



Contribution of annexin 2 to the architecture of mature endothelial adherens junctions.

Stéphanie Heyraud, Michel Jaquinod, Claire Durmort, Emilie Dambroise,
Evelyne Concord, Jean Patrick Schaal, Philippe Huber, Danielle
Gulino-Debrac

► To cite this version:

Stéphanie Heyraud, Michel Jaquinod, Claire Durmort, Emilie Dambroise, Evelyne Concord, et al.. Contribution of annexin 2 to the architecture of mature endothelial adherens junctions.: Annexin 2 stabilizes interendothelial junctions. Molecular and Cellular Biology, 2008, 28 (5), pp.1657-68. 10.1128/MCB.00695-07 . inserm-00433358

HAL Id: inserm-00433358

<https://inserm.hal.science/inserm-00433358>

Submitted on 19 Nov 2009

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Contribution of annexin 2 to the architecture of mature endothelial adherens junctions

Running title: **Annexin 2 stabilizes interendothelial junctions**

Stéphanie Heyraud¹, Michel Jaquinod², Claire Durmort³, Emilie Dambroise¹, Evelyne Concord¹, Jean Patrick Schaal⁴, Philippe Huber¹, and Danielle Gulino-Debrac^{1§*}

Key words: VE-cadherin; annexin 2; actin; adherens junctions

¹ Laboratoire de Physiopathologie vasculaire, INSERM U882; ² EDyP, INSERM UM201; ³ LIM, IBS, CNRS UMR 5075; CEA, Direction of Life Sciences, 38054 Grenoble, France; Joseph Fourier University, 38054 Grenoble, France

⁴ Département de Gynécologie Obstétrique, Centre Hospitalier Universitaire Michallon, BP 217, 38043 Grenoble Cedex 09, France

[§] corresponding author: D. Gulino-Debrac, iRTSV, APV, INSERM U882, CEA-Grenoble, 17 rue des Martyrs, 38054 Grenoble Cedex 9, France; email: danielle.gulino@cea.fr

Number of characters:

Title: 75

Condensed title: 86

Abstract: 155 words

Introduction: 906 words; Results: 2466 words; Discussion: 1575 words

Materials and methods: 978 words

Abstract (155 words)

The VE-cadherin-based complex is involved in the maintenance of vascular endothelium integrity. Using immunoprecipitation experiments, we have demonstrated that, in confluent human umbilical vein endothelial cells, the VE-cadherin-based complex interacts with annexin 2 and that annexin 2 translocates from the cytoplasm to the cell-cell contact sites as cell confluence is established. Annexin 2, 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. Moreover, we observed that the down-regulation of annexin 2 by siRNA induces a delocalization of VE-cadherin from adherens junctions and consequently a destabilization of these junctions. Furthermore, our data indicate that the decoupling of the annexin 2/p11 complex from the VE-cadherin-based junction, triggered by Vascular Endothelial Growth Factor treatment, facilitates the switch from a quiescent to an immature state.

1 Introduction

2 Vascular endothelium consists of a monolayer of endothelial cells which lines the whole
3 vascular tree. It forms an active boundary between the bloodstream and the underlying tissues
4 thus controlling the movement of circulating white cells between blood and inflamed tissues. The
5 endothelium is also at the origin of the extension of pre-existing vasculature through formation of
6 neo-vessels, a process named angiogenesis (43). Adherens and tight junctions which hold
7 together endothelial cells modulate leukocyte traffic and angiogenesis. Without diminishing the
8 importance of tight junctions, adherens junctions are particularly crucial in controlling the
9 formation and maintenance of interendothelial adhesion.

10 Endothelial cells express a cell-specific cadherin designated as Vascular Endothelial
11 cadherin (VE-cad) which constitutes the main component of interendothelial adherens junctions
12 (4, 13, 30). This transmembrane adhesive protein plays a crucial role in the maintenance of
13 endothelium integrity and in the modulation of its permeability (3, 20). As for other members of
14 the cadherin receptor family, VE-cad links endothelial cells together by homophilic interactions
15 mediated by its extracellular part and associates intracellularly with β - or γ -catenin in a mutually
16 exclusive fashion and with p120. Despite their sequence similarities, β -/ γ -catenins and p120 bind
17 to distinct sites on the cytoplasmic tail of VE-cad. While β -catenin or γ -catenin links to the distal
18 part of the VE-cad cytoplasmic tail, p120 interacts with the membrane proximal domain. These
19 three catenins also exhibit different biological cellular roles. Whereas p120 stabilizes VE-cad at
20 the plasma membrane (46, 47), β -/ γ -catenins interacts with α -catenin. Afterwards in the
21 manuscript, the complex formed by VE-cad and α -, β -, γ -, p120 catenins is designated as “VE-
22 cad-based complex” or “VE-cad complex”.

23 Until recently, it was commonly admitted that α -catenin binding to β - or γ -catenins
24 promotes connections between the VE-cad-based complex and the actin cytoskeleton, thus
25 strengthening the VE-cad ectodomain-based interactions (8, 35, 38). But this concept was
26 challenged since it was recently demonstrated that α -catenin cannot simultaneously bind to β -
27 catenin and the actin cytoskeleton (48).

28 Although it would be premature to dismiss a role for α -catenin, it is possible that some
29 other actin-binding proteins that bind to the different components of adherens junctions might
30 also be involved in the connection of the VE-cad complex with the actin cytoskeleton. In fact,
31 during the cadherin-mediated cell-cell adhesion, the actin cytoskeleton undergoes a drastic
32 reorganization. Thus, it was recently reported that, upon cadherin liganding, two actin
33 populations with a different spatial distribution are clearly distinguishable at early epithelial cell-

cell contacts (49). The first population is composed of thin circumferential actin bundles and the second is localized at cell-cell junctions. These two actin populations are regulated by distinct mechanisms. Indeed, the actin bundles are formed by reorganizing pre-existing actin filaments and the junctional filaments by *de novo* actin polymerization. To ensure the dynamic coordination between cadherin homophilic liganding and the remodelling of both actin populations, more regulatory proteins are required than assumed previously (33).

To bring some light on the connection existing between the VE-cad-based complex and the actin cytoskeleton, we examined the composition of the junctional VE-cad-based complex extracted from confluent endothelial cell monolayers by combining anti-VE-cad immunoprecipitation (IP) with proteomic tools, thereby identifying novel VE-cad partners (unpublished data). In these experiments, annexin 2 (A2) was identified as one of the most abundant VE-cad partners.

The superfamily of annexins forms a Ca^{2+} -dependent regulated class of proteins able to dock in a reversible manner onto the inner leaflet of the plasma membrane by interacting with acidic phospholipids (19). A2 interacts, *via* its N-terminal domain, with p11 (also called S100A10) to form a heterotetramer in which a central p11 dimer connects two A2 monomers (12, 26, 32). Moreover, A2 exhibits an F-actin binding site localized within the last 9 amino acid residues of its C-terminus (17). A2 is not only able to bind to actin but can also bundle actin filaments (22). It participates in the regulation of membrane organization and more particularly in the assembly of cholesterol rafts (2). These A2 containing- cholesterol rafts are highly dynamic membrane domains that serve as F-actin assembly platforms. The precise role of A2 in the dynamic remodelling of these platforms remains to be elucidated. Nevertheless, Hayes et al. recently demonstrated that A2 regulates actin polymerization by interfering with the barbed ends of growing actin filaments (23).

In the present manuscript, we examined the role played by A2 in the regulation and/or stabilization of endothelial adherens junctions. We have demonstrated by immunoprecipitation that A2 interacts directly with the junctional VE-cad-based complex. In addition, our results prove that A2 is absolutely required to maintain VE-cad at cell-cell junctions. Our data also indicate that A2 docks the VE-cad-based complex to cholesterol rafts and binds actin fibers to the complex as cells reach confluence. Thus, connection of the VE-cad-based complex to both cholesterol rafts and to A2-anchored actin filaments strengthens adherens junctions in the ultimate step of maturation.

Reciprocally, it can be assumed that the opening of adherens junction may result from A2 and VE-cad-based complex decoupling. To test this hypothesis, we evaluated effects of the pro-

1 angiogenic Vascular Endothelial Growth Factor (VEGF) on the A2/VE-cad-based complex
2 organization in HUVECs. We showed that VEGF induced a drastic perturbation of the VE-cad
3 localization, a release of A2 from the plasma membrane to the cytoplasm and a decoupling of A2
4 from the VE-cad-based complex. This dissociation leads to the disconnection of the VE-cad
5 complex from the actin cytoskeleton, an event that may constitute one of the earliest steps in
6 adherens junction destabilization favoring the increase of vascular permeability.

7

Materials and methods

Reagents and antibodies

The monoclonal anti-VE-cad antibody BV9 was from Santa Cruz Biotechnologies. The polyclonal antibody directed against human VE-cad anti-Cad3 was produced as previously described (24). Anti-annexin 2, anti-annexin 1, anti-annexin 5, anti- α -, β -, γ - catenins and anti- PECAM-1 antibody were purchased from BD Biosciences. Anti-N-cadherin antibody and rabbit non-immune IgG were purchased from DakoCytomation. Anti- β -actin, FITC- and TRITC-phalloidin, methyl β cyclodextrin (M β CD) and latrunculin B (La B) were from Sigma-Aldrich. Cy2- or Cy3-goat anti-rabbit antibodies and Cy2- or Cy3-goat anti-mouse antibodies were purchased from Immunotech. Cy5-goat anti-mouse and Cy5-goat anti-rabbit antibodies were provided by Amersham Biosciences. Alexa 488-labelled goat-anti-IgG1, Cy3-labelled goat-anti-IgM were purchased from Molecular Probes. Recombinant VEGF was from PeproTech Inc.

Cell culture

HUVECs were isolated and cultured as previously described (30). Only cells on passage 2 were used.

Immunofluorescence microscopy

After treatment, cells cultured on fibronectin-coated glass coverslips were fixed and permeabilized in methanol 85%, 15 mM PIPES pH 7.7, 2 mM MgCl₂ for 10 min at -20°C and blocked with 0.5% BSA in PBS. After several washing steps in PBS, cells were incubated with primary antibodies and then with the adequate secondary antibodies. The coverslips were mounted onto slides with DAPI-containing mowiol 40-88 (Sigma-Aldrich). Cells were then observed using an Axioplan 2 microscope (Zeiss) equipped with an Achroplan x 50 objective. Images were captured with an AxioCam MR camera using Axiovision software.

When specified, cells were observed on a confocal microscope TCS-SP2 (Leica) with a x63/1.4 objective. For image acquisition (1024x1024, 8bit), Alexa 488, TRITC, Cy5 and DAPI fluorescence were excited and collected sequentially (400 Hz line by line) by using the 488nm wavelength of an argon laser for Alexa 488, the 543nm wavelength of an helium-neon laser for TRITC, the 633nm wavelength of an helium-neon laser for Cy5 excitation and the 405nm wavelength of a photodiode for DAPI. Fluorescence emission was collected from 498 to 540nm for Alexa488, from 573 to 630 nm for TRITC, from 644 to 720nm for Cy5 and from 410 to 460nm for DAPI.

Immunoprecipitation (IP)

Affinity beads were prepared as followed: 60 µg of affinity-purified anti-Cad 3 or 6 µg of anti-A2 antibodies were mixed with 30 µL of a suspension of protein A- or protein G- Sepharose beads respectively (Sigma-Aldrich). After a 2 hour incubation time at 4°C, centrifugation at 200 g during 5 min and several washs in PBS, grafted beads were equilibrated in the lysis buffer L (10 mM PIPES pH 7, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EDTA, 2 mM PMSF, 5 µg/mL leupeptine, 2 µg/mL aprotinine) containing 0.5% IGEPAL CA-630. HUVEC monolayers were washed twice with PBS, lysed in 1 mL of 0.5% IGEPAL-containing buffer L for 20 min at 4°C. After lysate centrifugation at 15 000 g for 10 min, supernatants were incubated overnight at 4°C under continuous mixing with antibody-grafted protein A-/G-Sepharose beads. IPs were each time performed on a 78 cm² cell monolayer. To elute immunoprecipitated proteins, beads were washed 3 times in lysis buffer and then boiled in 50 µL Laemmli buffer for 10 min. Whole elutions were loaded on SDS-PAGE for Western blot analysis.

Cell treatments

After brief washing in PBS, HUVEC monolayers were incubated in culture medium containing either 3.8 mM MβCD for 30 min or 0.5 µM La B for 5 min. To determine whether VE-cad was internalized following La B treatment, La B-treated cells were incubated with trypsin-EDTA at 0.5 g/L for 2 min prior cell lysis and Western blot analysis. HUVECs were starved between 3 and 6h before a 30 min stimulation with either VEGF (50 ng/mL) in serum-free medium.

Subcellular fractionations

To separate cytoplasm from membrane, HUVEC monolayers were lysed in detergent-free buffer L. After centrifugation at 1000 g for 3 min at 4°C to remove unbroken cells, supernatants were collected and recentrifuged at 150 000 g for 30 min at 4°C. The resulting supernatants corresponded to the non-membrane fractions, designated as the cytoplasmic fraction afterwards in the manuscript, and pellets, resuspended in 0.5% IGEPAL-containing buffer L, to the membrane fractions. To isolate cholesterol raft-associated proteins, membrane pellets were resuspended in buffer L containing 0.01% digitonin. After a 20 min incubation on ice, homogenates were centrifuged at 150 000 g for 30 min at 4°C. The resulting supernatants

1 contained cholesterol raft-associated proteins while the pellets contained the other membrane-
2 associated proteins.

4 **LC-MS/MS**

5 Cholesterol raft-associated proteins were separated on 4-12% gradient gel (Criterion™ XT
6 Precast, BioRad) and gels were stained by Coomassie blue (Bio-Safe Coomassie G250 stain,
7 BioRad). The Coomassie-blue stained protein bands were in-gel digested with trypsin and the
8 recovered peptides were separated by liquid chromatography on silica C18 column (Dionex)
9 prior to analysis on an integrated nanoLC-MS/MS system. Peptide identification from the
10 resulting MS/MS dataset was achieved using an in-house MASCOT server (Matrix Sciences,
11 London).

13 **Western blotting**

14 Cell extracts were analyzed under reducing conditions by SDS-PAGE using either 10% Tris-
15 Glycine or 15% Tris-Tricine home-made gels, or 4-12% gradient Criterion™ XT gels and
16 electro-transferred onto a pure nitrocellulose membrane (BioRad). After blocking with 5% non-
17 fat dry milk, proteins were detected by specific primary antibodies and horseradish peroxidase-
18 conjugated goat anti-mouse or anti-rabbit antibodies (Sigma-Aldrich). The immunoreactive
19 bands were revealed using the ECL Western blotting detection kit (Amersham Biosciences).
20 Signals recorded on autoradiography films (Amersham Biosciences) were quantified using a Gel
21 Doc EQ apparatus and Quantity One software (BioRad).

23 **siRNA interference**

24 21-nt siRNA duplex 5'-UCAUCCACACCUUUGGUCUUU-3' and 5'-
25 UCAGCAUCAAGUUAGUAUUU-3' targeting sequences for human A2 were purchased from
26 Dharmacon. HUVECs were plated a day before siRNA transfection in medium without
27 antibiotics. 75 pmoles of siRNA were transfected with Oligofectamin (Invitrogen) according to
28 the manufacturer's instructions. Transfection was performed twice at an interval of 24 hours.
29 Then, siRNA-transfected HUVECs were plated on glass coverslips in complete medium and used
30 for various experiments 24 and 48h after plating. Cells were then fixed at 4 or 5 day post-
31 transfection.

Results

Cell density-dependent localization of annexin 2 to endothelial cell-cell junctions

By combining immunoprecipitation with proteomic studies, we recently determined the protein composition of confluent HUVEC adherens junctions (unpublished data). Numerous actin-binding proteins, not previously thought to localize at adherens junctions, were thus revealed. Among them, A2 was found. Here, we examined the possible role of A2 in the regulation and/or stabilization of VE-cad-based adherens junctions.

First, the subcellular localization of A2 was explored in density-increasing HUVEC monolayers. To do so, the membrane and cytoplasmic fractions were separated from subconfluent and confluent HUVECs by ultracentrifugation and analyzed by Western blot (**Fig 1A**). As expected, in subconfluent as well as in confluent cells, VE-cad was found in the membrane fraction (**Fig 1A**, lanes 2 and 4). The absence of VE-cad in the cytoplasm attested that this fraction was not contaminated by membranes (**Fig 1A**, lanes 1 and 3). On the contrary, it was observed that A2, which was exclusively found in the cytoplasmic fraction in subconfluent cells, distributed between the cytoplasm and the membranes in confluent cells (**Fig 1A**). These results showed that A2 partially translocated from cytoplasm to membranes as cell confluence increased.

To demonstrate A2 translocation, an immunocytochemical localization of A2 was performed on HUVECs plated at different densities. **Fig 1B** shows that A2 is exclusively cytoplasmic in subconfluent cells (Subconfluent, A2, and Magn., magnified image) and accumulates at the plasma membrane as cell confluence increases (Confluent, A2, and Magn., magnified image). To assess whether VE-cad and A2 co-localize at cell-cell contacts, confocal microscopy analysis was performed on 650nm optical sections (**Fig 1C**, larger areas are also shown in supplementary data **Fig S1**). This revealed that VE-cad and A2 effectively co-localized along the whole cell-cell junction (**Fig 1C**, white). Nevertheless, some minor restricted areas exhibit juxtaposed red and green dots, indicating thus that a few amount of A2, even though localized at the plasma membrane, was not associated with the VE-cad-based junction (**Fig 1C**, merge). Moreover, at cell-cell contacts, A2 and VE-cad co-localize with the actin cytoskeleton (**Fig 1B**, Confluent, A2, and Magn., magnified image).

It is notable that the adherens junction architecture changes with increasing cell density. Actually, subconfluent cells possess an elongated shape and adherens junctions appear immature exhibiting a thin, discontinuous and zigzag staining for VE-cad (**Fig 1B**, VE-cad). As the cell density increased, cells adopted a cobblestone morphology and adherens junctions presented an intense linear VE-cad staining (**Fig 1B**, VE-cad). Moreover, phalloidin staining indicated that

parallel actin stress fibers visible in subconfluent HUVECs progressively re-orientated to constitute the actin cortical ring in confluent cells (**Fig 1B**, actin). This indicates that, as cell confluence is established, the actin cytoskeleton undergoes a major remodelling which correlates with the arrival of A2 at cell-cell junctions.

Connection of annexin 2 to the VE-cadherin-based complex

To assess whether A2 is associated with the VE-cad/catenin complex, A2 immunoprecipitates were analyzed by Western blot. As reported in **Fig 2 A**, antibodies directed against VE-cad, p120, α -, β - and γ -catenins recognized bands of 150, 120-100, 110, 93 and 83-80 kDa respectively. Besides VE-cad and catenins, p11, the protein known to form a heterotetrameric complex with A2 at the plasma membrane (**39**), was also detected in the anti-A2 immunoprecipitates (**Fig 2 A**). Conversely, anti-VE-cad immunoprecipitation showed association with A2 only when cells have reached confluence (**Fig 2 B**). Other annexins expressed in endothelial cells such as annexin 1 (A1) and annexin 5 (A5) (**Table 1**), were not detected in anti-VE-cad IPs (**Fig 3 A**). Moreover, VE-cad was detected neither in anti-A1 (**Fig 3 B**) nor anti-A5 (**Fig 3 C**) IPs performed on confluent HUVECs. Altogether, these data indicate that the A2/p11 complex is associated with the VE-cad/catenin complex at adherens junction sites in confluent cells. It is noteworthy that N-cadherin, the other cadherin expressed by endothelial cells, and PECAM1, a transmembrane receptor that localizes at interendothelial cell-cell contacts, were not detected in anti-A2 IPs (**Fig 2A**). Altogether, the results attested that A2 interacts specifically with the VE-cadherin-based complex.

Based on these results, A2 and the VE-cad-based complex may be either directly connected or associated *via* actin filaments. To resolve this question, the actin cytoskeleton was depolymerized in confluent HUVECs with La B (**11**). Once the membrane and cytoplasm fractions were separated from cells either treated or not with La B, the subcellular localization of A2, actin and VE-cad was analyzed by Western blot (**Fig 4 A**). Quantification of the blotted bands indicated that the amount of membrane-associated A2 remained approximately constant before and after La B treatment (**Fig 4 A**). By contrast, a loss of 40% of the amount of membrane-associated actin was observed after La B treatment (**Fig 4 A**). This indicated that actin depolymerization did not induce a release of A2 from membranes. This is in agreement with confocal and phase contrast microscopy analyses performed on untreated and La B-treated HUVECs (**Fig 4 B**). The drug-induced depolymerisation of actin filaments lead to cell retraction and cell-cell junction disruption (**Fig 4 B, PC**). In spite of this drastic treatment, A2 remained co-localized with VE-cad at the cell edge, suggesting that VE-cad and A2 interact without requiring

actin filaments. Furthermore, anti-VE-cad and anti-A2 IP experiments were performed on HUVECs treated or not with La B (**Fig 4 C**) and the blotted bands were quantified (data not shown). Similar amounts of A2 were detected in VE-cad immunoprecipitates isolated from either La B- or non-treated cells. Conversely, in A2 immunoprecipitates, the amount of VE-cad co-immunoprecipitated remained unchanged between treated and untreated cells (**Fig 4 C**). These experiments revealed that, after actin cytoskeleton depolymerization, A2 did not decouple from the VE-cad-based complex. It could be concluded that, in confluent endothelial cells, the association of the A2/p11 complex to the junctional VE-cad-based complex does not require actin filaments.

Contribution of cholesterol raft-associated annexin 2 to the stability of adherens junctions

To evaluate the contribution of A2 in the localization of VE-cad at cell-cell junctions, A2 was knocked-down in HUVECs using a pool of two selected small interfering RNA (siRNA). Practically, one day after the second siRNA transfection, HUVECs were plated on glass coverslips and allowed to grow for additional 24 or 48 h periods of time prior to fixation. Four days after A2 siRNA transfection, immunoblot analysis indicated that 50 to 70% of A2 expression was abrogated while a scrambled siRNA had no effect (**Fig 5 A**). The effect observed with the A2 siRNA was specific since the expression of proteins such as actin, VE-cad and annexins 1 and 5 was not down-regulated following A2 siRNA transfection (**Fig 5 A**).

siRNA transfection induced no cell retraction as attested by phase contrast microscopy (data not shown). The effect of A2 down-regulation on VE-cad localization was then analyzed by immunofluorescence microscopy 24 and 48h after cell seeding on glass coverslips. At 24 or 48 h post seeding time, the immunofluorescence signal for A2 was undetectable in some cells while unaffected in others. This heterogeneity reflected the difficulty to transfect siRNA in HUVECs. As expected, transfection of the scrambled siRNA had no effect on A2 and VE-cad localization. At 24 h post-cell seeding time, A2 siRNA induced a drastic loss of VE-cad at adherens junctions between adjacent transfected cells (**Fig 5 B**). At 48 h post-cell seeding time, VE-cad began to reappear at cell-cell junctions exhibiting a zigzag staining pattern, a characteristic feature of immature junctions. Thus, it could be concluded that A2 down-regulation significantly disturbs the localization of VE-cad at cell-cell junctions.

Knock-down by siRNA affected the whole pool of cellular A2. Consequently, it was difficult to state whether either membrane-associated A2 or cytoplasmic A2 was involved in the observed phenotype. To discriminate between these two possibilities, the cholesterol raft-disrupting drug M β CD (**10**) was used to release A2 from the plasma membrane. Subcellular

1 fractionation combined with immunoblotting analysis indicated that, following M β CD treatment,
2 60% of the membrane-bound A2 was released to the cytoplasm whereas, as expected, in
3 confluent untreated HUVECs, A2 was mainly associated to membranes (**Fig 6 A and B**). 82% of
4 actin remained associated with membranes after M β CD treatment indicating that actin was
5 weakly affected by the drug (**Fig 6 A and B**).

6 As revealed by immunofluorescence microscopy, treatment of confluent HUVECs with
7 M β CD provoked a disruption of adherens junctions leading to the formation of large gaps in the
8 monolayer (**Fig 6 C**). Some cell-cell junctions resisted this treatment possibly due to poor
9 accessibility of M β CD to cholesterol rafts. This cell-cell contact destabilization might result from
10 adherens junction disruption possibly caused by VE-cad internalization or degradation. To
11 determine whether VE-cad internalization took place subsequently to M β CD treatment, HUVECs
12 were trypsinized prior to lysis. This trypsinization was used to remove cell surface-expressed
13 VE-cad molecules while leaving intact internalized ones. Western blot analysis revealed that A2
14 and β -catenin, the loading control, showed no appreciable variation in protein levels (**Fig 7 A**).
15 By contrast, trypsinization degraded the totality of VE-cad in both M β CD-treated and untreated
16 HUVECs attesting that VE-cad remained at the cell surface following M β CD treatment (**Fig 7 A**,
17 lanes 2 and 4). This experiment also revealed that following M β CD, VE-cad did not undergo
18 degradation (**Fig 7 A**, compare lanes 1 and 3). It could be concluded that destabilization of
19 adherens junctions observed subsequently to M β CD treatment was neither due to the loss of
20 membrane-associated VE-cad nor to its degradation.

21 To search for the intracellular event involved in such a phenotype, the VE-cad-based
22 complex was isolated by IP from either M β CD-treated or untreated HUVECs and its composition
23 analyzed by Western blotting (**Fig 7 B**). Anti-VE-cad IP showed that following M β CD treatment,
24 the VE-cad/catenin complex remained intact. On the contrary, a partial release of A2 from the
25 VE-cad-based complex was observed (**Fig 7 B**). Anti-A2 IP confirmed the partial decoupling of
26 A2 from the VE-cad/catenin complex in M β CD-treated cells. Consequently, it could be assumed
27 that the M β CD-induced destabilization of adherens junctions resulted from the release of
28 cholesterol raft associated-A2 and its disconnection from the VE-cad-based complex.

29 Actin was detected in both anti-VE-cad and anti-A2 IP isolated from confluent
30 endothelial cells (**Fig 7 B**). But, after M β CD treatment, actin association was decreased in both
31 VE-cad and A2 immunoprecipitations, therefore A2 exclusion from cholesterol rafts paralleled
32 VE-cad-actin dissociation (**Fig 7 B**). Moreover, a drastic reshaping of actin filaments was
33 observed in M β CD-treated cells by immunofluorescence microscopy (**Fig 7 C**). Indeed, in
34 control untreated cells, cortical actin fibers were parallel to the cell-cell junctions as defined by

A2 and VE-cad labelling. On the contrary, after M β CD treatment, which is known to induce actin fibers re-organization (28), A2 disappeared from most cell-cell contact sites and is so unable to link actin fibers to VE-cad-based complex (Fig 7 C, magnified boxed area). Consequently to M β CD treatment, actin fibers adopted a radial orientation and VE-cad exhibited a zig-zag pattern staining at cell-cell junction reminiscent of the immature junction pattern.

Altogether, these results suggest that by connecting the VE-cad-based complex, A2 is a major actor in the maintenance of endothelial adherens junction integrity.

Partners of cholesterol raft-associated annexin 2 potentially involved in the connexion with VE-cadherin

To determine whether VE-cad and the other proteins constituting the interendothelial adherens junctions were associated with cholesterol rafts, cholesterol-associated proteins were extracted from confluent HUVEC membranes using digitonin, a detergent able to bind exclusively to membrane-linked cholesterol (15). Identification of proteins present within cholesterol rafts was achieved after in-gel digestion by trypsin and analysis of the resulting peptides by mass spectrometry (Table 1 and complete results available on request to the corresponding author). As expected, clathrin and caveolin-1, two markers of cholesterol rafts, were detected thus attesting that the fractionation was correct. Although this protein extraction method enabled the solubilization of transmembrane proteins such as PECAM-1 and integrin β -1, VE-cad was not detected in the cholesterol rafts (Table 1). β -catenin, α -catenin, vinculin and α -actinin were the only proteins belonging to adherens junctions extracted by digitonin treatment (Table 1). Actin, tubulin and cytoskeleton-associated proteins such as actin-related protein 3 were also detected. In addition to A2, A1, A5 and annexin 6 were also found as well as AHNAK and moesin, two proteins known to belong to cholesterol rafts in other cell types (18, 21).

Western blot analysis mostly confirmed most of the proteomic study i.e. the presence of A2, moesin, α -actinin, β -catenin, PECAM-1 and the absence of VE-cad, γ -catenin and p120 in cholesterol rafts (Fig 8). Additionally, the phosphatase SHP2 and the A2 partner p11 were also detected using specific antibodies. The only divergence observed between proteomic and Western blotting analyzes concerned α -catenin that was detected in cholesterol rafts by proteomic study (Table 1) and not by Western blotting (Fig 8). Other relevant proteins not present in the cholesterol raft fraction (lane 4) were found either in the cytoplasmic (lane 1) and total membrane fractions (lane 2) or in the fraction containing all the membrane-associated proteins not solubilized by digitonin (lane 3).

Furthermore, the fractionation method illustrated in **Fig 8** allowed a quantitative comparison of the amounts of proteins present in each fraction. It could be evaluated by Western blot analysis that approximately 70% of membrane-tethered-A2 and -moesin were concentrated in the cholesterol rafts. As expected, VE-cad, α - and γ -catenins appeared essentially present in the membrane fractions (**Fig 8, lanes 2 and 3**) whereas p120 distributed between the membrane and the cytoplasm fractions in a 80:20 ratio.

IPs of cholesterol raft-associated A2 were performed to assess whether, within rafts, the cholesterol-associated proteins interacted with A2. Among the cholesterol raft-associated proteins, only α -actinin, β -catenin, SHP2, actin and p11 appeared to be connected to cholesterol-associated A2 (**Fig 8, lane 5**). Moreover, compared to the raft fraction, the A2 immunoprecipitates were enriched in α -actinin and SHP2 (**Fig 8**, compare lanes 4 and 5). These last proteins constitute partners of A2 potentially able to establish the connection between the VE-cad-based complex and the cholesterol rafts.

Effects of VEGF on A2 localization at cell-cell contacts

VEGF is a cytokine known for its capacity to increase vascular permeability. We examined the localization of VE-cad and A2 in HUVECs in the presence or absence of VEGF. After a 30 min treatment with VEGF, VE-cad staining was greatly disturbed compared to control (**Fig 9 A**). At some cell-cell junction sites (**Fig 9**, VEGF, full arrow heads), VE-cad labelling exhibited a spike pattern reminiscent of what was observed in M β CD-treated cells (**Fig 7 C**). Furthermore, at these particular sites, no A2 was detected at cell-cell contacts. These data suggest that treatment of HUVECs by physiological agonists of vascular permeability such as VEGF may induce the decoupling of the A2/p11 complex from the VE-cad-based junction. To verify this hypothesis, anti-VE-cad IPs were performed on confluent HUVECs treated or not with VEGF (**Fig 9 B**). It appeared that, following VEGF treatment, A2 was totally released from the VE-cad-based complex. Concomitantly, the amount of actin co-immunoprecipitated with VE-cad was drastically reduced. Altogether, these data indicated that VEGF treatment results in a VE-cad-based complex/A2 decoupling leading to a partial disconnection of the VE-cad-based complex from the actin cytoskeleton.

Discussion

Until recently, the VE-cad-based adherens junction was thought to be basically composed of the transmembrane adhesive receptor VE-cad, β -catenin and α -catenin bound directly to actin filaments (13, 36). This model was elaborated only by considering indirect evidence. Recently, it was established that α -catenin cannot bind simultaneously to the cadherin/catenin complex and to actin filaments demolishing the idea that α -catenin constitutes the direct link between the actin cytoskeleton and adherens junctions (48). Nevertheless, the actin cytoskeleton appears to play a crucial role in the regulation of adherens junction stability (33, 44). In fact, the interactions between the VE-cad-based complex and the underlying actin cytoskeleton are very dynamic and probably modulated by several intermediate proteins (45). Obviously, it is of great interest to determine the nature of the proteins involved in this controversial connection. Using proteomic tools, we recently discovered that the VE-cad based complex is associated with several acting-binding proteins. Among them, A2 constituted one of the most abundant proteins. In the present manuscript, we established the role played by A2 in the stabilization of interendothelial adherens junctions.

Firstly, we observed that A2 translocates from the cytoplasm to the plasma membrane and more particularly to cell-cell contact sites as HUVECs become confluent (Fig 1A and 1B). This correlates with the fact that A2 docks to these specific sites when VE-cad-mediated adherens junctions reach maturity. Although the mechanism underlying the A2 targeting to membrane as cell confluence establishes remains to be elucidated, it probably requires the formation of the heterotetramer (A2)₂(p11)₂ (12). Indeed, immunofluorescent labelling of p11 and A2 perfectly overlapped with that of VE-cad at cell-cell junctions (data not shown).

Secondly, using immunoprecipitation experiments, we demonstrated that both A2 and p11 subunits interact with the VE-cad-based complex composed at least of VE-cad, α -, β -, γ -catenins and p120. This interaction is independent of actin filament binding since it is not abolished by drug-induced actin depolymerization treatment (Fig 4). By contrast, no interaction between A2 and N-cadherin, the other cadherin present in endothelium, was detected. Similarly, A2 does not interact with PECAM1, a receptor expressed at endothelial adherens cell-cell junctions. Moreover, among annexins expressed in the endothelium, only A2 possesses the ability to interact with the VE-cad-based complex.

The fact that the A2/p11 complex interacts with the VE-cad complex when cells reach confluence suggests that it may be involved in the maturation of interendothelial adherens junctions. This hypothesis is supported by the fact that abrogation of the expression of A2 in

HUVECs by specific siRNA (**Fig 5 A**) drastically disturbs the localization of VE-cad at cell-cell junctions (**Fig 5 B**).

Furthermore, our experiments performed on confluent endothelial cells pre-treated with M β CD, a cholesterol raft-disrupting drug (**10, 28**), established that approximately 60% of the membrane-tethered pool of A2 are released into the cytoplasm (**Fig 6 B**). This results in a destabilization of adherens junctions with formation of intercellular gaps at some cell-cell contact sites (**Fig 6 C**). This destabilization is neither due to internalization nor degradation of VE-cad as attested by trypsin digestion experiments (**Fig 7A**) but from a delocalization of VE-cad from cell-cell junctions (**Fig 6 C**). siRNA down-regulation of A2 and M β CD-mediated depletion of membrane-associated A2 lead to very similar cellular effects i.e. partial disappearance of VE-cad from cell-cell junctions and a corollary destabilization of adherens junctions. The mechanism by which the disconnection of A2 from the VE-cad complex is induced remains unknown. It can not be excluded that this event may result from the release of signalling proteins usually located in the cholesterol rafts (**36**). This signalling pathway needs further investigations.

As previously observed, the actin cytoskeleton is absolutely needed to maintain the adherens junction architecture (**44**). Nevertheless, the connexion between the VE-cad-based complex and actin fibers remains elusive. Our experiments showed that the M β CD-induced disruption of A2 from the VE-cad complex was accompanied by a partial loss of actin in the immunoprecipitation (**Fig 7C**). Considering furthermore that A2 is known to strongly interact with actin fibers (**23**), we assume that the connexion of the VE-cad-based complex might be in part mediated by A2.

In confluent HUVEC, approximately 70% of membrane-associated A2 are localized in cholesterol rafts (**Fig 8**). Therefore, connection with the A2/p11 complex indirectly anchors the VE-cad complex to cholesterol rafts. Due to its stoichiometry, the AII/p11 complex may dock several VE-cad-based complexes. Using electron microscopy and biochemical studies, we recently established that each VE-cad complex is formed of hexameric structures in which six molecules of VE-cad associate in an anti-parallel manner (**5, 25, 31**). The multiplicity of interactions between the self-associated VE-cad molecules and the A2/p11 complex may lead to the elaboration of a supercomplex embedded in cholesterol rafts. Furthermore, at membrane cholesterol raft sites, the A2/p11 complex mediates in part interactions with the actin cytoskeleton (**17, 34, 37**). Consequently, once connected to the cholesterol raft-anchored pool of A2, the VE-cad-based complexes may be indirectly bound to actin cytoskeleton. We propose a model of VE-cad-actin interaction through A2. In subconfluent endothelial cells, the VE-cad-based complex freely diffuses laterally in the plasma membrane. Simultaneously with the arrival

of A2 at the membrane, the VE-cad-based complex is progressively docked to the A2-containing cholesterol rafts and indirectly linked to the actin cytoskeleton. This entrapment provides a rigid structure that considerably restricts lateral diffusion of the VE-cad-based complex thus strengthening and stabilizing adherens junctions in confluent endothelial cells (**Fig 10**). This correlates with observations made in myoblasts in which N-cadherin lateral diffusion is increased when raft structures are disrupted (**9**).

To determine how the VE-cad-based complex and A2 are inter-connected, the cholesterol raft-associated proteins were isolated by digitonin extraction of cholesterol-bound proteins combined with ultracentrifugation. Identification of these proteins was obtained after their in-gel digestion by trypsin and analysis of the resulting peptides by mass spectrometry (**Table 1 and Fig 8**). Strikingly, VE-cad was not detected within the cholesterol raft-associated protein fraction whereas A2 was found. This suggests that the connection between the VE-cad-based complex and A2 is mediated through intermediate proteins possibly identified as those associated with A2 within cholesterol rafts. Our proteomic analysis reveals that, in cholesterol rafts, A2 interacts with actin, β -catenin, α -actinin, p11 and SHP2 (**Fig 8, lane 5**). This suggests that these last three proteins constitute candidates able to potentially connect A2 to the VE-cad/ β -catenin complex.

Among the identified proteins, the phosphatase SHP2 is an interesting candidate. Indeed, on one hand, it was demonstrated to co-immunoprecipitate with the VE-cad-based complex by selectively interacting with β -catenin in confluent quiescent HUVECs (**40**). This interaction maintains β -catenin under its dephosphorylated form (**6, 27**). On the other hand, immunoprecipitation experiments performed on confluent cow pulmonary aortic endothelial cells revealed that SHP2 co-immunoprecipitates with A2 (**7**). Moreover, tyrosine phosphorylation of SHP2 promotes its dissociation from the VE-cad-based complex when HUVECs were activated by agonists able to increase vascular permeability such as thrombin (**40**). This correlates with a drastic increase in tyrosine-phosphorylation of β -catenin. Consequently, the ability to associate with the VE-cad-based complex in a tyrosine phosphorylation-dependant manner may confer the capacity to regulate the interaction between A2 and the VE-cad-based complex to the cholesterol raft-tethered pool of SHP2.

During the angiogenesis process, endothelial cell-cell junctions become leaky and endothelial cells acquire the capacity to migrate, two events leading to the constitution of new vessels (**41, 43**). The increase of vascular permeability might be correlated with the disconnection of A2 from the VE-cad-based complex. To test this hypothesis, we submitted confluent HUVEC monolayers to VEGF, a cytokine able to induce *in vivo* and *in vitro* angiogenesis processes (**14, 29**). Following this treatment, the junctional localization of A2 was

1 dramatically disturbed (**Fig 9 A**). In fact, we showed that A2 and actin are decoupled from the
2 VE-cad-based complex subsequently to VEGF treatment (**Fig 9 B**). This suggests that the VEGF-
3 induced increase in vascular permeability might be a consequence of the disconnection of the
4 actin cytoskeleton from the VE-cad-based complex.

5 It could be assumed that, consecutively to M β CD or VEGF treatments, VE-cad and the
6 catenins become phosphorylated (**16**) either by protein kinases such as Src (**1, 42**) or by the loss
7 of SHP2 from the VE-cad-based complex thus initiating the junction disabling. Indeed, results in
8 the literature indicated that membrane cholesterol depletion from confluent endothelial cells with
9 M β CD induces the phosphorylation of p120 and γ -catenin (**10**). In both cases, phosphorylation of
10 the adherens junction components is induced by loss of SHP2 or destabilisation of cholesterol
11 rafts, two phenomena that implicate the plasma membrane-associated pool of A2. Whether the
12 VE-cad/A2 disconnection is linked to the phosphorylation status of VE-cad-based complex
13 remains to be explored.

14 In the present manuscript, we provide evidence for the existence of a novel mechanism
15 able to promote the maturation of the interendothelial adherens junctions. By connecting the VE-
16 cad-based complex to the cholesterol raft-tethered pool of A2, a stabilization of adherens
17 interendothelial junctions occurs allowing the switch from an immature to a mature state.

Footnotes

Abbreviations used in this paper: A1: annexin 1; A2: annexin 2; A5: annexin 5; HUVEC: Human Umbilical Vein Endothelial Cell; IP: immunoprecipitation; latrunculin B: La B; Methyl- β -cyclodextrin: M β CD; siRNA: small interfering RNA; VE-cad; Vascular Endothelial-cadherin; VEGF: Vascular Endothelial Growth Factor; mAb: monoclonal antibody; pAb: polyclonal antibody.

Acknowledgments

We are indebted to staff of the maternity hospital from Hôpital Nord (Grenoble, France) for kindly collecting umbilical cords for these experiments. We thank Wallez Y. and Mannic T. for their help in VEGF use, Hewat E. for critical reading of the manuscript, and D. Grunwald for taking all confocal microscopy images.

S. H. is a recipient of a fellowship from Association de Recherche sur la Polyarthrite. This work was supported by grants from CNRS (ACI PCV and DRAB), Association pour la Recherche sur le Cancer (ARC N°4447 and 3775) and Alliance des Recherche sur le Cancer (ARECA-ARC).

References

1. **Angelini, D. J., S. W. Hyun, D. N. Grigoryev, P. Garg, P. Gong, I. S. Singh, A. Passaniti, J. D. Hasday, and S. E. Goldblum.** 2006. TNF{alpha} Increases Tyrosine Phosphorylation of Vascular Endothelial-Cadherin and Opens the Paracellular Pathway Through Fyn Activation in Human Lung Endothelia. *Am J Physiol Lung Cell Mol Physiol*.
2. **Babiychuk, E. B., and A. Draeger.** 2000. Annexins in cell membrane dynamics. Ca(2+)-regulated association of lipid microdomains. *J Cell Biol* **150**:1113-24.
3. **Bazzoni, G., and E. Dejana.** 2004. Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. *Physiol Rev* **84**:869-901.
4. **Bazzoni, G., E. Dejana, and M. G. Lampugnani.** Endothelial adhesion molecules in the development of the vascular tree: the garden of forking paths [In Process Citation].
5. **Bibert, S., M. Jaquinod, E. Concord, C. Ebel, E. Hewat, C. Vanbelle, P. Legrand, M. Weidenhaupt, T. Vernet, and D. Gulino-Debrac.** 2002. Synergy between extracellular modules of vascular endothelial cadherin promotes homotypic hexameric interactions. *J Biol Chem* **277**:12790-801.
6. **Biswas, P., J. Zhang, J. D. Schoenfeld, D. Schoenfeld, D. Gratzinger, S. Canosa, and J. A. Madri.** 2005. Identification of the regions of PECAM-1 involved in beta- and gamma-catenin associations. *Biochem Biophys Res Commun* **329**:1225-33.
7. **Burkart, A., B. Samii, S. Corvera, and H. S. Shpetner.** 2003. Regulation of the SHP-2 tyrosine phosphatase by a novel cholesterol- and cell confluence-dependent mechanism. *J Biol Chem* **278**:18360-7.
8. **Butz, S., J. Stappert, H. Weissig, and R. Kemler.** 1992. Plakoglobin and beta-catenin: distinct but closely related [letter]. *Science* **257**:1142-4.
9. **Causeret, M., N. Taulet, F. Comunale, C. Favard, and C. Gauthier-Rouviere.** 2005. N-cadherin association with lipid rafts regulates its dynamic assembly at cell-cell junctions in C2C12 myoblasts. *Mol Biol Cell* **16**:2168-80.
10. **Corvera, S., C. DiBonaventura, and H. S. Shpetner.** 2000. Cell confluence-dependent remodeling of endothelial membranes mediated by cholesterol. *J Biol Chem* **275**:31414-21.
11. **Coue, M., S. L. Brenner, I. Spector, and E. D. Korn.** 1987. Inhibition of actin polymerization by latrunculin A. *FEBS Lett* **213**:316-8.
12. **De Seranno, S., C. Benaud, N. Assard, S. Khediri, V. Gerke, J. Baudier, and C. Delphin.** 2006. Identification of an AHNAK binding motif specific for the Annexin2/S100A10 tetramer. *J Biol Chem* **281**:35030-8.
13. **Dejana, E.** 2004. ENDOTHELIAL CELL-CELL JUNCTIONS: HAPPY TOGETHER. *Nature Reviews Molecular Cell Biology* **5**:261-270.
14. **Dejana, E., R. Spagnuolo, and G. Bazzoni.** 2001. Interendothelial junctions and their role in the control of angiogenesis, vascular permeability and leukocyte transmigration. *Thromb Haemost* **86**:308-15.
15. **Elias, P. M., J. Goerke, D. S. Friend, and B. E. Brown.** 1978. Freeze-fracture identification of sterol-digitonin complexes in cell and liposome membranes. *J Cell Biol* **78**:577-96.
16. **Esser, S., M. G. Lampugnani, M. Corada, E. Dejana, and W. Risau.** 1998. Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells [In Process Citation]. *J Cell Sci* **111**:1853-65.
17. **Filipenko, N. R., and D. M. Waisman.** 2001. The C terminus of annexin II mediates binding to F-actin. *J Biol Chem* **276**:5310-5.

18. **Foster, L. J., C. L. De Hoog, and M. Mann.** 2003. Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. *Proc Natl Acad Sci U S A* **100**:5813-8.
19. **Gerke, V., C. E. Creutz, and S. E. Moss.** 2005. Annexins: linking Ca²⁺ signalling to membrane dynamics. *Nat Rev Mol Cell Biol* **6**:449-61.
20. **Gulino, D., E. Delachanal, E. Concord, Y. Genoux, B. Morand, M. O. Valiron, E. Sulpice, R. Scaife, M. Alemany, and T. Vernet.** 1998. Alteration of endothelial cell monolayer integrity triggers resynthesis of vascular endothelium cadherin. *J Biol Chem* **273**:29786-93.
21. **Harder, T., R. Kellner, R. G. Parton, and J. Gruenberg.** 1997. Specific release of membrane-bound annexin II and cortical cytoskeletal elements by sequestration of membrane cholesterol. *Mol Biol Cell* **8**:533-45.
22. **Hayes, M. J., U. Rescher, V. Gerke, and S. E. Moss.** 2004. Annexin-actin interactions. *Traffic* **5**:571-6.
23. **Hayes, M. J., D. Shao, M. Bailly, and S. E. Moss.** 2006. Regulation of actin dynamics by annexin 2. *Embo J*.
24. **Hermant, B., S. Bibert, E. Concord, B. Dublet, M. Weidenhaupt, T. Vernet, and D. Gulino-Debrac.** 2003. Identification of proteases involved in the proteolysis of vascular endothelium cadherin during neutrophil transmigration. *J Biol Chem* **278**:14002-12.
25. **Hewat, E. A., C. Durmort, L. Jacquamet, E. Concord, and D. Gulino-Debrac.** 2007. Architecture of the VE-cadherin Hexamer. *J Mol Biol* **365**:744-51.
26. **Hubaishy, I., P. G. Jones, J. Bjorge, C. Bellagamba, S. Fitzpatrick, D. J. Fujita, and D. M. Waisman.** 1995. Modulation of annexin II tetramer by tyrosine phosphorylation. *Biochemistry* **34**:14527-34.
27. **Ilan, N., and J. A. Madri.** 2003. PECAM-1: old friend, new partners. *Curr Opin Cell Biol* **15**:515-24.
28. **Kwik, J., S. Boyle, D. Fooksman, L. Margolis, M. P. Sheetz, and M. Edidin.** 2003. Membrane cholesterol, lateral mobility, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin. *Proc Natl Acad Sci U S A* **100**:13964-9.
29. **Lambeng, N., Y. Wallez, C. Rampon, F. Cand, G. Christe, D. Gulino-Debrac, I. Vilgrain, and P. Huber.** 2005. Vascular endothelial-cadherin tyrosine phosphorylation in angiogenic and quiescent adult tissues. *Circ Res* **96**:384-91.
30. **Lampugnani, M. G., M. Resnati, M. Raiteri, R. Pigott, A. Pisacane, G. Houen, L. P. Ruco, and E. Dejana.** 1992. A novel endothelial-specific membrane protein is a marker of cell-cell contacts. *J Cell Biol* **118**:1511-22.
31. **Legrand, P., S. Bibert, M. Jaquinod, C. Ebel, E. Hewat, F. Vincent, C. Vanbelle, E. Concord, T. Vernet, and D. Gulino.** 2001. Self-assembly of the vascular endothelial cadherin ectodomain in a Ca²⁺-dependent hexameric structure. *J Biol Chem* **276**:3581-8.
32. **Lewit-Bentley, A., S. Rety, J. Sopkova-de Oliveira Santos, and V. Gerke.** 2000. S100-annexin complexes: some insights from structural studies. *Cell Biol Int* **24**:799-802.
33. **Mege, R. M., J. Gavard, and M. Lambert.** 2006. Regulation of cell-cell junctions by the cytoskeleton. *Curr Opin Cell Biol* **18**:541-8.
34. **Oliferenko, S., K. Paiha, T. Harder, V. Gerke, C. Schwarzler, H. Schwarz, H. Beug, U. Gunthert, and L. A. Huber.** 1999. Analysis of CD44-containing lipid rafts: Recruitment of annexin II and stabilization by the actin cytoskeleton. *J Cell Biol* **146**:843-54.
35. **Ozawa, M., H. Baribault, and R. Kemler.** 1989. The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. *EMBO J* **8**:1711-7.

36. **Pokutta, S., and W. I. Weis.** 2000. Structure of the dimerization and beta-catenin-binding region of alpha-catenin. *Mol Cell* **5**:533-43.
37. **Rescher, U., D. Ruhe, C. Ludwig, N. Zobiack, and V. Gerke.** 2004. Annexin 2 is a phosphatidylinositol (4,5)-bisphosphate binding protein recruited to actin assembly sites at cellular membranes. *J Cell Sci* **117**:3473-80.
38. **Rimm, D. L., E. R. Koslov, P. Kebriai, C. D. Cianci, and J. S. Morrow.** 1995. Alpha 1(E)-catenin is an actin-binding and -bundling protein mediating the attachment of F-actin to the membrane adhesion complex. *Proc Natl Acad Sci U S A* **92**:8813-7.
39. **Schafer, B. W., and C. W. Heizmann.** 1996. The S100 family of EF-hand calcium-binding proteins: functions and pathology. *Trends Biochem Sci* **21**:134-40.
40. **Ukropec, J. A., M. K. Hollinger, S. M. Salva, and M. J. Woolkalis.** 2000. SHP2 association with VE-cadherin complexes in human endothelial cells is regulated by thrombin. *J Biol Chem* **275**:5983-6.
41. **Vila, E., and M. Salaices.** 2005. Cytokines and vascular reactivity in resistance arteries. *Am J Physiol Heart Circ Physiol* **288**:H1016-21.
42. **Wallez, Y., F. Cand, F. Cruzalegui, C. Wernstedt, S. Souchelnytskyi, I. Vilgrain, and P. Huber.** 2006. Src kinase phosphorylates vascular endothelial-cadherin in response to vascular endothelial growth factor: identification of tyrosine 685 as the unique target site. *Oncogene*.
43. **Wallez, Y., I. Vilgrain, and P. Huber.** 2006. Angiogenesis: The VE-Cadherin Switch. *Trends Cardiovasc Med* **16**:55-9.
44. **Waschke, J., F. E. Curry, R. H. Adamson, and D. Drenckhahn.** 2005. Regulation of actin dynamics is critical for endothelial barrier functions. *Am J Physiol Heart Circ Physiol* **288**:H1296-305.
45. **Weis, W. I., and W. J. Nelson.** 2006. Re-solving the cadherin-catenin-actin conundrum. *J Biol Chem* **281**:35593-7.
46. **Xiao, K., D. F. Allison, M. D. Kottke, S. Summers, G. P. Sorescu, V. Faundez, and A. P. Kowalczyk.** 2003. Mechanisms of VE-cadherin processing and degradation in microvascular endothelial cells. *J Biol Chem* **278**:19199-208.
47. **Xiao, K., J. Garner, K. M. Buckley, P. A. Vincent, C. M. Chiasson, E. Dejana, V. Faundez, and A. P. Kowalczyk.** 2005. p120-Catenin regulates clathrin-dependent endocytosis of VE-cadherin. *Mol Biol Cell* **16**:5141-51.
48. **Yamada, S., S. Pokutta, F. Drees, W. I. Weis, and W. J. Nelson.** 2005. Deconstructing the cadherin-catenin-actin complex. *Cell* **123**:889-901.
49. **Zhang, J., M. Betson, J. Erasmus, K. Zeikos, M. Bailly, L. P. Cramer, and V. M. Braga.** 2005. Actin at cell-cell junctions is composed of two dynamic and functional populations. *J Cell Sci* **118**:5549-62.

Table and figure legends

Table 1: Examples of proteins identified in cholesterol rafts

(Cholesterol raft-associated proteins, isolated as previously described in Materials and Methods, were separated by electrophoresis prior to be in-gel digested by trypsin. Identification of the trypsin peptides was achieved by MS/MS mass spectrometry.)

a: Only proteins relevant for this particular study are listed. Complete results are available on request to the corresponding author

Fig 1: Cell density-dependent accumulation of A2 at cell-cell junctions

(A): Redistribution of A2 to membranes in confluent HUVECs. Subcellular fractionation of subconfluent (lanes 1 and 2) and confluent (lanes 3 and 4) HUVECs was performed as described in Materials and Methods. Proteins isolated from cytoplasm (lanes 1 and 3) and membrane (lanes 2 and 4) fractions were analyzed on 10% Tris-Glycine gels prior to immunoblotting for VE-cad, and A2. Molecular weight markers (kDa) are given at the left margin of each blot.

(B): Subcellular distribution of A2, VE-cad and actin. HUVECs were plated on glass coverslips at two different densities and cultured for three days. Density of confluent cells (Confluent) was 8 times higher than that of subconfluent cells (Subconfluent). Cells were then stained with mAb anti-A2, pAb anti-Cad3, Cy5-labelled goat-anti-mouse and Cy3-labelled goat-anti-rabbit antibodies, FITC-labelled phalloidin, and DAPI. Right panels are magnified images of the areas boxed in left panels (Magn.). Bar: 20 μ m.

(C): Confocal analysis of A2 and VE-cad in confluent HUVECS. Cells were plated on glass coverslips, cultured for three days, and then stained with mAb anti-A2, pAb anti-Cad3, Alexa 488-labelled goat-anti-mouse and Cy5-labelled goat-anti-rabbit antibodies. Co-localization was determined with the “CF2D” software from Leica. Images correspond to an optical section (\approx 650nm). The diagram (Co-localization) represents the relative intensity of the pixels in the red and the green images. Dots gated in the region of interest (yellow), corresponding to the co-localization, are represented in white in the images. Bar: 20 μ m.

Fig 2: Association of annexin 2 to the VE-cad-based complex

(A): Anti-A2 IPs (IP A2), performed on confluent HUVEC, were resolved on 10% Tris-Glycine gels, electro-transferred and probed for VE-cad, N-cadherin (N-cad), PECAM-1, p120, α -, β -, γ -catenins (α -, β -, γ -cat) and actin and on 15% Tris-Tricine gels and probed for A2 and p11. For comparison, aliquots of whole cell lysates (Lys) were analyzed in parallel.

(B): Anti-VE-cad IPs were performed on subconfluent (-) and confluent (+) HUVEC and resolved on 4-12% gradient precast CriterionTM XT gels. As negative controls, whole cell lysate (Lys) and IPs performed on confluent HUVEC lysate using rabbit non-immune IgG (NI) were analyzed in parallel. Parallel blots were probed for VE-cad and A2. Molecular weight markers (kDa) are given at the left margin of each panel.

Fig 3: Specificity of the association between the VE-cad-based complex and the endothelial annexins

Anti-VE-cad (A), anti-A1 (B) and anti-A5 (C) IPs were performed on confluent HUVEC and were resolved on 12% Tris-Glycine gels. Anti-VE-cad IPs were immunoblotted for VE-cad and annexins 1, 2 and 5. Anti-A1 and anti-A5 IPs were probed for VE-cad and either A1 or A5 respectively. For comparison, aliquots of whole cell lysates (Lys) were analyzed in parallel. Molecular weight markers (kDa) are given at the left margin of each blot.

Fig 4: No requirement of actin filaments in the connection between A2 and the VE-cad-based complex

(A): Confluent HUVECs were treated or not with La B for 5 min. Membrane (M) and cytoplasm (C) fractions were separated from La B- treated (+) or untreated (-) cells. Proteins isolated from M and C fractions were analyzed on 10% Tris-Glycine gels and immunoblotted for VE-cad, actin, and A2. Molecular weight markers (kDa) are given at the left margin of the blot. The amounts of A2 and actin associated to membrane were quantified after scanning of immunoreactive bands (n=3 ± standard error). The histograms represent the mean percentages of A2 (grey bar) and actin (black bar) in treated compared to untreated cells (100%, white bars).

(B): Confocal microscopy analysis of VE-cad, A2 and actin in HUVEC before (Control) and after La B treatment. Cells plated on glass coverslips were stained with pAb Anti-Cad3, mAb anti-A2, Cy2-labelled goat-anti-rabbit antibodies, Cy5-labelled goat-anti-mouse, TRITC-labelled phalloidin and DAPI. Images correspond to a ≈ 650nm confocal optical section. The efficiency of La B treatment was verified by phase contrast (PC). In the merging panels, arrow heads point out areas where VE-cad and A2 perfectly co-localize. Bar: 20 μm.

(C): Anti-VE-cad and anti-A2 IPs were performed on lysates extracted from confluent La B-treated (+) or untreated (-) HUVECs. Anti-VE-cad and anti-A2 IPs were separated on 4-12% gradient precast Criterion XT and 10% Tris-Glycine gels respectively and immunoblotted for VE-cad and A2. Molecular weight markers (kDa) are given at the left margin of the blot.

Fig 5: Delocalization of VE-cad from adherens junctions induced by siRNA-mediated A2 down-regulation

(A): siRNA were used to knock-down A2 level in HUVECs. Western-blot analysis was performed to determine VE-cad, actin, A1, A2 and A5 levels in HUVECs transfected with control siRNA (lane 1) or A2 siRNA (lane 2). Lysates were resolved on 10% Tris-Glycine gels and probed for VE-cad, actin, A1, A2, and A5. Molecular weight markers (kDa) are given at the left margin of the blot.

(B): One day after the second siRNA transfection, HUVECs were plated on glass coverslips, cultured for 24h (upper panel, 4 days after transfection) or 48h (lower panel, 5 days after transfection) prior fixation. Cells were then labelled with mAb anti-A2, pAb anti-Cad3, Cy3-labelled goat-anti-mouse and Cy2-labelled goat-anti-rabbit antibodies, and DAPI. Empty arrow heads indicate adherens junctions where A2 and VE-cad co-localized and full arrow heads areas where adherens junctions are missing. Bar: 20 μ m.

Fig 6: Disruption of adherens junctions induced by M β CD treatment

(A): Confluent HUVECs were treated with M β CD (+) for 30 min or untreated (-). Membrane (M) and cytoplasm (C) fractions were separated from M β CD-treated (+) or untreated (-) cells. Proteins isolated from M and C fractions were analyzed on 10% Tris-Glycine gels and probed for VE-cad, actin, and A2. Molecular weight markers (kDa) are given at the left margin of the blot.

(B): Quantification of the membrane-associated-A2 and -actin after scanning of immunoreactive bands in **(A)** (n=3 \pm standard error). To consider any variation in loading, the actin and A2 amounts of were normalized to the corresponding VE-cad immunoreactive bands of the same lanes. The histograms represent the percentages of A2 (grey bar) and actin (black bar) in treated compared to untreated cells (100%, white bars).

(C): HUVECs plated on glass coverslips were treated with M β CD for 30 min or not (Control). VE-cad, A2 and nuclei were labelled as previously described in **Fig 5 B**. Asterisks indicate gaps in the cell monolayers. Bar: 20 μ m.

Fig 7: Decoupling of A2 from the VE-cad-complex induced by M β CD treatment

(A): Confluent HUVECs were treated with M β CD for 30 min (lanes 3 and 4) or not (lanes 1 and 2) and then submitted (lanes 2 and 4) or not (lanes 1 and 3) to trypsin digestion for 2 min prior lysis. Cell lysates were separated on 4-12% gradient precast CriterionTM XT gels and immunoblotted for VE-cad, β -catenin (β -cat), and A2. Molecular weight markers (kDa) are given at the left margin of the blot.

(B): Anti-VE-cad and anti-A2 immunoprecipitations were performed on confluent HUVECs treated for 30 min with M β CD (+) or untreated (-). Immunoprecipitates were separated on 10% Tris-Glycine gels and probed for VE-cad, α -, β -, γ -catenins (α -, β -, γ -cat), actin and A2. Molecular weight markers (kDa) are given at the left margin of the blot.

(C): HUVECs were plated on glass coverslips, treated with M β CD for 30 min (M β CD) or untreated (Control), and stained for pAb anti-Cad 3, mAb anti-A2, Cy2-labelled goat-anti-rabbit and Cy3-labelled goat-anti-mouse antibodies, FITC-labelled phalloidin, and DAPI. Right panels are magnified images of the areas boxed in left panels (Magn.). Bar: 20 μ m.

Fig 8: Identification of the proteins associated with cholesterol rafts

Subcellular fractionations performed on confluent HUVECs allowed the isolation of proteins contained in the cytoplasm (lane 1), in the total membranes (lane 2), in the digitonin-extracted cholesterol rafts (lane 4) and in the digitonin-insoluble membranes (lane 3). Proteins of each fraction were separated on 4-12% gradient precast Criterion XT gels and probed for A2, VE-cad, α -, γ -, β -catenins (α -, β -, γ -cat), p120, moesin, α -actinin, actin, SHP2 and PECAM-1. Samples of each fraction were also resolved on 15% Tris-Tricine gels and probed for p11. Anti-A2 immunoprecipitations were performed on the digitonin-extracted cholesterol raft fraction (lane5). After separation on 4-12% gradient precast Criterion XT gels, immunoprecipitates were probed for A2, VE-cad, α -, γ -, β -catenins (α -, β -, γ -cat), p120, moesin, α -actinin, actin, SHP2, and PECAM-1. Immunoprecipitations were also resolved on 15% Tris-Tricine gels and probed for p11. Proteins unambiguously identified were labelled with arrows. Molecular weight markers (kDa) are given at the left margin of the blots.

Fig 9: VEGF- induced delocalization of A2 and VE-cad from adherens junctions

(A): HUVECs plated on glass coverslips were treated with VEGF or not treated (Control). VE-cad, A2 and nuclei were labelled as previously described in **Fig 4 B**. Empty arrows pointed out adherens junctions where A2 and VE-cad co-localized and full arrows missing or perturbed junctions. Bar: 20 μ m.

(B): Anti-VE-cad IPs were performed on confluent HUVECs treated for 30 min with VEGF (+) or untreated (-). Immunoprecipitates were separated on 10% Tris-Glycine gels and probed for VE-cad, β - catenins (β -cat), actin and A2. Molecular weight markers (kDa) are given at the left margin of the blot. For comparison, aliquots of whole cell lysates (Lys) were analyzed in parallel.

Fig 10: Potential mechanism of stabilization of VE-cad-mediated adherens junctions

1 In subconfluent HUVECs, adherens junctions are immature and poorly associated with the actin
2 cytoskeleton. The VE-cad-based complex is free to move laterally in the plasma membrane.
3 Once HUVECs reach confluence and adherens junctions become mature, the A2/p11 complex,
4 which accumulates underneath the plasma membrane at cholesterol raft sites (CR), associates
5 with the VE-cad-based complex. Consequently, the complex is docked to cholesterol rafts. Thus,
6 by providing a link between the VE-cad/catenin complex and the actin cytoskeleton, the
7 cholesterol raft-embedded A2 stabilizes the adherens junctions, thereby allowing the
8 development of stable intercellular connections.

9 **Supplementary data**

11 **Fig S1: Co-localization of A2 and VE-cad in confluent HUVECS**

12 Cells were plated on glass coverslips, cultured for three days, and then stained with mAb anti-
13 A2, pAb anti-Cad3, Alexa 488-labelled goat-anti-mouse and Cy5-labelled goat-anti-rabbit
14 antibodies. Nuclei were counterstained with DAPI (blue). Images correspond to a $\approx 650\text{nm}$
15 confocal optical section. Bar: 20 μm .

Table 1:

Description ^a	Accession	pI	Score	Mass	Coverage	# peptides
Cytoplasmic proteins						
actin, alpha	gi 178027	5,24	328,07	42081	22,28	6
actin, beta	gi 4501885	5,29	757,83	41710	37,33	10
actinin, alpha 1	gi 4501891	5,25	423,84	102993	9,19	6
actinin, alpha 4	gi 2804273	5,27	621,67	102204	13,01	8
actin-related protein 3	gi 27806335	5,61	32,64	47341	1,91	1
AHNAK nucleoprotein isoform 1	gi 61743954	5,80	180,05	628699	1,04	4
annexin 1	gi 442631	7,77	311,53	35018	24,52	5
annexin 2, isoform 2	gi 12655075	7,57	274,61	38580	22,12	5
annexin 5	gi 809190	4,94	104,72	35783	8,46	2
annexin 6, isoform 2	gi 71773415	5,46	81,98	75229	4,35	2
catenin, alpha	gi 1092190	6,06	47,65	100018	1,32	1
catenin, beta 1	gi 4503131	5,53	258,73	85442	9,48	4
moesin	gi 4505257	6,08	717,50	67778	26,34	11
tubulin, alpha	gi 340021	4,94	318,10	50120	12,86	4
tubulin, class I beta	gi 4580988	4,78	327,73	49639	17,57	6
vinculin isoform VCL	gi 4507877	5,83	544,83	116649	10,60	10
Membrane proteins						
PECAM-1	gi 129747	6,55	91,59	82484	3,25	2
integrin beta 1 (isoform 1B)	gi 19743815	5,35	62,12	87389	2,15	1
Raft markers						
clathrin heavy chain 1	gi 4758012	5,48	191,18	191493	2,87	4
caveolin 1	gi 4972627	5,85	46,24	13066	8,85	1