molecular mass markers is indicated on the right. *B*, detection of  $\alpha$ -catenin-associated proteins of endothelial cells (EC), wild type (VE), and truncated (T) VE-cadherin transfectants. Samples containing an identical amount of  $\alpha$ -catenin immunocomplexes were sequentially immunoblotted with antibodies to VE-cadherin (TEA 1.31) and  $\alpha$ - and  $\beta$ -catenin. VE-cadherin and  $\beta$ -catenin were associated with  $\alpha$ -catenin expressed by endothelial cells (EC) and wild type VE-cadherin transfectants (VE) but no signal for these proteins was detected in truncated VE-cadherin transfectants (T) and control cells (C). Migration of the molecular mass markers is indicated on the right. *C*, detergent extraction of VE-cadherin polypeptides from wild type transfectants (VE) truncated VE-cadherin transfectants (T1 and T2), and endothelial cells (EC). Cells growing at high density were treated with 1% Nonidet P40, and the soluble (s) and insoluble (i) fractions were resolved by gel electrophoresis, transferred to nitrocellulose membranes, and blotted with mAb TEA 1.31. VE-cadherin was detected in the insoluble fraction only in endothelial cells and wild type VE-cadherin transfectants. Migration of the molecular mass markers is indicated on the right.



**FIG. 4. Immunofluorescent staining of VE-cadherin and  $\alpha$ -catenin in CHO-transfected cells.** The different cell lines were grown to confluence on glass coverslips, fixed, and immunostained with mAb TEA 1.31 (*a*, *b*, and *c*), mAb against integrin  $\beta$ 1 chain (*d*), or polyclonal antibody to  $\alpha$ -catenin (*e*–*h*). VE-cadherin was localized at intercellular contacts in wild type (*b*) and truncated (*c*) VE-cadherin transfectants, whereas no specific staining was observed in control cells (*a*).  $\beta$ 1 was not enriched at cell to cell contacts in  $\beta$ 1-transfected cells (*d*).  $\alpha$ -catenin codistributes at cell-cell junctions only in wild type VE-cadherin transfectants (*f*), whereas there was a diffuse distribution in the truncated VE-cadherin (*g*), integrin  $\beta$ 1 (*h*), and control transfectants (*e*). Bar, 30  $\mu$ m.

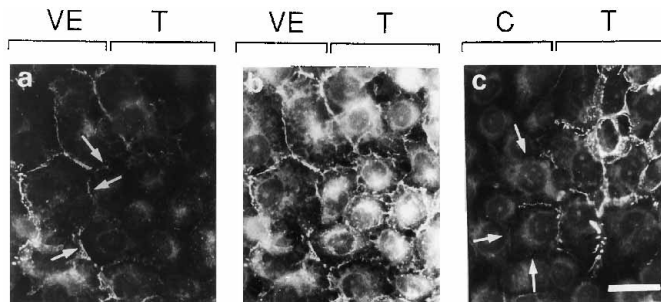
**FIG. 5. Immunofluorescent localization of VE-cadherin after EGTA and cytochalasin D treatment.** Cells grown to confluence were treated with EGTA (5 mM) for 30 min (*a*, *b*, *e*, and *f*) or with cytochalasin D, 1  $\mu$ g/ml for 1 h (*c*, *d*, *g*, and *h*), before fixation and immunofluorescent staining. Wild type (*a*, *c*, *e*, and *g*) and truncated (*b*, *d*, *f*, and *h*) VE-cadherin transfectants were double-labeled with VE-cadherin mAb (TEA 1.31) followed by a secondary rhodamine-labeled anti-mouse antibody (*a*–*d*) and fluorescein-phalloidin for actin staining (*e*–*h*). After EGTA treatment both wild type and truncated VE-cadherin showed a disperse localization on the cell surface (*a* and *b*) without a significant cell retraction as seen by actin staining (*e* and *f*). After 1 h of cytochalasin D treatment, both wild type and truncated VE-cadherin maintained cell-cell contact localization (*c* and *d*, *g* and *h*). Bar, 30  $\mu$ m.

VE-cadherin corresponding to the amino-terminal domain of the molecule (Cad 1, see “Materials and Methods”). Aggregation of both wild type and truncated VE-cadherin transfectants was inhibited by 100  $\mu$ g/ml Cad 1 ( $87.3 \pm 2\%$  for wild type and  $98.5 \pm 1\%$  for truncated, mean values  $\pm$  S.D. from triplicates;  $p < 0.01$  versus control by analysis of variance and Duncan test). In contrast, a GpIIIa subunit fragment was ineffective at the same concentration.

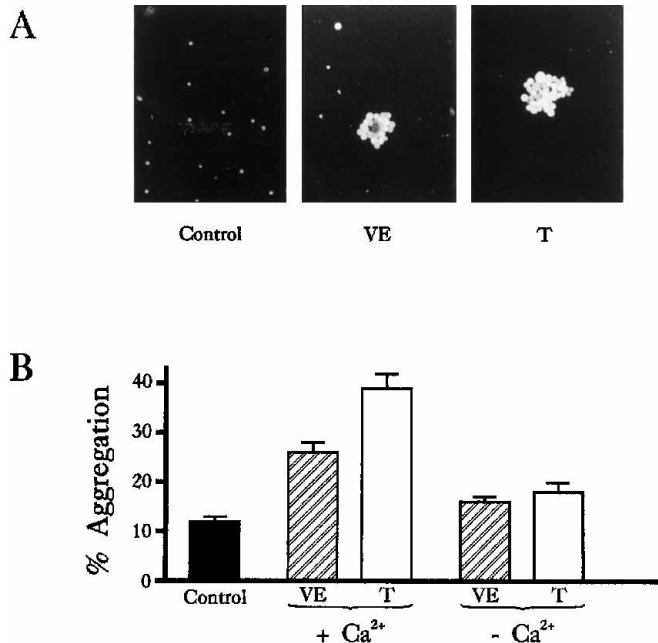
Treatment of the cells with cytochalasin D in order to disrupt actin cytoskeleton did not affect both wild type and truncated VE-cadherin transfectant aggregation (not shown)

**Effect of Truncated VE-cadherin Transfection on Cell Migration and Monolayer Permeability**—As a further indication of the cohesion of intercellular contacts we tested the capacity of transfectants to detach from the neighboring cells and migrate into a “wounded” area produced in the cultured monolayer. The distance covered by the migration front was measured at different times after the lesion. As reported in Fig. 8, transfection of both wild type (VE) and truncated (T) VE-cadherin reduced cell migration in the wounded area. Wild type VE-cadherin was slightly but significantly more effective in inhibiting cell detachment and migration from the monolayer.

To further analyze whether wild type and truncated VE-cadherin could modify the strength of cell to cell contacts, we

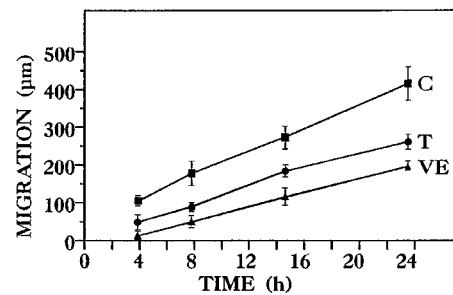


**FIG. 6. Truncated and wild type VE-cadherin distribution in mixed cultures.** Truncated VE-cadherin transfectants (*T*) were cocultured with wild type VE-cadherin (*VE*) (*a* and *b*) or control transfectants (*C*) (*c*). Cells were grown to confluence on glass coverslips and fixed. The following antibodies were used for immunofluorescence: a rabbit anti-serum to a conserved region of cytoplasmic domain of cadherins (pan-cadherin) which recognizes only wild type VE-cadherin but not the truncated molecule (*a*) and mAb TEA 1.31 which recognizes both wild type and truncated VE-cadherin (*b* and *c*). Arrows in *a* show heterotypic cell-cell contact between wild type and truncated VE-cadherin transfectants (*a* and *b*). No VE-cadherin staining was detected at intercellular contacts between truncated VE-cadherin and control transfectants (*c*, arrows). Bar, 30  $\mu$ m.



**FIG. 7. Effect of truncated VE-cadherin transfection on cell aggregation.** Control transfectants (*Control*), wild type (*VE*), or truncated (*T*) VE-cadherin transfectants were dissociated into single cells by treatment with trypsin in the presence of 1 mM calcium chloride, labeled with the fluorescent probe 2',5'-bis[2-carboxyethyl]-5[ and -6]carboxyfluorescein acetoxymethylester and allowed to aggregate for 90 min in the presence of 5 mM calcium chloride or 5 mM EGTA. *A*, wild type and truncated VE-cadherin transfectants form cell aggregates in the presence of calcium, while control cells remain mostly single. *B*, quantification of aggregation in the presence of 5 mM calcium chloride (+Ca<sup>2+</sup>) or 5 mM EGTA (-Ca<sup>2+</sup>) was done as described under "Materials and Methods." The data showed are from a representative experiment out of four performed. Values are the mean  $\pm$  S.D. from triplicates. The differences in calcium-dependent cell aggregation of wild type or truncated VE-cadherin versus control cells were statistically significant ( $p < 0.01$ , Duncan test).

measured the permeability of the cell monolayers to a high molecular weight soluble marker. Cells were grown on Transwell filters, and the passage of horseradish peroxidase conjugated to goat immunoglobulin was quantified (see "Materials and Methods"). In preliminary experiments we established the conditions to ensure a comparable number of cells on filters and a similar degree of confluency. Cells on the filters essentially



**FIG. 8. Effect of wild type and truncated VE-cadherin transfection on wound repair.** Time course of the migration distance covered by control (*C*) wild type (*VE*), and truncated (*T*) VE-cadherin transfectants. Cells layers were wounded and migration of the cells out of the wounded edge was recorded at different times. Truncated VE-cadherin transfectants migrated slower than control but slightly faster than wild type transfectants. The results showed are the mean of three different experiments  $\pm$  S.D. performed in triplicate. At all times considered, migration values of *VE* and *T* were statistically different ( $p < 0.01$ ) from the corresponding values of *C*; in addition, at all times, migration values of *VE* were statistically significant from the corresponding values of *T* by analysis of variance and Duncan test ( $p < 0.01$ ).

remained in monolayers when fixed and stained with crystal violet and examined by phase contrast microscopy. As reported in Fig. 9, Panel A, transfection of wild type VE-cadherin (*VE*) reduced monolayer permeability to values comparable to those obtained with human endothelial cells (*EC*). In contrast, transfection of truncated VE-cadherin (*T*) did not significantly change monolayer permeability in comparison to control transfectants (*C*). The effect of wild type VE-cadherin transfection was lost after treatment of the cells with EGTA (Fig. 9, Panel B) or disruption of actin microfilaments by cytochalasin D (Fig. 9, Panel C). It is important to emphasize that in our experimental conditions the increase in monolayer permeability was not due to changes either in the cell number or significant cell retraction (see also Fig. 5).

#### DISCUSSION

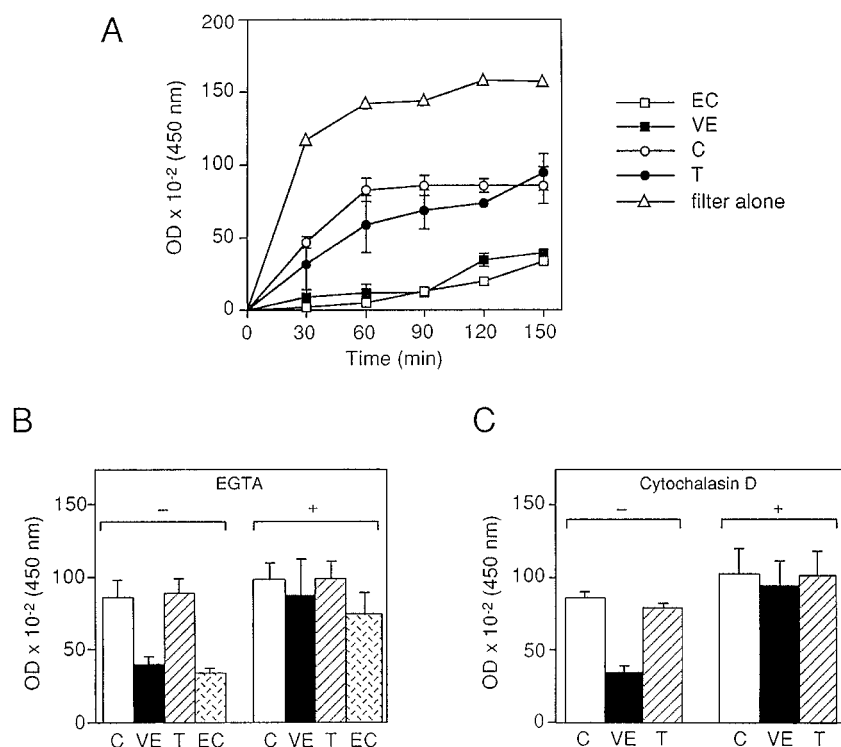
VE-cadherin is an endothelial specific cadherin which seems to play a role in junction organization and permeability properties of these cells (21, 22, 28, 47). We explored the effects of blocking VE-cadherin binding to cytoskeletal proteins. To this end, a deletion of 82 amino acids of the cytoplasmic tail was performed as this region has been previously shown to be responsible for cadherin interaction with catenins (12, 16). Indeed, the truncated VE-cadherin mutant was unable to bind to catenins and to associate with the detergent-insoluble fraction of cell extracts.

As for other cadherins (11), the lack of interaction with catenins did not prevent the processing and exposure of the molecule on the cell surface in amounts comparable to the wild type and native form. Recognition by three separate mAbs strongly suggests that mutant VE-cadherin also retained a correct conformation.

Truncated VE-cadherin localized at intercellular junctions. The specificity of junction accumulation of truncated VE-cadherin was controlled in different ways.

First, it did not seem to be related to unspecific increase in fluorescence intensity by membrane overlapping, since another transfected membrane protein in the same cells (integrin  $\beta$ 1) (35) did not present a similar pattern of distribution.

Truncated VE-cadherin localization at junctions was calcium-dependent. After treatment of the cells with EGTA, truncated and wild type VE-cadherin disappeared from cell to cell contacts. Previous work has shown that EGTA did not cause a significant internalization of VE-cadherin (21, 28), indicating that its disappearance from cell junctions is due to a diffuse



**FIG. 9. Effect of wild type and truncated VE-cadherin transfection on cell monolayer permeability.** *A*, time course of permeability values of endothelial cells (*EC*), control (*C*), wild type (*VE*), and truncated (*T*) VE-cadherin transfectants. Confluent cell monolayers grown on Transwell filters were tested for permeability to horseradish peroxidase conjugated to goat immunoglobulin at different time points. Monolayers of wild type VE-cadherin transfectants significantly reduces permeation of peroxidase in comparison to control transfectants ( $p < 0.01$  by analysis of variance and Duncan test). In contrast, transfection of truncated VE-cadherin was without a significant effect in comparison to control cells. Filters alone showed permeability values always much higher than in the presence of any type of cell. *B*, effect of EGTA treatment on cell permeability. As reported in *Panel A*, transfection of wild type VE-cadherin (*VE*) but not of the truncated mutant (*T*) reduced CHO permeability to values comparable to those obtained using endothelial cells (*EC*). Treatment of cells with EGTA (5 mM) abolished this difference. *C*, effect of cytochalasin D on cell permeability. Confluent cells grown on Transwell filters were treated with 1  $\mu\text{g/ml}$  cytochalasin D. The reduction of permeability induced by transfection with wild type VE-cadherin was lost after disruption of actin network by cytochalasin D treatment. A cell count performed by detaching the cells from the filter at the end of the permeability assay did not show any significant change after any of the treatments described in the figure. Data showed in *Panels A, B, and C* are a representative experiment out of four. The values are means  $\pm$  S.D. from triplicates. OD, optical density at 450 nm (see "Materials and Methods").

redistribution on the cell surface.

Furthermore, truncated VE-cadherin clustering does not occur in response to adhesion to cells that do not bear VE-cadherin. Therefore truncated VE-cadherin clustering is not simply a response to cell contact formation but must involve the specific recruitment of the molecule in response to the proximity of another intact or truncated VE-cadherin bearing cell.

Finally, electron microscopy analysis showed that truncated VE-cadherin was concentrated at cell to cell contacts as the wild type and the endothelial native form (not shown).

An important question is how VE-cadherin is brought to the junctions, if its interaction with catenins and actin cytoskeleton is blocked. One possible explanation is that it can still bind other cytoskeletal molecules able to drive it at cell to cell contacts. Cadherins have been shown to interact with members of the membrane cytoskeleton such as fodrin, ankyrin, radixin, and zyxin (45, 48). However, truncated VE-cadherin is absent in the detergent-insoluble fraction of cell extracts where these proteins are concentrated (45).

Another possibility is that, if VE-cadherin is mobile on the cell membrane, homotypic interactions should act as "diffusion traps" for the molecule with a consequent increase in its concentration in areas of cell to cell contact. This model has been previously proposed for NCAM clustering at cell to cell junctions (49) and for other adhesive molecules (50, 51). The possibility of a diffusion trap of VE-cadherin at cell junctions is supported by the observation that the loss of interaction with catenins does not inhibit some VE-cadherin adhesive proper-

ties (see below) and homotypic recognition.

Indeed, a surprising observation of this work is that truncated VE-cadherin is able to promote calcium-dependent cell aggregation even more efficiently than the wild type form. Cytochalasin D treatment of wild type VE-cadherin transfectants did not alter their aggregating properties, supporting the concept that interaction with an intact actin cytoskeleton is not required for VE-cadherin aggregating activity.

In support of this, a recombinant soluble fragment of VE-cadherin, corresponding to the extracellular domain of the molecule, was able to counteract VE-cadherin-mediated cell aggregation, suggesting that cadherin-cadherin binding can occur independent of the cytoplasmic and membrane domains. Other authors have shown that soluble extracellular domains of other cadherins (52, 53) were able to bind to the corresponding native cadherins on cells.

Tanihara *et al.* (54) reported that VE-cadherin is unable to promote homotypic cell aggregation. However, in this work the transfectant cells were detached with trypsin and EDTA, a procedure known to digest VE-cadherin from the cell surface (21). Since no analysis of the residual or *de novo* synthesized molecule was reported, a possible explanation for this discrepancy is that the amount of VE-cadherin on the cell surface was not enough to sustain cell aggregation. In this study the cell detachment procedure was such to preserve at least 80% (see "Materials and Methods") of the total amount of the protein on the cell membrane.

In contrast to cell aggregation, association of VE-cadherin

with catenins and the cytoskeleton seems to be relevant in other assays of cell junction cohesion.

We studied the effect of wild type and truncated VE-cadherin transfection on the capacity of the cells to detach from a confluent monolayer and to migrate into a wounded area. Wild type VE-cadherin transfection significantly reduces cell migration. Truncated VE-cadherin was slightly but significantly less effective than the native molecule. Furthermore, permeation of cell monolayers to high molecular weight molecules was reduced by VE-cadherin but not by its truncated mutant. Interestingly, in this case, actin microfilament disruption by cytochalasin D or calcium ion chelation by EGTA treatment blocked the effect of wild type VE-cadherin.

We do not know the exact mechanism of regulation of junction permeability through cadherins. Cadherins have been found to act as architects of epithelial cell junctions. Their engagement is necessary for gap junction and tight junction organization (48, 55, 56). Since these activities require cadherin cytoskeletal interaction, a possibility is that permeability is controlled through the organization of more complex cell to cell contacts. However, in VE-cadherin transfectants we could find evidence for the presence of neither tight nor gap junctions by morphological and functional studies.<sup>2</sup>

Alternatively, the binding of the cytoplasmic domain of VE-cadherin to cytoskeletal proteins might change the conformation of the extracellular domain and of cadherin-cadherin complexes making them tighter and more stable.

Taken together the data reported in this study suggest that there might be different degrees of complexity and strength of cell junction. The VE-cadherin extracellular domain might be sufficient to establish the first contact and clustering of the molecule. Full junction organization and strength might be then regulated by the interaction of the cytoplasmic domain with catenins and the actin cytoskeleton.

This possibility is supported by previous data (28) showing that, when endothelial cell reach confluency, VE-cadherin becomes concentrated in areas of cell to cell contacts without apparent association with detergent-insoluble cytoskeletal molecules. Evolution of junction organization leads to an increase in plakoglobin synthesis and localization at cell contacts. This is accompanied by redistribution of VE-cadherin in the detergent-insoluble fraction. These last observations are in agreement with previous work by McNeill *et al.* (57) in Madin-Darby canine kidney cells following E-cadherin localization. The capacity of VE-cadherin to promote cell adhesion and recognition in the absence of catenin interaction partially distinguishes this molecule from other cadherins (58–62). However, T-cadherin, a cadherin naturally lacking the cytoplasmic domain, is able to promote cell aggregation and to partially localize at cell to cell junctions (20). In addition, a deletion mutant of N-cadherin, in which all but three amino acids of the cytoplasmic domain are eliminated, was still able to localize at cell to cell junctions (63). Overall, these data suggest that the functional pattern of cadherins might not be identical for all of the members of the family. Different cadherins might present a distinct behavior possibly related to their sequence and conformational properties.

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<sup>2</sup> P. Navarro, L. Caveda, F. Breviario, I. Mândoteanu, M.-G. Lampugnani, and E. Dejana, unpublished results.



## **Catenin-dependent and -independent Functions of Vascular Endothelial Cadherin**

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