Synergy between Extracellular Modules of Vascular Endothelial Cadherin Promotes Homotypic Hexameric Interactions*

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Vascular endothelial (VE) cadherin is an endothelial specific cadherin that plays a major role in remodeling and maturation of vascular vessels. Recently, we presented evidence that the extracellular part of VE cadherin, which consists of five homologous modules, associates as a Ca2+-dependent hexamer in solution (Legrand, P., Bibert, S., Jaquinod, M., Ebel, C., Hewat, E., Vincent, F., Vanbelle, C., Concord, E., Vernet, T., and Gulino, D. (2001) J. Biol. Chem. 276, 3581–3588). In an effort to identify which extracellular modules are involved in the elaboration and stability of this hexameric structure, we expressed various VE cadherin-derived fragments overlapping individual or multiple successive modules as soluble proteins, purified each to homogeneity, and tested their propensity to self-associate. Altogether, the results demonstrate that, as their length increases, VE cadherin recombinant fragments generate increasingly complex self-associating structures; although single module fragments do not oligomerize, some two or three module-containing fragments self-assemble as dimers, and four module-containing fragments associate as hexamers. Our results also suggest that, before elaborating a hexameric structure, molecules of VE cadherin self-assemble as intermediate dimers. A synergy between the extracellular modules of VE cadherin is thus required to build homotypic interactions. Placed in a cellular context, this particular self-association mode may reflect the distinctive biological requirements imposed on VE cadherin at adherens junctions in the vascular endothelium.

Adhesion between cells of identical phenotypes is mediated by receptors belonging to the cadherin superfamily (1, 2). This protein family contains 50 different classic cadherins in both vertebrates and invertebrates and the recently described human protocadherins (3, 4). Based on their amino acid sequence, classic cadherins were first classified into two subgroups, type I and type II (5, 6). More recently, cadherin-5 and -15 that share little sequence similarity with other cadherins or among themselves were considered as two distinct non-type I or II cadherins (7).

Classic cadherin molecules exhibit a similar organization, in particular in their extracellular domain, that consists of five homologous repeats designated EC1 to EC5 and numbered from the N to the C terminus. Type I members show a high degree of protein sequence similarity when compared with the E cadherin sequence and possess, in their N-terminal extracellular module EC1, the HAV cell adhesion recognition sequence (8). By contrast, the HAV sequence is absent in the extracellular domain of either type II members or non-type I or II cadherins (9).

The highly conserved cytoplasmic domain of cadherins interacts with β-catenin or γ-catenin (also called plakoglobin) in a mutually exclusive fashion (10, 11). Moreover, β- or γ-catenins also interact with α-catenin which links the cadherin-adhesion complex either directly or indirectly to the actin cytoskeleton (12, 13). Cell-cell adhesion is constantly rearranged suggesting that the cadherin-catenin complex is dynamically remodeled.

By mediating homotypic interactions, cadherins are responsible for segregation of different cell types and, consequently, are fundamental for the establishment and maintenance of multicellular structures. Several results demonstrate that the homotypic binding regions reside in the extracellular part of cadherins (14). High resolution structure determination sheds light on the molecular determinants and organization of homotypic cadherin interactions at cell-cell junctions. Based on the first structure derived from the N-terminal domain of neural cadherin (N-EC1 fragment) (15), a model for cadherin-mediated homophilic interactions was proposed (the zipper model) (16). It suggests the formation of parallel dimer interfaces (cis dimers) and anti-parallel alignments (trans dimers). Both types of association may reflect interactions occurring between cadherins at the cell surface. Cis dimers involving the five extracellular modules of cadherins may mimic the alignment of two molecules emerging from the same cell, whereas trans dimers mediated by the N-terminal EC1 module may be elaborated by molecules protruding from adjacent cells. Structural data obtained for the two module fragments of E (E-EC1–2) (17, 18) or N (N-EC1–2) (19) cadherin revealed that the length of a single cadherin extracellular module is ~45 Å. It can be deduced that the cell to cell distance in the zipper model is about 405 Å, a value incompatible with the dimension of cell-cell interactions.
adherens junctions that ranges from 200 to 250 Å based on electron microscopy analysis.

By using direct-force measurements, Leckband and co-workers (20, 21) proposed a new model for homotypic interactions in which cadherin molecules emerging from adjacent cells elaborate antiparallel, completely interdigitated contacts. These structures, which exhibit multiple adhesive contacts involving successive domains along the extracellular region of the protein, possess a length in the range of 250 Å.

Here we investigate the mechanism of homotypic interactions mediated by the non-type I or II cadherin 5 (or VE1 protein, possess a length in the range of 250 Å that involve VE cadherin is also required for vascular morphogenesis. Furthermore, in contrast to other cadherin family members, VE cadherin is both connected to the actin cytoskeleton and to intermediate filaments (27, 28).

Recently, we have presented evidence (29) for the Ca2+-dependent hexameric association of the extracellular region of VE cadherin. This type of oligomerization is difficult to reconcile with structural data obtained for shorter fragments of other cadherins. We have now extended our previous study by analyzing a series of VE cadherin-derived arrays of modules. We show that the number of modules within the array indicating that each module acts in synergy with the neighboring ones during the homotypic assembly of VE cadherin. Altogether, our results suggest that VE cadherin molecules first self-assemble as intermediate dimers involving extracellular modules EC3 and EC4 before elaborating mature hexameric structures.

MATERIALS AND METHODS

Recombinant Fragments—The single module fragments were named VE-E1, with i corresponding to the position of the respective extracellular module within the extracellular domain of VE cadherin. The multidomain fragments were designated VE-E1-i-j, where i and j correspond to the positions of the most N- and the most C-terminal modules, respectively. For instance, VE-E1-3 starts at the N-terminus of module EC1, ends at the C-terminus of module EC3, and consequently overlaps the three modules EC1, EC2, and EC3 (Fig. 1A).

The oligonucleotide pairs used to produce the cDNA fragments encoding the VE cadherin-derived proteins are summarized in Table 1. PCR-amplified products were cloned into different expression vectors. The single module fragments, cloned into the vector pET-30b (Novagen), were expressed as native proteins. The resulting pET-VE cadherin plasmids allowed the expression of VE cadherin multiple module fragments fused to an N-terminal methionine. The coding sequence of all VE cadherin-derived constructs was verified by sequence analysis.

The abbreviations used are: VE, vascular endothelial; MALDI, matrix-assisted laser desorption ionization; Rh, hydrodynamic radius; EDC, N-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; GST, glutathione S-transferase; MES, 4-morpholineethanesulfonic acid.

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### Table I

| Fragments | Oligonucleotides |
|-----------|------------------|
| VE-EC1    | 5' GGA TCC GAT TGG ATT TGG AAC CAG ATG CAC 3' |
|           | BamHI Asp1 1 |
|           | 3' GAA TTC TTA CCG GTG CAG GAA CAC AGG CCA GTT GTC 3' |
|           | EcoRI stop Arg1 |
| VE-EC1-2  | 5' TATA CAT ATG GAT TGG ATT TGG AAC CAG ATG CAC 3' |
|           | NdeI Asp1 1 |
|           | 3' GAA TTC TCA CCG GTT CTG GGG CTC ATG TGG GCC 3' |
|           | EcoRI stop Arg4 |
| VE-EC1-3  | 5' TATA CAT ATG GAT TGG ATT TGG AAC CAG ATG CAC 3' |
|           | NdeI Asp1 1 |
|           | 3' GAA TTC TCA CTC GTC CAC ATC TGG GTT GAT 3' |
|           | EcoRI stop Glu122 |
| VE-EC1-4  | 5' TATA CAT ATG GAT TGG ATT TGG AAC CAG ATG CAC 3' |
|           | NdeI Asp1 1 |
|           | 3' GAA TTC TCA CTC GTC CAC ATC TGG GTT GAT 3' |
|           | EcoRI stop Glu31 |
| VE-EC4    | 5' CTT GGA TCC CCA TAC ATG AGC CCT CCC GCG GGA AAC 3' |
|           | BamHI Arg500 |
|           | 3' GAA TTC TCA CTC GGG GTC ATT GTC ATT CTC ATC 3' |
|           | EcoRI stop Glu431 |
| VE-EC3-4  | 5' TATA CAT ATG ACC CAG ACC AAC TAC ACA TTT GTC 3' |
|           | NdeI Thr12 |
|           | 3' GAA TTC TCA CTC GGG GTC ATT GTC ATT CTC ATC 3' |
|           | EcoRI stop Glu431 |
| VE-EC2-4  | 5' TATA CAT ATG ACC CAG CAT TGG TTT TGC AAT GCG TCC 3' |
|           | NdeI Thr105 |
|           | 3' GAA TTC TCA CTC GGG GTC ATT GTC ATT CTC ATC 3' |
|           | EcoRI stop Glu431 |
| VE-EC1-4m | 5' GTT CAT GAG GTG GCC GCC AAC TGG CCT GTG 3' |
|           | Ala39 Ala39 |
|           | 3' CAC AGG CCA GTT GCC GCC GAC ATG AAC 3' |
|           | Ala99 Ala99 |

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1 The abbreviations used are: VE, vascular endothelial; MALDI, matrix-assisted laser desorption ionization; Rh, hydrodynamic radius; EDC, N-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; GST, glutathione S-transferase; MES, 4-morpholineethanesulfonic acid.
The modified fragment VE-EC1–4m was elaborated using the Quick-
Change site-directed mutagenesis kit (Stratagene Europe, Amsterdam, 
The Netherlands). The mutations N98A and D99A were introduced 
using the synthetic oligonucleotide primers shown in Table I.

**Purification of Recombinant Fragments**—The single module frag-
ments VE-EC1–4 derived from E. coli were purified using the pGEX-
4x (Amersham Biosciences) vector. The GST fusion proteins were puri-
fied directly from bacterial lysates using the affinity matrix glutathione-
Sepharose 4B (Amersham Biosciences). Proteins were eluted from the 
GST fusion proteins with glutathione by changing the pH from 8.0 to 
2.0. The recovery was >95%. The purity of the VE cadherin fragments 
was verified by SDS-PAGE analysis (Fig. 3). N-terminal sequencing reve-
ed that cleavage by thrombin occurred at the N-terminus of fragment VE-
EC1 (Table II). The boundary of the fragments was defined according to 
the cadherin domain organization proposed by Tanihara et al. (6). To improve 
the purity of the fragments, the boundaries of the fragments were 
also delineated by means of limited proteolysis experiments. Individual modules or arrays of modules are depicted in Fig. 1A.

The single module fragments VE-EC1 and VE-EC4 were 
expressed as N-terminal glutathione S-transferase (GST) fu-
sion proteins and purified by affinity chromatography using 
glutathione-Sepharose prior to proteolytic cleavage of the GST 
tag. The multimodule fragments were purified from inclusion 
figs by refolding and diluted as described previously (29).

**Matrix-assisted Laser Desorption-Mass Spectrometry (MALDI)**—
Mass spectra of the recombinant fragments, cross-linked or not, were 
determined as described previously (29).

**RESULTS**

**Recombinant VE Cadherin Fragments**—To evaluate the rel-
ative contribution of each extracellular module of VE cadherin 
to homotypic binding, several recombinant fragments, 
comprising variable parts of the extracellular region of human VE 
cadherin, were expressed in *Escherichia coli*. The boundary of 
these fragments was defined according to the cadherin domain 
organization proposed by Tanihara et al. (6). To improve the 
 purity of some of them, the boundaries of the fragments were 
also delineated by means of limited proteolysis experiments. Individual modules or arrays of modules are depicted in Fig. 1A.

The single module fragments VE-EC1 and VE-EC4 were 
expressed as N-terminal glutathione S-transferase (GST) fu-
sion proteins and purified by affinity chromatography using 
glutathione-Sepharose prior to proteolytic cleavage of the GST 
tag. The multimodule fragments were purified from inclusion 
figs by refolding and diluted as described previously (29).

**Oligomeric States of the VE Cadherin Recombinant Frag-
ments**—The capacity of the fragments to self-associate was 
analyzed by gel filtration chromatography, chemical cross-link-
ing, and analytical ultracentrifugation experiments.

As illustrated in Fig. 2, the chromatographic profiles differ 
dramatically for the native and cross-linked fragments. Thus, fragments 
VE-EC1, VE-EC4, VE-EC1–2 (not shown), and VE-EC1–3 gave 
single elution peaks for cross-linked concentrations tested up to 
200 μM. In contrast, double distributions (peaks I and II) were 
observed for the fragments VE-EC3–4, VE-EC2–4, and VE-
EC1–4, even at the relatively low concentration of 10 μM, 
indicating that they possess two different oligomeric states.
Synergistic Self-assembly of VE Cadherin Extracellular Modules

From the elution volumes of the peaks observed on the chromatograms, the hydrodynamic radii of the different species of each fragment were deduced (Table III and "Materials and Methods"). To determine the oligomeric states of the fragments, they were first cross-linked using the heterobifunctional reagent EDC that covalently couples primary amino to carboxyl groups located in close proximity. Fig. 3 shows the electrophoretic separation of the cross-linked products. Cross-linked fragments VE-EC3–4 and VE-EC2–4 exhibited a two band pattern. The upper bands have molecular masses of ~50 and 72 kDa for the cross-linked fragments VE-EC3–4 and VE-EC2–4, respectively, indicating that both fragments form dimers. By using similar cross-linking experimental conditions, a pattern of six bands was obtained with VE-EC1–4 (29). Altogether, these data confirm our gel filtration chromatography results from which it can be deduced that, in Fig. 2, peaks I and II observed with both VE-EC3–4 and VE-EC2–4 correspond to the dimeric and monomeric forms of these fragments, respectively, and that peak I of the fragment VE-EC1–4 represents its hexameric association.

In contrast, under similar cross-linking conditions, fragments VE-EC1–2 and VE-EC1–3 gave single bands of 24 and 36 kDa, respectively, confirming the absence of oligomerization seen by gel filtration experiments for these fragments. Similar results were obtained for fragments VE-EC1 and VE-EC4 (results not shown). These observations were confirmed when high concentrations of fragment VE-EC1–3 were analyzed by analytical ultracentrifugation. Indeed, sedimentation velocity experiments performed using VE-EC1–3 at concentrations ranging from 1 to 108 μM showed a single species (Fig. 4). The sedimentation coefficient remained constant as the concentration of the fragment increased and was measured to 2.2 Svedberg. Combined with the hydrodynamic radii determined by gel filtration chromatography (Table III), these values allowed the determination of the molar mass of the fragment VE-EC1–3 (36 kDa) and confirmed that this fragment does not self-associate even at high protein concentrations.

We have established a standard curve connecting the Rh to the corresponding molecular masses (Mₐ) of the VE cadherin fragments. The hydrodynamic radii were determined from gel filtration chromatography as mentioned above (Table III), whereas the molecular weights were calculated from the degree of self-association deduced from either cross-linking or analytical centrifugation experiments. A linear relationship connecting log(Mₐ) to Rh was observed for the different oligomeric states of the various VE cadherin fragments (Fig. 5). All the points for VE cadherin fragments are down-shifted when compared with the curve established using globular proteins reflecting a difference in the molecular shape between globular proteins and VE cadherin fragments. This can be explained by the elongated form adopted by VE cadherin fragments as observed for VE-EC1–4 in cryoelectron microscopy (29). These data confirm, for each fragment, the multimerization results obtained either by gel filtration chromatography or cross-linking or ultracentrifugation experiments.

**Equilibria between the Monomeric and the Multimeric Forms**—We have demonstrated recently (29) the existence of an equilibrium between the monomeric and the hexameric forms of fragment VE-EC1–4. Here this study was extended to fragments VE-EC3–4 and VE-EC2–4 that self-associate as dimers. Indeed, gel filtration chromatography experiments showed that the intensities of peak I increased with concomitant decrease of those of peak II for increasing concentrations of the fragments (Fig. 6A). This result demonstrates the existence of an equilibrium between the monomeric and the dimeric species of both fragments VE-EC3–4 and VE-EC2–4. For comparison, results concerning the equilibrium between the monomeric and hexameric forms of fragment VE-EC1–4 were also included in Fig. 6A.

To study the parameters governing the monomer-oligomer equilibria, time courses of the multimer dissociation, established by diluting the fragments from 150 to 20 μM, were followed by comparing the areas of peaks I and II obtained by gel filtration chromatography. The time required to reach the equilibrium at 4 °C was estimated to 24 h for the fragments VE-EC3–4 and VE-EC2–4 and to be longer than 2 weeks for VE-EC1–4 (results not shown). Thus, during the chromatographic runs, which lasted 30 min, the dissociation of multimers was negligible (results not shown). Consequently, the dissociation constants Kₐ calculated from the ratios [dimer]/
monomer]2 can be directly evaluated from the areas of peaks I and II. The KD values are thus estimated to 80 and 25 \( \mu \text{M} \) for the fragments VE-EC3–4 and VE-EC2–4, respectively.

To establish a comparison with the fragment VE-EC1–4, which self-associates as a hexamer, the concentrations [C]50% for which 50% of the fragments are multimeric are estimated using the following equation:

\[
[C]_{50\%} = \frac{2}{K_Dn^{1/n}}K_D^{1/n-1}
\]

where \( n \) represents the oligomeric state of the fragment. For the fragments that self-associate as dimers, \( C_{50\%} = K_D \) and for the fragment VE-EC1–4, \( C_{50\%} = \frac{\alpha(2\beta\gamma)^{1/5}}{K_D^{1/5}} \), with \( K_D \) being expressed in this case in \( \mu \text{M} \). As indicated in Table III, the \( C_{50\%} \) values are calculated to be 80, 25, and 0.5 \( \mu \text{M} \) for the fragments VE-EC3–4, VE-EC2–4, and VE-EC1–4, respectively. Clearly, as the length of the fragments increases, the affinity for self-association increases. This reflects a synergy existing between the extracellular modules to elaborate VE cadherin homotypic interactions.

As illustrated in Fig. 6B, oligomerization of the fragments was demonstrated, by gel filtration chromatography experiments, to be \( \text{Ca}^{2+} \)-dependent. Indeed, upon elution of fragments, previously equilibrated in the presence of \( \text{Ca}^{2+} \), using an EDTA-containing buffer, the multimers of fragments VE-

### Table II

**Characterization of VE cadherin recombinant fragments**

| Fragments   | N-terminal sequencing | Mass Theoretical | Mass Experimental |
|-------------|-----------------------|-----------------|-------------------|
| VE-EC1      | GS DWIWNQM            | 12,552          | 12,554            |
| VE-EC1–2    | MDWIWNQM              | 27,455          | 27,473            |
| VE-EC1–3    | MDWIWNQM              | 36,278          | 36,269            |
| VE-EC1–4    | MDWIWNQM              | 48,941          | 48,948            |
| VE-EC4      | GSPEFQQP              | 12,890          | 12,892            |
| VE-EC3–4    | MTQTKTFV              | 25,198          | 25,198            |
| VE-EC2–4    | MTHRFLNAS             | 36,514          | 36,816            |

\( ^a \) Determined using the computer program ProtParam Tools of the Expasy server (www.expasy.ch/tools/protparam.html).

\( ^b \) Measured by MALDI mass spectrometry.
Synergistic Self-assembly of VE Cadherin Extracellular Modules

TABLE III

Oligomeric states of different VE cadherin fragments

The oligomeric states of the different fragments were determined either by analytical centrifugation or by cross-linking experiments. For each fragment, the first number corresponds to the monomeric form, and the second one corresponds to the multimeric form. Hydrodynamic radii (Rh) and sedimentation coefficients (s) were determined from gel filtration chromatography and ultracentrifugation experiments, respectively. C_{50%} values indicate the concentration at which 50% of the respective fragments is oligomeric.

| VE cadherin fragments | Rh (kDa) | s (Svedberg) | Molar masses | Multimer | C_{50%} |
|-----------------------|----------|--------------|--------------|----------|---------|
| VE-EC1–4 | 38; 67 | 2.9; 9.8 | 49; 301⁵ | Monomer, hexamer | 0.5 |
| VE-EC1–3 | 33 | 2.2 | 36⁴ | Monomer | |
| VE-EC1–2 | 26 | | 12⁴ | Monomer | |
| VE-EC1 | 18.5 | | 15⁴ | Monomer | |
| VE-EC4 | 20.5 | | 30; 60⁸ | Monomer, dimer | 80 |
| VE-EC3–4 | 30; 40 | | 36; 67⁷ | Monomer, dimer | 25 |

⁴ Determined by analytical centrifugation.
⁵ Determined by cross-linking experiments.

FIG. 3. Determination of the oligomeric states of VE cadherin-derived fragments using cross-linking experiments. The purified recombinant fragments VE-EC1–2 (50 μM), VE-EC3–4 (50 μM), VE-EC1–3 (70 μM), and VE-EC2–4 (50 μM) were cross-linked for 2 h using molar EDC/fragment ratios of 37.5 (lane 1), 75 (lane 2), 150 (lane 3), 300 (lane 4), and 600 (lane 5). Cross-linked products were analyzed by SDS-PAGE using 4–15% gradient Phast gels (Amersham Biosciences). Arrows in the right margin of the gels indicate different oligomeric states of each cross-linked product (x1, monomer; x2, dimer). Molecular mass markers (lane Mw) are given in kDa in the left margin of the gels.

FIG. 4. Determination of the oligomeric states using ultracentrifugation analysis. Sedimentation velocity experiments were performed at 42,000 rpm using the fragment VE-EC1–3 (at 2.5, 5, 21, 34, 84, and 108 μM). The s values were calculated from sedimentation profiles obtained at 20 °C after 120 min using the computer program Svedberg (50). The path length was 1.2 cm for the 1 μl sample and 0.3 cm for the other samples. The slight decrease of the sedimentation coefficient is due to a nonideality term observed in such experiments as described (14).

EC3–4 (panel 1), VE-EC2–4 (panel 2), and VE-EC1–4 (panel 3) dissociated during the chromatographic runs.

Folding of VE Cadherin Fragments—The varying capacity of the recombinant VE cadherin fragments to self-associate may reflect differences in their efficiency to adopt a correctly folded conformation. Consequently, their stability was studied by limited trypsin digestion. Proteolytic experiments were performed at concentrations at which recombinant VE cadherin fragments were mainly oligomeric.

Fig. 7A shows the electrophoretic separation of the digestion products. The single module fragment having its proximal inter-module region VE-EC1 (Asp¹–Arg¹⁰⁷) appeared resistant to trypsin (Fig. 7A). In contrast, the short single domain fragments VE-EC1 (Asp¹–Phe¹⁰⁴) and VE-EC4 (Gln¹¹⁰–Phe¹⁵²), which did not possess inter-module extensions, were almost completely digested with very low amounts of trypsin (data not shown). Moreover, digestion patterns for the various multimodule fragments exhibited a limited number of bands. It could be deduced that most of the potential cleavage sites were not accessible to the protease indicating that the fragments of VE cadherin are folded (Fig. 5B).

Cleavage sites were accurately localized using an approach that combines limited enzymatic proteolysis, in-gel proteolytic digestion, and MALDI mass spectrometry (33, 34). Results are given in Tables II and IV.

Following limited trypsinolysis, VE-EC1 (Asp¹–Arg¹⁰⁷), extended by the EC1-EC2 inter-module region exhibited two distinct proteolytic bands (band b, Ile²⁴–Arg¹⁰⁷; band c, Tyr³⁵–Arg¹⁰⁷) (Fig. 7A). This means that, among the 13 putative
trypsin-susceptible sites, only two (Lys\textsuperscript{23} and Lys\textsuperscript{34}) remain effectively accessible to the enzyme.

For VE-EC1–2, one cleavage site, localized in the EC1-EC2 linker region at position Arg\textsuperscript{107}, yielded two bands of 15 and 12 kDa corresponding to fragments Leu\textsuperscript{108}–Arg\textsuperscript{244} and Asp\textsuperscript{1}–Arg\textsuperscript{107}, respectively (bands d and e, Fig. 7, A and B, and Table IV). In addition, two N-terminal products with molecular masses of 25 and 23 kDa resulting from cleavage sites at residues Lys\textsuperscript{23} and Lys\textsuperscript{34} (bands b and c, Fig. 7, A and B, and Table IV) were also detected. Similarly, N-terminal truncations of fragment Asp\textsuperscript{1}–Arg\textsuperscript{107} at positions Lys\textsuperscript{23} and Lys\textsuperscript{34} generated the 10- and 9-kDa bands (bands f and g, Fig. 7, A and B, and Table IV). The intensity of band d decreased with increasing enzyme to fragment ratios indicating the presence of a new...
FIG. 7. Trypsin digestions of purified VE cadherin fragments. A, SDS-PAGE analysis of digestion products. Fragments were digested for 20 min at 22 °C by trypsin using enzyme/fragment ratios of 0 (lane 2), 0.162 (lane 3), 0.324 (lane 4), 0.648 (lane 5), and 1.3 units/μg (lane 6). Lane 1, molecular mass markers. Trypsin-digested fragments were run through 8–25% gels (Phast System, Amersham Biosciences). Following migration, proteins were detected by Coomassie Blue staining. Each band detected in lane 5 of the different gels was individually excised and in-gel digested. The resulting peptides were extracted from the obtained gel bands and analyzed by MALDI mass spectrometry (see under “Materials and Methods”). For the nomenclature of the protein bands, see under “Results” and Table III. B, cleavage sites generated by trypsin digestion. Trypsin cleavage positions were deduced from the molecular weights of in-gel-digested peptides. Small black tic marks represent theoretical trypsin cleavage sites, and highly accessible cleavage sites are indicated by black arrows, and moderately accessible cleavage sites are represented by open arrowheads.
cleavage site located at position Arg<sup>183</sup> (band g). Nevertheless, compared with fragment Asp<sup>1</sup>–Asp<sup>203</sup> (results not shown), fragment Asp<sup>1</sup>–Asp<sup>244</sup> exhibits a higher trypsin resistance indicating that addition of the EC2-EC3 inter-domain improves the stability of the fragment VE-EC1–2.

Limited proteolysis of the dimeric VE-EC3–4 fragment revealed cleavage sites at Arg<sup>200</sup> and Arg<sup>244</sup>, yielding four distinct bands migrating at 21, 15, 10, and 6 kDa (bands b–e, Fig. 7A, and Table IV). Even at the highest trypsin concentrations used, the intensity of band c remained unchanged indicating that the amino acid stretch Tyr<sup>301</sup>–Glu<sup>431</sup> (<b>7</b>) was not cleaved by trypsin. Consequently, within VE-EC3–4, module EC4 is probably correctly folded.

Moreover, when compared with VE-EC3–4, VE-EC2–4 exhibited two additional highly accessible cleavage sites at positions Arg<sup>107</sup> and Arg<sup>309</sup> within module EC2 (Fig. 7A and B, and Table IV).

Limited proteolysis of the monomeric VE-EC1–3 fragment generated a major 27-kDa band corresponding to peptide Asp<sup>1</sup>–Arg<sup>244</sup> (band b, Fig. 7A, and Table IV). In addition, multiple minor fragments with molecular masses between 33 and 30 kDa (bands c–f, Fig. 7A) appear for a trypsin/VE cadherin fragment ratio of 1.3 units/μmol, indicating secondary cleavage events at residues Lys<sup>323</sup>, Lys<sup>334</sup>, and Arg<sup>300</sup> (lane 6, Fig. 7B, and Table IV).

Concerning VE-EC1–4, limited proteolysis, performed using enzyme to fragment ratios ranging from 0.162 to 0.650 units/μmol, yielded two bands of 35 (band b) and 14 kDa (band c), respectively (Fig. 7A, lanes 3–5). With a larger amount of trypsin (enzyme/fragment ratio of 1.3 units/μmol), an additional band of 22 kDa (band d) appeared (Fig. 7A, lane 6). The 35- and 14-kDa bands were identified by mass spectrometry to be the N-terminal Asp<sup>1</sup>–Arg<sup>209</sup> part and the C-terminal Arg<sup>309</sup>–Glu<sup>431</sup> part, respectively (Fig. 7B and Table IV). Cleavage of Asp<sup>1</sup>–Arg<sup>209</sup> at position Arg<sup>107</sup> generated the secondary 22-kDa product corresponding to the sequence Lys<sup>108</sup>–Arg<sup>309</sup> (band d, Fig. 7B, and Table IV).

Fig. 7B summarizes the theoretical and experimental trypsin cleavage sites for the different fragments. It can be concluded that, in general, neighboring inter-module regions seem to mutually limit access to potential trypsin cleavage sites for each domain, probably by interactively favoring correct folding or by masking sites due to homotypic association.

**Ca<sup>2+</sup>-binding Site Mutations at the EC1-EC2 Linker Region Impair Hexamer Formation**—The tridimensional structure of N and E cadherin-derived EC1-EC2 fragments revealed that Ca<sup>2+</sup> promotes dimerization of cadherins (17, 19). It is therefore possible that formation of a dimer represents a step in the VE cadherin assembly pathway. We have investigated the role of Ca<sup>2+</sup>-binding sites of VE cadherin by introducing mutations within the EC1-EC2 inter-region domain.

As indicated by amino acid sequence alignment, most of the amino acids involved in Ca<sup>2+</sup>-binding (17) are conserved among E and VE cadherins and particularly the VE cadherin amino acids Asn<sup>98</sup> and Asp<sup>99</sup> (Fig. 8A). Consequently, two mutations, N98A and D99A, were simultaneously introduced within the Ca<sup>2+</sup>-binding region of the VE cadherin EC1-EC2 inter-domain to generate a new fragment designated as VE-EC1–4m (Fig. 8A).

The capacity of the fragment VE-EC1–4m to self-associate was analyzed by gel filtration chromatography (Fig. 8B). Chromatographic profiles exhibited one major (peak III) and two minor peaks (peaks I and II) indicating that the fragment possesses three different oligomeric states. By comparing the chromatographic profiles of the wild-type to those of the mutated VE-EC1–4m fragment, it could be deduced that the minor peaks I and II correspond to the hexameric and monomeric states, respectively. The major peak corresponds to a species intermediate between a monomer and a hexamer. The hydrodynamic radius of this intermediate species, evaluated from its elution volume to 50.5 Å, allowed the estimation of its molecular mass to be 95 kDa using the standard curve from Fig. 5. This result demonstrated that the intermediate species (peak III) is a dimer (theoretical molecular mass of 98 kDa). We can conclude that the ability of the mutant protein to form a hexamer is greatly impaired by introducing mutations within the EC1-EC2 interdomain. Indeed, at 0.5 μM, 50% of the wild-type fragment VE-EC1–4 is hexameric, whereas for the mutated fragment at 22 μM, the dimer is the preponderant species. Altogether, the results are consistent with the existence of a dimeric intermediate appearing when the fragment VE-EC1–4 is deprived of interactions involving Ca<sup>2+</sup>-binding sites between modules EC1 and EC2.

**DISCUSSION**

Suzuki and co-workers (6) classified VE cadherin as a class II cadherin because the tripeptide HAV, involved in cellular adhesion of classical group I cadherins, is missing within its N-terminal module EC1. Based on low sequence similarity scores, Shimoyama et al. (7) recently attributed VE cadherin to a separate phylogenetic class. To analyze whether this classification can be justified by a specific self-association mechanism, possibly different from that of the classical group I and II cadherins, various recombinant fragments encompassing one

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**Table IV**

MALDI mass spectrometry identification of tryptic peptides from in gel-digested VE cadherin fragments

After SDS-PAGE separation of digested VE cadherin fragments, each protein band was individually excised before trypsin digestion (Fig 7A). From these peptide mixtures, precise molecular mass determination enabled the identification of cleavage sites. Major proteolytic products are shown in bold.

| Fragment   | Electrophoretic band | Mass       | Corresponding peptide          |
|------------|----------------------|------------|--------------------------------|
| VE-EC1–4   | a                    | 48,958     | Met-[Asp<sup>1</sup>–Glu<sup>31</sup>] |
|            | b                    | 34,985     | Met-[Asp<sup>1</sup>–Arg<sup>200</sup>] |
|            | c                    | 13,992     | Ala<sup>31</sup>–Glu<sup>31</sup> |
|            | d                    | 21,472     | Leu<sup>31</sup>–Arg<sup>200</sup> |
| VE-EC1–3   | a                    | 36,296     | Met-[Asp<sup>1</sup>–Glu<sup>21</sup>] |
|            | c                    | 33,350     | Ile<sup>24</sup>–Glu<sup>31</sup> |
|            | d                    | 32,150     | Tyr<sup>35</sup>–Glu<sup>41</sup> |
|            | e                    | 31,065     | Ile<sup>44</sup>–Arg<sup>200</sup> |
|            | f                    | 29,865     | Tyr<sup>35</sup>–Arg<sup>200</sup> |
| VE-EC1–2   | a                    | 27,473     | Met-[Asp<sup>1</sup>–Arg<sup>244</sup>] |
|            | b                    | 27,473     | Met-[Asp<sup>1</sup>–Arg<sup>244</sup>] |
|            | c                    | 24,527     | Ile<sup>24</sup>–Arg<sup>244</sup> |
|            | d                    | 14,934     | Leu<sup>24</sup>–Arg<sup>244</sup> |
|            | e                    | 12,557     | Gly-[Ser–Asp<sup>1</sup>–Arg<sup>107</sup>] |
|            | f                    | 9,611      | Ile<sup>44</sup>–Arg<sup>200</sup> |
| VE-EC1–3   | a                    | 12,557     | Met-[Asp<sup>1</sup>–Arg<sup>107</sup>] |
|            | b                    | 9,611      | Ile<sup>24</sup>–Arg<sup>244</sup> |
|            | c                    | 8,411      | Tyr<sup>35</sup>–Arg<sup>244</sup> |
|            | d                    | 8,245      | Leu<sup>105</sup>–Glu<sup>431</sup> |
| VE-EC2–4   | a                    | 36,816     | Thr<sup>105</sup>–Glu<sup>431</sup> |
|            | b                    | 36,419     | Leu<sup>105</sup>–Glu<sup>431</sup> |
|            | c                    | 28,192     | Asp<sup>184</sup>–Glu<sup>431</sup> |
|            | d                    | 21,442     | Leu<sup>108</sup>–Arg<sup>244</sup> |
|            | e                    | 21,503     | Met<sup>108</sup>–Glu<sup>431</sup> |
|            | f                    | 14,934     | Thr<sup>105</sup>–Glu<sup>431</sup> |
| VE-EC3–4   | a                    | 25,198     | Thr<sup>105</sup>–Glu<sup>431</sup> |
|            | b                    | 20,509     | Gly<sup>205</sup>–Glu<sup>431</sup> |
|            | c                    | 14,966     | Thr<sup>105</sup>–Glu<sup>431</sup> |
|            | d                    | 10,250     | Thr<sup>105</sup>–Arg<sup>300</sup> |
|            | e                    | 5,562      | Gly<sup>205</sup>–Arg<sup>300</sup> |
or several extracellular modules of VE cadherin were recombinantly produced, and their propensity to self-assemble in solution was analyzed.

Limited proteolysis experiments show that single module fragments devoid of their adjacent inter-module regions are unstable most likely because they lack Ca\(^{2+}\)-binding sites that are known to rigidify cadherin molecules (17). Extension by adding inter-module regions at the C or N terminus stabilizes the single module fragments. This correlates with the fact that all multidomain fragments are stable. Although they possess well conserved overall structures, some subtle structural differences are detected around positions Lys\(^{23}\), Lys\(^{34}\), Arg\(^{244}\), Arg\(^{252}\), Arg\(^{300}\), and Arg\(^{309}\) that are digested within some but not all fragments. This may reflect differences in the capacity of the fragments to self-assemble or in the relative orientation of modules as their number increases.

Fragment VE-EC1–4 appears to be the most stable among the VE cadherin fragment series. Cleavage sites at positions Arg\(^{244}/\text{Arg}^{252}\) and Lys\(^{23}/\text{Lys}^{34}\) observed on shorter fragments were not accessible to trypsin on VE-EC1–4, probably because they were masked by its particular multimerization capacity (see below) (29).

VE cadherin single module fragment EC1 is not able to self-associate. This is in good agreement with results obtained for the N-terminal module EC1 of E cadherin, which also remains monomeric in solution (14, 35). By contrast, the single N-terminal fragment of N cadherin self-associates as dimers as shown by x-ray crystallography (15). However, the intermolecular contacts seen in this single domain structure might be artificially induced by crystal packing (36).

Whereas the single module fragment VE-EC1 is not able to self-associate, some of the fragments consisting of two (VE-EC3–4) or three modules (VE-EC2–4) form dimers. Moreover, the fragment overlapping the four N-terminal extracellular modules (VE-EC1–4) was recently demonstrated to associate as a hexamer in solution (29). Thus, a synergy between the extracellular modules of VE cadherin is necessary to build homotypic interactions. This cooperative effect is confirmed by the \(C_{50\%}\) values for which 50% of the fragments are present under their respective oligomeric form. These \(C_{50\%}\) values are 0.5, 25, and 80 \(\mu\)M for the hexameric and dimeric self-associations of the fragments VE-EC1–4, VE-EC2–4, and VE-EC3–4, respectively. It can be deduced that, as the length of the fragment increases, the affinity of self-association increases concomitantly. VE cadherin homotypic interactions therefore require multiple inter-module interactions to elaborate tight cell-cell adhesion. These results are in agreement with those presented in the paper of Chappuis-Flament et al. (37) on C cadherin who demonstrated that homophilic binding and adhesion are mediated by multiple cadherin extracellular repeats.

VE-EC1–2 and VE-EC1–3 do not self-associate. Specific regions necessary for homotypic association of VE cadherin are possibly missing on these fragments. In fact, only fragments containing the EC3-EC4 tandem module such as VE-EC3–4, VE-EC2–4, and VE-EC1–4 are able to self-assemble. We therefore propose that module EC4 and/or the hinge region between modules EC3 and EC4 are required for elaborating VE cadherin multimerization. This is in agreement with the results of Corada et al. (38) who mapped the epitope of a monoclonal anti-VE cadherin antibody able to increase endothelial permeability between amino acids 296 and 301, therefore possibly interfering with the self-assembling process described here.

Whereas VE-EC2–4 is able to form dimers, VE-EC1–4 self-associates as a hexamer. This underlines the particular role of module EC1 in homotypic VE cadherin association, thus confirming results obtained on other cadherin members in particular for E (17) and N cadherins (15, 19).

In contrast to VE cadherin, E (14, 17) and N cadherins (19) dimerize via their EC1-EC2 inter-module region. X-ray structure determination demonstrated that within these dimers, cadherin molecules are not only connected by their EC1-EC2 linker region but also bridged by an arrangement of three calcium ions that are associated with modules EC1 and EC2. Cadherin sequence alignments predicted that calcium is similarly bound to the successive extracellular EC3 and EC4 modules of VE cadherin and thus might play a similar stabilizing role in VE cadherin multimers. In fact, formation of VE cadherin multimers is shown to be \(\text{Ca}^{2+}\)-dependent because, in the presence of EGTA, the VE-EC1–4 hexamer and both the VE-
EC3–4 and VE-EC2–4 dimers dissociate. This is compatible with results of Baumgartner et al. (32) who demonstrated that the extracellular domain of VE cadherin oligomers dissociates into monomers in the absence of Ca\(^{2+}\) in vitro.

Altogether, the results presented here concerned the self-associating behavior of VE cadherin are different from those published previously on type I E (14) and N cadherins (19). For instance, fragments EC1–2 of E and N cadherins form dimers, whereas the VE cadherin equivalent fragment does not self-associate. In contrast, the VE-EC3–4 fragment forms dimers with a C_{50}\% of 80 \(\mu\)m comparable with that of E-EC1–2 suggesting that modules EC3 and EC4 are the basic interacting modules of VE cadherin. This result is confirmed by the fact that the mutated fragment VE-EC1–4m, whose EC1-EC2 hinge region was destabilized by introducing mutations within its Ca\(^{2+}\)-binding domain, forms mainly dimers. This reveals the existence of a transient dimeric intermediate involving interactions between modules EC3 and EC4. Once formed, these dimers can elaborate hexameric structures by establishing new inter-module interactions involving the EC1-EC2 interdomain Ca\(^{2+}\)-binding sites of VE cadherin.

Cryoelectron microscopy images obtained with VE cadherin EC1–4 allowed us to determine the length of the hexameric structure to be 233 ± 10 Å (Fig. 9C) (29), a value compatible with that determined by others (32) for VE cadherin. This value also correlates with the 200–250-Å intercellular gap distance measured on electron micrographs of endothelial cell adherens junctions (39). The hexameric structure possesses a length equivalent to the length of five cadherin modules and therefore probably results from inter-digitation of six molecules of VE cadherin as proposed in Fig. 9, D and E.

Our results cannot distinguish between cis or trans interactions within the hexamer. Nevertheless, as illustrated in Fig. 9, due to symmetry only two orientations of VE cadherin molecules within the hexamer are possible, either the six molecules adopt a parallel orientation (A and B) or they are alternatively arranged in an anti-parallel manner (D and E). Constitution of parallel hexamers would need, as a prerequisite, the formation of parallel intermediate dimers. Consequently, parallel hexamers would have to assemble into anti-parallel multimers to constitute a cell-cell adhesive interface. The lack of any detectable multimers of hexamers for the fragment VE-EC1–5 seems to exclude this possibility. Therefore, we favor models Fig. 9, D and E, where VE cadherin molecules within the hexamer form trans interactions that lead directly to cell-cell binding.

The model for trans-binding proposed in Fig. 9E depicts six molecules of VE cadherin on apposing cells aligned anti-parallel so that extracellular modules EC1 are paired with EC5, EC2 with EC4, and EC3 with EC3. This face-on view of the hexameric VE cadherin structure is simplified for reasons of clarity and does not reflect the overall complexity of the interdigitation of the extracellular modules. Understanding the detailed interface will be possible when the tridimensional structure of the hexamer is solved. Moreover, VE cadherin homotypic binding is influenced by polysialic acids (40) suggesting that the overall mechanism is more complex and may involve additional interactions. Our interaction model is compatible with a model proposed by Sivasankar and co-workers (20, 21) who demonstrated by direct-force measurements that the ectodomain of C cadherin exhibits multiple adhesive contacts involving successive domains along the extracellular region of the protein. The proposed anti-parallel alignment of the six molecules within the hexamer favors trans interactions, a prerequisite for cadherin-mediated cell-cell adhesion (17, 18, 41–44).

Formation of VE cadherin hexameric structures described here may correspond to an early clustering event occurring during the formation of endothelial cell-cell contacts. In fact, these hexamers that probably correspond to the cylinder-shaped particles observed on freeze-etch images of adherens junctions may first act as discrete units (45–47). Binding of catenins to the cytoplasmic domain of VE cadherin molecules may result in indirect connection of these hexameric structures to each other by anchoring them to the actin and/or intermediate filament cytoskeletons. By increasing the local concentration of VE cadherin in the plane of the cellular membrane, these clusters first increase cell adhesion as demonstrated previously for C cadherin (48) and, second, may promote the generation of different intracellular signals as observed for N cadherin (49).
Synergistic Self-assembly of VE Cadherin Extracellular Modules

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