Self-assembly of the Vascular Endothelial Cadherin Ectodomain in a Ca\(^{2+}\)-dependent Hexameric Structure*§

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Vascular endothelial cadherin (VE-cadherin) is a transmembrane protein essential for endothelial cell monolayer integrity (Gulino, D., Delachanal, E., Concord, E., Genoux, Y., Morand, B., Valiron, M. O., Sulpice, E., Scaife, R., Alemany, M., and Vernet, T. (1998) J. Biol. Chem. 273, 29786–29793). This molecule belongs to the cadherin family of cell-cell adhesion receptors, for which molecular details of homotypic interactions are still lacking. In this study, a recombinant fragment encompassing the four N-terminal modules of VE-cadherin (VE-EC1–4) was shown to stabilize, in solution, as a stable Ca\(^{2+}\)-dependent oligomeric structure. Cross-linking experiments combined with mass spectrometry demonstrated that this oligomer is a hexamer. Gel filtration chromatography experiments and analytical ultracentrifugation analyses revealed the existence of an equilibrium between the hexameric and monomeric species of VE-EC1–4. The concentration at which 50% of VE-EC1–4 is in its hexameric form was estimated as 1 \(\mu\)M. The dimensions of the hexamer, measured by cryoelectron microscopy to be 233 ± 10 \(\times\) 77 ± 7 Å, are comparable to the thickness of adherens endothelial cell-cell junctions. Altogether, the results allow us to propose a novel homotypic interaction model for the class II VE-cadherin, in which six molecules of cadherin form a hexamer.

The endothelium is a semipermeable barrier that controls the passage of solutes and circulating cells between the bloodstream and the neighboring tissues. The junctions between endothelial cells play a crucial role in controlling this traffic. An adhesion receptor belonging to the cadherin superfamily, vascular endothelial cadherin (VE-cadherin)† (1) is specifically localized at these cell-cell junctions.

VE-cadherin is involved in the maintenance and restoration of endothelial integrity (1–4). In fact, opening of VE-cadherin-mediated endothelial cell contacts was suggested to be a crucial step in neutrophil extravasation (5–7). VE-cadherin is also involved in determining the vascular architecture since recent studies demonstrated that targeted inactivation of VE-cadherin in transgenic mice leads to embryonic mortality due to severe vasculogenic defects (8, 9).

Cadherins are Ca\(^{2+}\)-dependent cell-cell adhesion receptors that are able to bind cells together by means of homotypic interactions. In these interactions, only cadherin types interact. Consequently, cells expressing various cadherins segregate into like groups when mixed. Due to this selective cell-cell recognition property, cadherins are important regulators of morphogenesis (10) by promoting clustering of cells with identical phenotypes.

The extracellular part of cadherins consists of five homologous protein modules designated EC1–EC5, numbered from the N terminus to the C terminus. Various lines of evidence have implicated the N-terminal module EC1 in determining homotypic binding specificity (11, 12). Fixation of Ca\(^{2+}\) on sites generally located between two consecutive extracellular modules (13, 14) reduces cadherin sensibility to proteolytic degradation, rigidifies the elongated structure of cadherins (15, 16), and is required for homotypic interactions (17). The cytoplasmic domains of cadherins are homologous and interact either with \(\beta\)- or \(\gamma\)-catenins in a mutually exclusive fashion. By connecting cadherins to the actin-based cytoskeleton, binding of \(\alpha\)-catenin to \(\beta\)- or \(\gamma\)-catenins (18) strengthens cadherin-mediated cell adhesion.

Comparison of cadherin sequences provides evidence that VE-cadherin is structurally different from class I cadherins, which include N- and E-cadherins. For example, the EC1 module of VE-cadherin lacks the sequence motif HAV (1) involved in the adhesion activity of class I cadherins (11, 12). Consequently, VE-cadherin was classified in the class II cadherin group (19), inferring that it might have particular adhesive properties. The elucidation of how the extracellular region of VE-cadherin assembles is fundamental to understanding the mechanism of cell-cell adhesion in the endothelium and to clarifying the process by which VE-cadherin modulates the transmigration of leukocytes. In this study, we focus on the behavior in solution of VE-EC1–4, a fragment consisting of the four N-terminal modules of VE-cadherin.

The molecular determinants at the basis of homotypic interactions and organization of cadherins in the adherent struc-
Hexameric Assembly of VE-cadherin Extracellular Domains

...ures at cell-cell junctions have been studied by analyzing the behavior in solution of cadherin fragments and by determining their high resolution structures. Some of the results are conflicting. For example, the E-cadherin N-terminal module (E-EC1) remains monomeric in solution (20, 21), whereas the equivalent fragment of N-cadherin (N-EC1) self-associates as dimers (14), suggesting that the homoaassembly mechanism is different according to the cadherin type. Comparison of the crystal structures from various cadherin fragments is also ambiguous. Thus, the N-EC1 crystal structure revealed the presence of parallel dimer interfaces (cis-dimers) and antiparallel alignments (trans-dimers) (14). It was suggested that both types of association reflect the interactions occurring between cadherins at the cell surface. cis-Dimers were proposed to mimic the alignment of two molecules emerging from the same cell surface, whereas trans-dimers may correspond to molecules protruding from opposing cell surfaces. The presence of these parallel and antiparallel dimers inspired Shapiro et al. to build a zipper model for the molecular organization of cadherins at the cell surface, in which each extracellular module contributes to lateral dimerization (14, 22). Until now, no direct evidence for lateral dimerization of the EC2–EC5 modules has been presented.

This model is in conflict with conclusions drawn from the crystal structures of the two module fragments of E-cadherin (E-EC1–2) (23, 24) and N-cadherin (N-EC1–2) (25). In fact, both EC1–2 fragments adopt twisted X structures maintained (E-EC1–2) (23, 24) and N-cadherin (N-EC1–2) (25). In fact, crystal structures of the two module fragments of E-cadherin build a zipper model for the molecular organization of cadherins. We show that the recombinant fragment including the aamipathic adhesion for the class II VE-cadherins (Asp1–Glu431). The DNA template was isolated from (Novagen, Madison, WI) using a 100 μM EDTA and a protease inhibitor mixture (Complete tablets, Roche Molecular Biochemicals, Meylan, France). After sonication, the insoluble proteins were collected by centrifugation and washed four times with 0.5 M urea and 0.5% Triton X-100 dissolved in Tris-HCl (pH 7.5). The insoluble proteins were then dissolved in TE buffer containing 8 M urea and chromato-...
When \( n \) is equal to 2, the \( C_{s\text{eq}} \) value corresponds to the \( K_d \) for a monomer ↔ dimer equilibrium. In contrast, when \( n \) is equal to 6, the \( C_{s\text{eq}} \) value becomes equal to \( 1.4 \times \sqrt{6} K_{a} \) for a monomer ↔ hexamer equilibrium.

Matrix-assisted Laser Desorption Ionization (MALDI) Mass Spectrometry—Mass spectra of the recombinant fragment VE-EC1–4, cross-linked or not, were obtained with a time-of-flight mass spectrometer (VoyagerElite XI, Perseptive Biosystems, Framingham, MA) equipped with a 337-nm nitrogen laser. Aliquots of 0.5 \( \mu \)l of the protein solution and 0.5 \( \mu \)l of the matrix solution were mixed on a stainless steel sample plate and dried in the air prior to mass spectrometry analysis. External calibration was performed with bovine serum albumin (m/z 66452; Sigma). All experiments were performed using a saturated solution of 2,5-dihydroxybenzoic acid (Mol. wt 197 g/mol) in acetonitrile and aqueous 0.1% trifluoroacetic acid. The accuracy of MALDI molecular mass determinations is between 0.01 and 0.05%.

Cross-linking of VE-EC1–4—The recombinant fragment VE-EC1–4 was partially cross-linked using N-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC; Sigma) or ethylene glycol bis(succinimidy succinate) (EGS). The molar ratio between the recombinant fragment and the cross-linker reagent was adjusted by varying the concentrations of the cross-linking reagents from 3 to 30 \( \mu \)M and from 0.1 to 3 \( \mu \)M for EDAC and EGS, respectively. In fact, these experiments were performed at 20 °C in MES (pH 7.0) for 2 h and in 2 mM Tris (pH 8.5) for 15 min for EDAC and EGS, respectively; and the concentration of VE-EC1–4 was maintained at 100 \( \mu \)M. The molar ratio was calculated and the cross-linker reagent was adjusted by varying the concentrations of the cross-linking reagents from 0.1 to 10 electrons/Å².

Expressed and Purified—A recombinant fragment of human VE-cadherin encompassing the four extracellular N-terminal modules (EC1–EC4) was expressed in E. coli in sufficient amounts for biophysical and biochemical studies (Fig. 1). The boundary of this fragment, designated VE-EC1–4, was essentially defined according to the domain organization proposed by Tanahara et al. (19). To avoid cross-linking and the associated problems of insolubility, the fifth module of VE-cadherin (EC5), the only module that may contain disulfide bridges, was excluded (Fig. 1). Moreover, to increase solubility of the fragment, the hydrophobic amino acid Phe432 in the C-terminal region of VE-EC1–4 was substituted into Trp by site-directed mutagenesis. The replacement of Phe432 with Trp increased the solubility of the fragment, indicated by several criteria such as centrifugation and gel filtration chromatography experiments, the molar mass (\( m \)) of the multimer could be calculated using the following equation:

\[
S = n(1 - p\%\text{af} N/2)^2 + n(1 - p\%\text{af} N/2)(1 - p\%\text{af} N/2) + E.
\]

For each profile, \( A_{i}(r_{i}) \), the absorbance for monomers at the radial position \( r_{i} \) (the first point of each data set), and \( E \), the residual base line, were fitted as follows: \( A = A_{i}(r_{i})\exp(1/1 - p\%\text{af} N/2)^2T + A_{i}(r_{i})\exp(1/1 - p\%\text{af} N/2)^2T + E \), where \( m_{i} \) is the molecular mass of the monomer, \( p \) is the angular velocity in \( s^{-1} \), \( R_{i} \) is 8.31 J·K^{-1}·mol^{-1} (the gas constant), and \( T \) is the temperature in Kelvin. The dissociation constant \( K_{d\text{app}} \) was calculated from \( K_{d\text{app}} \) using an extinction coefficient of 4,843 cm^{-1}·mol^{-1}·liter. The 48.9-kDa recombinant fragment VE-EC1–4 overlaps the four N-terminal modules of VE-cadherin (EC1–EC4). The N-terminal sequence of VE-EC1–4 is marked. CYT, cytoplasmic tail of VE-cadherin; \( \bullet \) signal peptide of VE-cadherin.
correctly folded (data not shown).

**Self-association of VE-EC1–4**—The capacity of the recombinant fragment to self-associate was evaluated by gel filtration chromatography (Fig. 2). Purified VE-EC1–4 was first injected at a concentration of 1 μM onto an analytical gel filtration column (Fig. 2A). Two peaks with elution volumes equal to 12.4 ± 0.1 ml (peak I; \( R_h = 68 ± 1 \, Å \)) and 15.74 ± 0.05 ml (peak II; \( R_h = 38.6 ± 0.8 \, Å \)) were detected on the chromatograms. SDS electrophoresis analysis of the chromatographic fractions corresponding to elution peaks I and II showed the presence of a unique 49-kDa band (Fig. 2B). This attested that both elution peaks correspond to the VE-EC1–4 fragment, thus excluding the possibility that peak II was due to a contaminating protein. It can be predicted that peaks I and II contain the oligomeric and monomeric VE-EC1–4 forms, respectively.

**Oligomeric State of VE-EC1–4**—To determine the oligomeric state of VE-EC1–4 in solution, it was cross-linked using the homobifunctional cross-linking reagents EGS (which reacts covalently with amino groups) and EDAC (which reacts covalently with amino and carboxylic groups). Before adding EGS or EDAC, the concentrations of both VE-EC1–4 and \( \text{Ca}^{2+} \) were adjusted so that the fragment was mostly in its monomeric form. Electrophoresis analysis of the cross-linked product revealed the formation of a multiple band pattern that varied with the concentration of the cross-linking reagent. In the presence of 0.1 mM EGS, six bands clearly appeared on the gel (Fig. 3A, lane 1). As the concentration of EGS was increased, the intensity of the five lower molecular mass bands decreased, whereas that of the upper band increased (Fig. 3A, lane 2). At 3 mM EGS, only the higher molecular mass band was detected (Fig. 3A, lane 3). Cross-linking with EDAC was comparatively less efficient since the six previously mentioned bands were observed at an EDAC concentration of 3 mM instead of 0.1 mM for EGS (Fig. 3A, lane 5), whereas three bands were observed with 1 mM EDAC (lane 4). The five lower molecular mass bands are partially cross-linked VE-EC1–4 products, and the highest one reflects the oligomeric association found in solution. By attributing a degree of oligomerization to each band (Fig. 3A), it was possible to relate the theoretical molecular mass to the electrophoretic mobility. Indeed, a linear curve was obtained by plotting the log theoretical molecular mass against the corresponding electrophoretic mobility for each band detected on the gel (data not shown). This suggests that the six discrete bands correspond to multimers containing one, two, three, four, five, or six covalently cross-linked molecules from the bottom to the top of the gel.

Using MALDI mass spectrometry, the molecular mass of the fragment cross-linked with 3 mM EGS was measured to be 315 kDa. There are 26 lysines on VE-EC1–4, all susceptible to cross-linking experiments. VE-EC1–4 at 110 μM was cross-linked using EGS (0.1 (lane 1), 0.300 (lane 2), and 3 (lane 3) mM) and EDAC (1 (lane 4) and 3 (lane 5) mM) at the indicated concentrations. Lane 6 shows the molecular mass markers. Cross-linked products were analyzed by SDS electrophoresis analysis using a 4–15% gradient Phast gel. The oligomeric states of each cross-linked product are indicated on the left, B, and C, analytical ultracentrifugation. Shown in B are the sedimentation velocity profiles of VE-EC1–4 at 1.2 (upper) and 26.2 (lower) μM. The first and last profiles presented here for each VE-EC1–4 concentration were obtained at 20 °C after 50 and 120 min of sedimentation at 42,000 rpm. The path length was 1.2 cm for the 1.2 μM sample and 0.3 cm for the 26.2 μM sample. Shown in C are the sedimentation coefficient distribution functions (\( g(s^*o) \)) for VE-EC1–4 at 1.2 (– – –) and 26.2 (– – – –) μM. The apparent sedimentation coefficient distribution functions (\( g(s^*o) \), i.e., normalized for loading concentrations, but without correction for diffusion effect) were evaluated using DCDT (32), considering six profiles of absorbance obtained for \( f_{o}r^2 \) between \( 1.1 \times 10^{11} \) and \( 1.4 \times 10^{11} \) s^{-1}. 

FIG. 2. Gel filtration analysis of VE-EC1–4 self-association. A, fast pressure liquid chromatography molecular exclusion profile for the VE-EC1–4 fragment at an initial loading concentration of 1 μM. Two distinct peaks (designated peaks I and II) are clearly detected on the chromatograms performed at 4 °C. B, SDS-polyacrylamide gel electrophoresis analysis of the fractions corresponding to peaks I and II. 40 μl of each fraction were set at the top of a 12.5% polyacrylamide gel. Following migration, the protein was detected by Coomassie Blue staining.

FIG. 3. Determination of the oligomeric state for VE-EC1–4. A, cross-linking experiments. VE-EC1–4 at 110 μM was cross-linked using EGS (0.1 (lane 1), 0.300 (lane 2), and 3 (lane 3) mM) and EDAC (1 (lane 4) and 3 (lane 5) mM) at the indicated concentrations. Lane 6 shows the molecular mass markers. Cross-linked products were analyzed by SDS electrophoresis analysis using a 4–15% gradient Phast gel. The oligomeric states of each cross-linked product are indicated on the left, B, and C, analytical ultracentrifugation. Shown in B are the sedimentation velocity profiles of VE-EC1–4 at 1.2 (upper) and 26.2 (lower) μM. The first and last profiles presented here for each VE-EC1–4 concentration were obtained at 20 °C after 50 and 120 min of sedimentation at 42,000 rpm. The path length was 1.2 cm for the 1.2 μM sample and 0.3 cm for the 26.2 μM sample. Shown in C are the sedimentation coefficient distribution functions (\( g(s^*o) \)) for VE-EC1–4 at 1.2 (– – –) and 26.2 (– – – –) μM. The apparent sedimentation coefficient distribution functions (\( g(s^*o) \), i.e., normalized for loading concentrations, but without correction for diffusion effect) were evaluated using DCDT (32), considering six profiles of absorbance obtained for \( f_{o}r^2 \) between \( 1.1 \times 10^{11} \) and \( 1.4 \times 10^{11} \) s^{-1}. 

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molecular mass of the VE-EC1–4 multimer can be calculated as \((49 + 26 \times 0.224) \times n\), where 49 corresponds to the mass of a VE-EC1–4 monomer, \(n\) is the degree of oligomerization, and 0.224 corresponds to the mass of an EGS molecule coupled to the fragment. Accordingly, the molecular masses were theoretically evaluated as 274 and 329 kDa for pentameric and hexameric associations, respectively. Due to the lack of accessibility to EGS of some lysines buried inside the core of the protein, these calculated values are likely overestimated. This suggested that the 315-kDa experimental value corresponds to a hexameric association.

The oligomeric state of VE-EC1–4 was determined independently by analytical centrifugation. Sedimentation velocity experiments were performed using VE-EC1–4 at 1.2 and 26 \(\mu\)M. At 1.2 \(\mu\)M, the concentration profiles exhibited bimodal boundaries (Fig. 3B). Consistent with this observation, \(g(s^w)\) versus \(s^w\), generated from this set of concentration profiles with the software DCDT, showed a double distribution (Fig. 3C). This reflected the heterogeneity existing within the molecular masses of the VE-EC1–4 protein at 1.2 \(\mu\)M. The curve fitting returned values of \(s = 2.9 \pm 0.1\) \(S (s_{20,w} = 3.0\) \(S)\) and 9.8 \(\pm 0.1\) \(S (s_{20,w} = 10.2\) \(S)\) for the left-hand and right-hand peaks, respectively. A good agreement was observed between the \(s\) values generated according to the programs SVEDBERG and DCDT. At 26 \(\mu\)M VE-EC1–4, the lightest species represented <5%, whereas the heaviest species migrated with a sedimentation coefficient of 10.2 \(S (s_{20,w} = 10.6\) \(S)\) (Fig. 3C).

The molar masses for each distribution were then calculated from the sedimentation coefficients combined with the hydrodynamic radius determined by gel filtration chromatography. Under these conditions, the molar masses were estimated to 49 and 301 kDa for the 2.9 \(S\) and 10.2 \(S\) distributions, respectively. These results indicate that the 2.9 \(S\) peak distribution corresponds to the monomeric form of VE-EC1–4, whereas the 10.2 \(S\) peak distribution corresponds to the hexameric form of VE-EC1–4.

To determine the molecular mass by a method that does not depend on the shape of the molecule, sedimentation equilibrium experiments were performed. VE-EC1–4 at various initial concentrations was equilibrated at 4500, 6000, and 12,000 rpm (Fig. 4, A and B). The mean molecular mass increased with increasing protein concentration and reached a plateau value of 285 kDa, compatible with a hexameric form (Fig. 4C).

Altogether, the results from gel filtration chromatography, analytical ultracentrifugation, and MALDI spectrometry demonstrate that the multimer of VE-EC1–4 is a hexamer. Moreover, variations in the relative amounts of VE-EC1–4 hexamer over monomer as a function of protein concentration show that both species are at equilibrium in solution.

**Dissociation Rate Constants of the Hexameric Form.—** To quantify the equilibrium between the monomer and hexamer, the stability of the hexamer was first analyzed by gel filtration chromatography. It was noticed that, following dilution of the VE-EC1–4 stock solution, the level of hexamer relative to monomer slowly decreased with time. Equilibrium between the monomeric and hexameric forms required at least 30 h at 20 °C (Fig. 5A). At 4 °C, the dissociation rate was considerably slowed down since equilibrium was reached 2 weeks after the dilution (data not shown). From this kinetic study, the hexamer dissociation rate constants \(k_d\) at 4 and 20 °C were determined from a plot of \(\ln([H]/[H])\) versus \(t\), assuming that the concentration of the monomer is negligible at the beginning of the time course (where \(t\), \([H]_0\), and \([H]\) are the dissociation time and the hexamer concentrations at time 0 and \(t\), respectively). The dissociation rate constants of the hexamer \(k_{d1}\) were calculated to be \(6 \times 10^{-6}\) and \(1 \times 10^{-6}\) \(s^{-1}\) at 20 and 4 °C, respectively.

**Apparent Dissociation Constant of the Hexamer.—** The parameters of the equilibrium between the monomeric and hexameric forms were further established by loading VE-EC1–4 at a range of initial concentrations onto the gel filtration column. The chromatographic runs were carried out at 4 °C, taking advantage of the low hexamer dissociation rate constant at this temperature. Indeed, we verified that, at 4 °C, the hexamer was not dissociated during the chromatographic runs despite the occurring dilution.

The chromatographic profiles showed that, as loading concentrations of VE-EC1–4 were increased, a simultaneous increase in the intensity of peak I and a decrease in that of peak II were observed (Fig. 2A). Based on the previous peak attribution, it was deduced that increases in VE-EC1–4 concentration favor formation of the hexamer, thus confirming results generated from sedimentation velocity experiments.

The monomer/multimer composition could be estimated from the areas of peaks I and II seen on the chromatographic profiles. The relationship between the percentage of multimer in solution and the initial concentration of VE-EC1–4 is then

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**Fig. 4. Global fitting of the sedimentation equilibrium data for VE-EC1–4.** A, the loading concentrations/angular velocities for the nine profiles presented are as follows: 40 \(\mu\)M/4500 rpm (○), 40 \(\mu\)M/6000 rpm (●), 40 \(\mu\)M/12,000 rpm (+), 12.5 \(\mu\)M/4500 rpm (□), 12.5 \(\mu\)M/6000 rpm (■), 12.5 \(\mu\)M/12,000 rpm (×), 3 \(\mu\)M/4500 rpm (△), 3 \(\mu\)M/6000 rpm (▲), and 3 \(\mu\)M/12,000 rpm (○). The global fit also takes into account data at a VE-EC1–4 concentration of 25 \(\mu\)M, which are not shown here for clarity. The lines correspond to the fitted curves, from which a dissociation constant of 0.5 \(\mu\)M\(^2\) was deduced. B, the differences between experimental and calculated values of absorbance (normalized by the statistical error of experimental absorbance (dA)) are presented. The separation between the resolutions is 1; for clarity, the curves are displayed with respect to each other by a value of 2. C, the apparent molar mass was calculated from \(d \ln \text{A} = dR^2\) as a function of the total concentration of VE-EC1–4. A is the absorbance.
formed an equilibrium between the monomeric and hexameric forms of VE-EC1–4. A kinetic study of the hexamer dissociation, VE-EC1–4 was diluted from 45 to 2 μM and stored at room temperature. The percentage of VE-EC1–4 present as hexamers was estimated from chromatographic profiles performed at different time intervals after dilution. B, relationship between hexamer formation and total concentration of VE-EC1–4. Multimer/monomer molar ratios were estimated from molecular exclusion profiles by integrating the area of peaks I and II (see “Materials and Methods”) for various loading concentrations of VE-EC1–4. C, Ca²⁺-dependent association of the hexameric VE-EC1–4 form. VE-EC1–4 at 18 μM (equilibrated in 5 mM Ca²⁺) was injected onto a Superdex S200 gel filtration column. Elutions were performed in Tris/saline (pH 7.4) running buffer containing either 5 mM Ca²⁺ (——) or 10 mM EGTA (—–). In the presence of 10 mM EGTA, the hexamer (peak I) dissociated into the monomer (peak II).

established (Fig. 5B). At VE-EC1–4 concentrations of 0.5 and 37.5 μM, 50 and 96% of the fragment was in the hexameric form, respectively. The apparent dissociation constant $K_{D(H)}$, as defined under “Materials and Methods,” was estimated as 0.3 μM⁻¹.

Sedimentation equilibrium experiments were also used to determine the apparent dissociation constants ($K_{D(H)}$) independently of the determination of $R_\lambda$. As shown in Fig. 4, our data were successfully described by the simplest model considering an equilibrium between the monomeric and hexameric forms of VE-EC1–4. A $K_{D(H)}$ value of 0.5 μM⁻¹, similar to that determined from gel filtration chromatography experiments, was deduced. From this $K_{D(H)}$ value, $C_{50\%}$ was estimated as 1 μM (see “Materials and Methods”). This value is comparable to that experimentally determined by gel filtration chromatography (0.5 μM).

Ca²⁺ Stabilization of the Hexamer—To study the Ca²⁺ dependence of the monomer ↔ hexamer equilibrium by gel filtration chromatography, a modified elution buffer containing 10 mM EGTA was used to elute VE-EC1–4 loaded in the presence of Ca²⁺. The resulting progressive removal of free Ca²⁺ prevented the formation of aggregates that clog the column. As illustrated in Fig. 5C, progressive Ca²⁺ depletion induced a large increase in peak II at the expense of peak I. This shows that the multimer dissociates into monomers in the absence of Ca²⁺.

Electron Microscopy of VE-EC1–4—In the presence of 5 mM Ca²⁺ at a protein concentration of 2 μM, conditions for which the hexameric form is the preponderant species, the electron micrographs of negatively stained VE-EC1–4 show a quite homogeneous population of particles with an elongated ellipsoidal shape (Fig. 6A). In the presence of EGTA, smaller objects with various ill defined shapes replaced these structures, indicating that formation of the hexamer is dependent on the presence of Ca²⁺ (data not shown).

A more precise picture of the shape of the hexamer was obtained by VE-EC1–4 in the frozen hydrated state (Fig. 6, B and C). It has a hollow cigar shape when viewed from the side (Fig. 6C) and appears as an annulus when viewed from the end (Fig. 6B). There is apparently a specific interaction between VE-EC1–4 and the air/water interface, so there are only two views of the molecule. In each view, the protein density of the hexamer is subdivided into smaller domains as expected for VE-EC1–4, but it is too variable from particle to particle to allow determination of the multimeric state from these images. The average dimensions of 100 hexameric VE-EC1–4 particles, visualized by cryoelectron microscopy, are 233 ± 10 × 77 ± 7 Å.

**DISCUSSION**

A recombinant fragment encompassing the four extracellular modules of VE-cadherin was produced in E. coli. Recently, this fragment was demonstrated to be functional since it is able to inhibit the aggregation of Chinese hamster ovary cells transfected with VE-cadherin in a concentration-dependent manner (27). Here, we demonstrated that this fragment spontaneously forms a unique well organized multimer. This study provides the first in vitro evidence that the extracellular part of cadherins can homoassociate in solution in a structure possessing an oligomeric order higher than 2. Furthermore, this calcium-dependent oligomer self-assembles at lower concentrations than those previously published for cadherin dimeric associations (20).
Analysis of the oligomeric state of the VE-cadherin VE-EC1–4 fragment both by analytical centrifugation and, after cross-linkage, by mass spectrometry revealed a hexameric association. This is the first description of such an organization in solution for a cadherin. Until now, the extracellular recombinant fragments of other cadherins, such as E-cadherin, were demonstrated either to stay monomeric (15, 33) or to form dimers, as for Xenopus C-cadherin (34). Oligomeric self-associations were observed for the extracellular region of E-cadherin; but in this case, the molecule was fused to the assembly domain of the cartilage oligomeric matrix protein to force oligomerization (24, 33). Removal of the fifth module (EC5) to the extracellular part of VE-cadherin does not change the properties of the molecule since we recently verified that the VE-EC1–5 fragment (Asp1–Asp542), which corresponds to the whole extracellular domain of the receptor, was also able to form a hexameric structure.

Gel filtration chromatography and ultracentrifugation experiments showed evidence of a concentration-dependent monomer ↔ hexamer equilibrium with a dissociation constant $K_{D_{(H)}} = 0.3–0.5 \mu M^{-1}$ Although the detailed mechanism resulting in the elaboration of the hexamer is not known, it can be assumed that this occurs via intermediate species such as dimers or trimers. These transient associations probably appear for VE-EC1–4 concentrations lower than those required for hexameric associations, suggesting that VE-EC1–4 dimers may be formed at concentrations lower than 0.5 μM. The unusual unit used for this $K_{D_{(H)}}$ value does not allow a direct comparison between our value and those previously published for other cadherins. To palliate this inconvenience, $C_{S_{0.5}}$ was evaluated for comparison with other cadherins. The VE-EC1–4 fragment associates as a hexamer with a $C_{S_{0.5}}$ of $0.5 \mu M$, whereas the E-EC1–2 fragment, containing the two N-terminal modules of E-cadherin, forms dimers with a $C_{S_{0.5}}$ ranging from 80 μM (20) to 170 μM (35). Differences in propensity to self-associate observed for E- and VE-cadherin fragments suggest that class I and II cadherins elaborate various types of homotypic interactions.

Our results also indicate that the hexamer is able to dissociate after dilution, with a very slow $k_d$ dissociation rate constant that increases from $10^{-6}$ to $6 \times 10^{-6} s^{-1}$ when the temperature is raised from 4 to 20 °C. This reflects the stability of the hexameric structure.

Formation of the hexameric structure observed for the VE-cadherin fragment is strictly dependent on the presence of Ca$^{2+}$. This result reflects the behavior of cadherins expressed at the cell surface and is in agreement with studies done on various cadherin-derived recombinant fragments. Indeed, except for the earliest works of Shapiro et al. (14) and Brieber et al. (34), multimers of cadherins were formed only in the presence of Ca$^{2+}$ (20, 23–25, 35).

Cryoelectron microscopy allows us to visualize the hexameric structures as elongated cigar-shaped particles. From these images, the average dimensions of the hexamers were evaluated as $233 \pm 10 \times 77 \pm 7 \ Å$ (Fig. 6, B and C). The length of the hexameric structure is comparable to the distance separating two adjacent endothelial cells, estimated by electron microscopy to be $200 \ Å$ (36). It is not clear from the cryoelectron microscopy images whether the six cadherin molecules adopt a parallel or an antiparallel orientation within the hexamer. Determination of the relative orientation of the six molecules of VE-cadherin within the hexamer is a key step in understanding how adherent junctions are formed between endothelial cells. A parallel orientation would indicate formation of adherens junctions consisting of molecules protruding from neighboring cells. The crystal structure of the hexameric VE-EC1–4 complex should resolve this question. Nevertheless, the fact that VE-cadherin was demonstrated, by atomic force microscopy, to be able to constitute multiple trans-interactions is compatible with an antiparallel orientation of the hexamer (37). This trans-orientation is also in agreement with results recently published demonstrating that formation of antiparallel dimers requires Ca$^{2+}$, in contrast to parallel dimers (38, 39). Moreover, as attested by the determination of the N-EC1 structure (14) and as deduced from the electron microscopy measurement for the E-EC1–5 cadherin construct (i.e. $220 \ Å$ (33)), the average dimension of a cadherin module is $45 \pm 1 \ Å$. Consequently, the length of the VE-EC1–4 hexamer ($233 \pm 10 \ Å$) corresponds approximately to the length of five cadherin modules. Thus, this is compatible with a model in which the six monomers are disposed in an antiparallel fashion to form a barrel with three overlapping modules along its length.

Due to its relatively high affinity constant, the hexamer appears at low concentrations. Placed in a cellular context, this result indicates that this hexameric structure may be physiologically relevant at the cell-cell junctions where VE-cadherin is concentrated. In fact, this hexameric assembly may constitute a basic association motif whose assembly may promote formation of the continuous sealing region between endothelial cells.

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REFERENCES

1. Lampugnani, M. G., Resnati, M., Rafteri, M., Pigotti, R., Pisacane, A., Houen, G., Ruco, L. P., and Dejana, E. (1992) J. Cell Biol. 118, 1511–1522
2. Delmas, A., Chailan, E., Concord, E., Genoux, Y., Morand, B., Valiron, M. O., Sulpczez, E., Scaliet, E., Alemany, L., and Vernet, T. (1998) J. Biol. Chem. 273, 29786–29793
3. Navarro, P., Caveda, L., Breviario, F., Mandoteanu, I., Lampugnani, M. G., and Dejana, E. (1995) J. Biol. Chem. 270, 30965–30972
4. Horlick, D. P., Chang, R., and Fasman, G. D. (1978) J. Biol. Chem. 253, 3429–3436
5. Vernet, T., Dejana, E., and Lampugnani, M. G. (1997) J. Biol. Chem. 272, 15281–15284
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27. Breviario, F., Caveda, L., Corada, M., Martin-Padura, I., Navarro, P., Golay, J., Introna, M., Gulino, D., Lampugnani, M. G., and Dejana, E. (1995) Arterioscler. Thromb. Vasc. Biol. 15, 1229–1239
28. Mach, H., Middaugh, C. R., and Lewis, R. V. (1992) Anal. Biochem. 200, 74–80
29. Le Maire, M., Aggerbeck, L. P., Monteilhet, C., Andersen, J. P., and Moller, J. V. (1986) Anal. Biochem. 154, 525–535
30. Philo, J. S. (1997) Biophys. J. 72, 435–444
31. Stafford, W. F., III (1997) Curr. Opin. Biotechnol. 8, 14–24
32. Stafford, W. F., III (1992) Anal. Biochem. 203, 295–301
33. Tomschy, A., Pauser, C., Landwehr, R., and Engel, J. (1996) EMBO J. 15, 3507–3514
34. Brieher, W. M., Yap, A. S., and Gumbiner, B. M. (1996) J. Cell Biol. 133, 487–496
35. Alattia, J. R., Ames, J. B., Porumb, T., Tong, K. I., Heng, Y. M., Ottensmeyer, P., Kay, C. M., and Ikura, M. (1997) FEBS Lett. 417, 405–408
36. Leach, L., and Firth, J. A. (1997) Microsc. Res. Tech. 38, 137–144
37. Baumgartner, W., Hinterdorfer, P., Ness, W., Raab, A., Vestweber, D., Schindler, H., and Drenckhahn, D. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4005–4010
38. Chitaev, N. A., and Troyanovski, S. M. (1998) J. Cell Biol. 142, 837–846
39. Shan, W. S., Tanaka, H., Phillips, G. R., Arndt, K., Yoshida, M., Colman, D. R., and Shapiro, L. (2000) J. Cell Biol. 148, 579–590
Self-assembly of the Vascular Endothelial Cadherin Ectodomain in a Ca\textsuperscript{2+}-dependent Hexameric Structure

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