Structure of artificial and natural VE-cadherin-based adherens junctions

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Abstract
In vascular endothelium, adherens junctions between endothelial cells are composed of VE-cadherin (vascular endothelial cadherin), an adhesive receptor that is crucial for the proper assembly of vascular structures and the maintenance of vascular integrity. As a classical cadherin, VE-cadherin links endothelial cells together by homophilic interactions mediated by its extracellular part and associates intracellularly with the actin cytoskeleton via catenins. Although, from structural crystallographic data, a dimeric structure arranged in a trans orientation has emerged as a potential mechanism of cell–cell adhesion, the cadherin organization within adherens junctions remains controversial. Concerning VE-cadherin, its extracellular part possesses the capacity to self-associate in solution as hexamers consisting of three antiparallel cadherin dimers. VE-cadherin-based adherens junctions were reconstituted in vitro by assembly of a VE-cadherin EC (extracellular repeat) 1–EC4 hexamer at the surfaces of liposomes. The artificial adherens junctions revealed by cryoelectron microscopy appear as a two-dimensional self-assembly of hexameric structures. This cadherin organization is reminiscent of that found in native desmosomal junctions. Further structural studies performed on native VE-cadherin junctions would provide a better understanding of the cadherin organization within adherens junctions. Homophilic interactions between cadherins are strengthened intracellularly by connection to the actin cytoskeleton. Recently, we have discovered that annexin 2, an actin-binding protein connects the VE-cadherin–catenin complex to the actin cytoskeleton. This novel link is labile and promotes the endothelial cell switch from a quiescent to an angiogenic state.

Introduction
Blood vessels are lined with a monolayer of cells that form a barrier between blood and underlying tissues. This monolayer, designated as vascular endothelium, plays a central role in regulating the recruitment of leucocytes at sites of injury or inflammation, and in the growth and repair of blood vessels. All of these processes are tightly controlled by adherens junctions formed between endothelial cells. In vascular endothelium, adherens junctions are mainly composed of VE-cadherin (vascular endothelial cadherin), an adhesive receptor that is able to self-associate at endothelial cell–cell contacts as illustrated in Figure 1 [1].

VE-cadherin is a member of a superfamily of Ca2+-dependent adhesive receptors known to bind cells together via homotypic interactions. By promoting clustering of cells with identical phenotypes, cadherins play a crucial role in the elaboration of various tissues during embryogenesis [2] and in the maintenance of adult tissue architecture. VE-cadherin is crucial for the proper assembly of vascular structures and maintenance of vascular integrity [3]. Inactivation of the VE-cadherin gene or truncation of the cytoplasmic domain of VE-cadherin was demonstrated to be embryonically lethal due to vascular insufficiency [4,5]. In adult mice, antibodies directed against the extracellular region of VE-cadherin led to an increase in microvessel permeability or impaired the assembly of new vascular structures caused by disruption of VE-cadherin-mediated cell–cell adhesion or by inhibition of adherens junction formation [6,7].

Basic molecular structure of cadherins
Classical cadherins possess a general modular structure consisting of five ECs (extracellular repeats) of approx. 110 amino acids, designated as EC1–EC5 from the N- to the C-terminus, a transmembrane domain and a well-conserved cytoplasmic tail [8,9]. Whereas the extracellular part of cadherin mediates homophilic interactions, their cytoplasmic regions bind intracellular partners, including catenins (α, β and γ) and p120, which in turn bind to the actin cytoskeleton [10].
Figure 1 | Confocal analysis of VE-cadherin and actin in confluent endothelial cell monolayer (HUVECs)

Cells were plated on to glass coverslips, cultured for 3 days and then stained with polyclonal anti-VE-cadherin antibody, Cy5 (indodicarbocyanine)-labelled goat anti-rabbit antibody (green), FITC-labelled phalloidin (red) and DAPI (4',6-diamidino-2-phenylindole) (blue).

Among the cadherin superfamily, classical cadherins comprise two major subfamilies, termed type I and type II cadherins. Both types have a conserved tryptophan residue (Trp²) at the N-terminus, whereas the type II cadherins, such as VE-cadherin, have an additional conserved tryptophan residue (Trp⁴). Despite this difference, type I and type II EC domains contain seven-stranded barrels (Greek-key sandwiches), revealing an unexpected structural similarity with the immunoglobulin fold [11–14]. The three-dimensional structure of the entire EC domain of C-cadherin (from Xenopus) has revealed an elongated structure containing Ca²⁺-binding sites located between two consecutive EC domains [13]. Fixation of three Ca²⁺ ions per site rigidifies the cadherin molecules, thus reducing their sensitivity to proteolytic degradation.

**Homophilic interactions mediated by the EC domain of cadherins**

A variety of experiments demonstrate that the adhesive binding site of cadherin is primarily localized within the EC1 domain. From crystallographic studies, pairs of molecules are formed through the exchange of an N-terminal β-strand between the partner EC1 domains, named the swapping strand [11,13,14]. For type 1 cadherins, the EC1–EC1 interaction results from the insertion of the conserved Trp³ side chain from swapping the strand of partner 1 into the hydrophobic core of the partner 2 molecule and vice versa. Within the E-cadherin (epithelial cadherin) pocket, the formation of a salt bridge between Asp¹ and Glu⁹⁰, and a hydrogen bond between Trp² and Asp⁹⁰, stabilizes the swapping interaction. The Pro⁵-Pro⁶ motif could contribute to the Trp² exchange [15]. For type II cadherins, the side chains of the critical conserved residues Trp³ and Trp¹ of the swapped strand insert into a large hydrophobic core of the partner molecule [14]. This swapping interaction involving the EC1 domain leads to the formation of antiparallel dimers that could mediate binding between cadherins exposed from opposing cells [13]. In addition to these structural data, the EC1 domain emerges as the determinant area involved in the specific recognition of classical cadherins [14,16].

**VE-cadherin hexamer- and artificial VE-cadherin-based junctions**

Junctional assembly is a complex process which requires various events such as the local concentration of cadherin molecules at the cell surface, the recruitment of both cytoskeleton-binding proteins and -regulating proteins and the reorganization of the cytoskeleton network. All of these processes are intimately interrelated and their individual contribution to the modulation of cell–cell junction strength remains difficult to define. Consequently, to explore the role of the VE-cadherin ectodomain clustering independently of the cellular events mediated by the cytoplasmic VE-cadherin–catenin complex, we have expressed in *Escherichia coli* a fragment overlapping the four N-terminal EC domains of VE-cadherin (EC1–EC4).

We have demonstrated that this fragment possesses the capacity to self-associate as hexamers in solution. Cryoelectron microscopy allowed us to visualize the EC1–EC4 hexamer as a hollow cylindrical structure 23 nm long and 7 nm wide [17,18]. This finding suggests that the hexameric units observed in solution may participate in the elaboration of endothelial adherens junctions. Therefore we performed cryoelectron microscopy studies to provide novel insights into the way endothelial cells adhere to each other at adherens junctions [19]. Thus a soluble VE-cadherin molecule made of the four N-terminal EC domains (EC1–EC4–His) was produced with a polyhistidine extension at its C-terminal end in order to allow its binding to membranes containing lipids functionalized with Ni²⁺-nitrilotriacetate groups and to mimic the natural orientation of cadherin molecules at cell membranes. It was shown that artificial adherens junctions can be reconstituted in vitro, by direct two-dimensional self-assembly of the EC1–EC4–His fragment sandwiched between two membranes (Figures 2A–2D). The molecular architecture of these artificial junctions exhibiting a 23 nm intermembrane distance consists in the juxtaposition of the hexameric units found in solution. It appears to be similar to native desmosomal cadherin-based junctions observed in vivo [20]. This study demonstrated that the minimal information required to build up an artificial adherens junction is contained within the extracellular moiety of cadherin molecules.
Furthermore, this finding underlines the propensity of the extracellular moiety of cadherin to form spontaneous clusters that may influence the adhesion. It was shown previously that the lateral clustering of cadherin mutants lacking the cytoplasmic tail increased the cadherin-mediated adhesive strength [21]. Although clustering mediated by the cadherin cytoplasmic part should contribute to a strong cadherin-based adhesion, self-assembly of the cadherin extracellular part can provide alternative modulations in the adhesive strength.

To determine the molecular architecture of the molecule, we calculated a three-dimensional reconstruction from negatively stained electron microscopy images and produced a homology model based on the known structure of C-cadherin EC1–EC5 (Figure 2E) [21]. The hexamer model consists of a trimer of dimers with each N-terminal EC1 module making an antiparallel dimeric contact, and the EC4 modules forming extensive trimeric interactions. Each EC1–EC4 molecule makes a helical curve that gives some torsional flexibility to the edifice [19,22]. Although there is no direct evidence for the existence of VE-cadherin hexamers at adherens junctions, this model is of particular interest since it is supported by some disparate results for adherens junctions. In particular, it is in agreement with the three-dimensional structure of C-cadherin [13], the trans-dimer at the base of cell–cell adhesion exhibits a 38.5 nm length, too long to fit within the measured intermembrane space. To take into account this geometrical constraint, Boggon et al. [13] proposed that the adhesive trans-dimer adopts a curved structure over the length of the EC domain of C-cadherin. This results in an end-to-end binding distance of 24.5 nm consistent with EM images of adherens junctions. Another model issued from electron tomography of mouse skin plastic sections proposed that adherens junction are composed of discrete groups of 10–20 cadherin molecules interacting via their tips, leading to the formation of trans-dimers [25].

Organization of native cadherin junction

Adherens junctions and desmosomes that densely associated with actin and intermediate filaments respectively are some of the most abundant types of intercellular adhesive junctions. They consist of classical and desmosomal cadherins. Owing to the sequence and organizational similarity between the extracellular domains of classical and desmosomal cadherins, both junctions adopt a similar architecture. As attested by electron-microscopic analyses, cadherin molecules within these adhesive junctions form a lattice that maintains the intermembrane space to a constant thickness of approx. 30 nm for desmosomes [20,24,25].

Although there is a consensus view about the involvement of EC1 in the primary formation of trans-dimers, the organization of the cadherin extracellular part within the intermembrane space remains a matter of debate. In the three-dimensional structure of C-cadherin [13], the trans-dimer at the base of cell–cell adhesion exhibits a 38.5 nm length, too long to fit within the measured intermembrane space. To take into account this geometrical constraint, Boggon et al. [13] proposed that the adhesive trans-dimer adopts a curved structure over the length of the EC domain of C-cadherin. This results in an end-to-end binding distance of 24.5 nm consistent with EM images of adherens junctions. Another model issued from electron tomography of mouse skin plastic sections proposed that adherens junction are composed of discrete groups of 10–20 cadherin molecules interacting via their tips, leading to the formation of trans-dimers [25].

However, the recent observations of desmosome junctions performed on 50-nm-thick human epidermal sections preserved in a hydrated state using the CEMOVIS (cryoelectron microscopy of vitreous sections) technique did not support those models of cadherin assembly [20]. Desmosome junctions exhibited, within the intercellular space, a regular organization of the cadherin molecules made of elongated densities perpendicular to the membrane. This indicates that cadherins are densely packed in the desmosome
junction and are not sparsely arranged. Our results are in agreement with the latter model of cadherin clustering in which cadherin molecules are well ordered and arranged perpendicularly to the cell membrane.

VE-cadherin native junction between HUVECs (human umbilical vein endothelial cells)

In endothelial cell monolayers, treatment with the actin-monomer-sequestering drug latrunculin B induces a depolymerization of the actin cytoskeleton accompanied by a drastic destabilization of cell–cell junctions. The removal of the drug promotes the reformation of the junctions. Altogether, these results indicate that the homophilic interactions mediated by the extracellular part of cadherins must be strengthened by intracellular interactions involving actin cytoskeleton to maintain cell–cell junctions. Intracellularly, cadherins associate with β- or γ-catenin which in turn interact with α-catenin. Until recently, it was commonly admitted that α-catenin binding to β- or γ-catenins promotes connections between cadherin-based complexes and the actin cytoskeleton. However, this concept has been challenged, since it was demonstrated recently that α-catenin cannot simultaneously bind to β-catenin and the actin cytoskeleton [26]. Recently, using proteomic tools, we have discovered new partners within the VE-cadherin-based junctional complex. Among them, the membrane-binding protein annexin 2 is the most abundant. We have observed that the alation of the expression of annexin 2 in HUVECs by siRNA (small interfering RNA) induces a drastic modification of the localization of VE-cadherin at cell–cell junctions. This protein, located in cholesterol rafts, binds both to the actin cytoskeleton and the VE-cadherin-based complex so the complex is docked to cholesterol rafts. These multiple connections prevent the lateral diffusion of the VE-cadherin-based complex, thus strengthening adherens junctions in the ultimate steps of maturation. In fact, the connection of annexin 2 to the junctional VE-cadherin-based complex is very labile and governs, at least in part, the switch between immature and mature states of endothelial cell–cell junctions [27].

Conclusion

Our results support an alternative model of cadherin clustering. In this model, the extracellular part of VE-cadherin is able to elaborate hexameric self-assemblies composed of three trans-dimers, each of them exhibiting a supertwisted structure. Once attached to the membrane, these hexamers pack in close arrays to build large two-dimensional edifices reminiscent of the organization of desmosomal cadherins in native tissues. Furthermore, as is evident by the involvement of the membrane-binding protein annexin 2, a more extended inventory of proteins associated to VE-cadherin-based junctions will allow a more comprehensive understanding of cadherin-mediated cell–cell interaction processes.

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