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Driving Rho GTPase activity in endothelial cells regulates barrier integrity

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Abstract

In the past decade understanding of the role of the Rho GTPases RhoA, Rac1 and Cdc42 has been developed from regulatory proteins that regulate specific actin cytoskeletal structures - stress fibers, lamellipodia and filopodia - to complex integrators of cytoskeletal structures that can exert multiple functions depending on the cellular context. Fundamental to these functions are three-dimensional complexes between the individual Rho GTPases, their specific activators and inhibitors, which greatly outnumber the Rho GTPases themselves, and additional regulatory proteins. By this complexity of regulation different vasoactive mediators can induce various cytoskeletal structures that enable the endothelial cell to respond adequately. In this review we have focused on this complexity and the consequences of Rho GTPase regulation for endothelial barrier function. The permeability inducers thrombin and VEGF are presented as examples of G-protein coupled receptor- and tyrosine kinase receptor-mediated Rho GTPase activation respectively. These mediators induce complex but markedly different networks of activators, inhibitors and effectors of Rho GTPases, which alter the endothelial barrier function. An interesting feature in this regulation is that Rho GTPases often have both barrier protecting and barrier disturbing functions. While Rac1 enforces the endothelial junctions, it becomes part of a barrier-disturbing mechanism as activator of reactive oxygen species generating NADPH oxidase. Similarly RhoA is protective under basal conditions, but becomes involved in barrier dysfunction after activation of endothelial cells by thrombin. The challenge and promise lies in unfolding this complex regulation, as this will provide leads for new therapeutic opportunities.

Index

1. Introduction
 - 1.1 Ligand-dependent Rho GTPases regulation involved in barrier dysfunction
 - 1.2 Regulation of Rho GTPase activities
 - 1.2.1 Activation of Rho GTPases by guanine nucleotide exchange factors (GEFs)
 - 1.2.2 Inactivation of Rho GTPases by GTPase activating proteins (GAPs)
 - 1.2.3 Rho GTPase regulation by guanine nucleotide dissociation inhibitors (GDIs)
2. Basal maintenance of the endothelial barrier
 - 2.1 Proteins and assembly of AJs
 - 2.2 Interactions of junctional proteins with Rho GTPases
 - 2.3 RhoA activity and gap closure
 - 2.4 Inactivation of RhoA by Rac1
 - 2.5 Summing up
3. Receptor-mediated cell activation by thrombin
 - 3.1 Direct and indirect (in) activation of Rho GTPases by thrombin
 - 3.1.1 G-protein-mediated activation of RhoA by thrombin
 - 3.1.2 Feedback mechanisms prolonging RhoA activation
 - 3.1.3 Modulation of Rac1 activity by thrombin
 - 3.1.4 Cdc42 modulation by thrombin
 - 3.1.5 Summing up
 - 3.2 Consequences for the regulation of permeability
4. VEGF receptor mediated activation involved in permeability
 - 4.1 VEGF/RTK-mediated Rac1 and ROS generation
 - 4.2 Additional effects of VEGF/RTK on RhoGTPases
 - 4.3 VEGF-induced Rho GEFs and GAPs
 - 4.4 Summing up
5. Rho GTPases in permeability regulation by other agents
6. Conclusion and perspectives

1. Introduction

During the late 1990s evidence accumulated that the small GTPase RhoA plays an essential role in many vascular pathologies including hypertension, vasospasm and vascular leakage.¹⁻⁴ Although RhoA was known to regulate many cellular processes including F-actin cytoskeleton and microtubule (MT) dynamics, cell polarity, gene regulation, as well as cell-cycle progression, it remained unclear how alterations in activity of this single molecule could lead to such diverse (biological) activities.^{5,6} Soon it became clear that a close interplay between RhoA and other members of the Rho GTPase family contribute to the fine-tuning of the biological effects of Rho GTPase activation. Rho GTPases belong to the Ras superfamily of proteins. So far, at least 20 members of the Rho superfamily have been identified.^{4,7,8} The best-studied members of the Rho GTPase family are RhoA, Rac1 and Cdc42. They contribute to many aspects of cell behaviour, such as cell migration, cell division and contraction.⁵ This is reflected in a variety of biological processes, such as axon growth, leukocyte infiltration and angiogenesis.^{5,6} For example, regulation of endothelial barrier integrity, which is disrupted in many vascular pathologies, not only involves RhoA, but also Rac1 and Cdc42.⁹⁻¹² Additionally, it was recognized that the individual Rho GTPases can exert both positive and negative effects on the endothelial barrier integrity, depending on their cellular context and mode of (pathological) activation, adding an additional level of complexity to fine-tuning of the biological response.^{13,14}

To date, it has been generally accepted that the combined actions of the Rho GTPases require a crucial spatio-temporal regulation of their activities. This regulation, which involves over 150 regulatory proteins, plays a significant role in many vascular pathologies. However, knowledge regarding the contribution of individual regulatory proteins to vascular dysfunction is still fragmental. Yet it is here, where a new area for specific therapeutic targeting opens up.

In this review we discuss the principles of regulation of Rho GTPase activity in vascular function. We focus on barrier dysfunction by the hyperpermeability inducers thrombin and vascular endothelial growth factor-A (VEGF) as main representatives of G-protein coupled receptor (GPCR)- and receptor tyrosine kinase (RTK)-mediated Rho GTPase activation respectively. After a short introduction about the role of Rho GTPases in regulation of the endothelial barrier and the nature of the regulatory proteins involved, we first describe the regulation of Rho GTPases during endothelial

barrier maintenance. Next, we describe thrombin-mediated Rho GTPase signaling, which results mainly in a contractility and inter-endothelial gap formation. This is followed by a discussion of VEGF-induced Rho GTPase regulation, which disrupts the junctional complexes and causes actin cytoskeleton rearrangement required for endothelial migration. Finally, we briefly discuss Rho GTPase regulation during permeability induced by other agents and indicate some future perspectives.

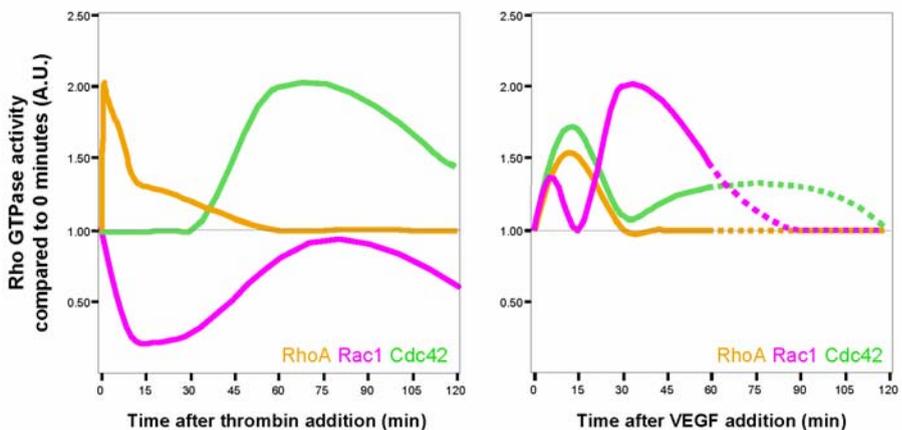
1.1 Ligand-dependent Rho GTPase regulation involved in barrier dysfunction

The F-actin cytoskeleton plays a major role in the maintenance of the endothelial barrier function, as it determines cell shape, facilitates cell adhesion to the sub-endothelial matrix, and participates in regulation of junctional complexes. The dynamics and structure of the F-actin filaments are mainly regulated by the Rho GTPases RhoA, Cdc42 and Rac1.^{7, 11} In addition, during barrier maintenance the regulation of Rac1 activity also involves the Rap GTPase Rap1.¹⁵ Several vasoactive agents, like β -adrenergic agonists, sphingosine-1-phosphate (S1P),^{16, 17} and adrenomedullin,¹⁸ improve the endothelial barrier integrity by enforcing the dense peripheral F-actin band. This process involves changes in the activities of several Rho GTPases. The activities of RhoA, Rac1, Cdc42 and Rap1 are not only involved in regulating barrier maintenance, but also in active enforcement and disruption of barrier integrity. Other vasoactive agents, such as thrombin and VEGF, can mediate barrier dysfunction via the regulation of often the same Rho GTPases, but activated in another context within the cell.

A reconstruction of the time courses of thrombin- and VEGF-mediated alterations in Rho GTPase activities based on several EC studies is shown in figure 1.^{17, 19-23} It is clear that the time course – and for Rac1 also the direction – of the activity changes are different after activation by these mediators. The profound increase of RhoA induced by thrombin is primarily involved in stress fiber (SF) formation and cell contraction, whereas VEGF-regulated Rho GTPases cause protrusions and cell migration. Simultaneously thrombin also weakens the junctional complexes by indirectly inhibiting Rac1 and relocating Cdc42.²⁰ *In vitro* as well as *in vivo* this results in a hyperpermeable endothelial monolayer.²⁴ *In vitro* the delayed activation of Rac1 and Cdc42 restores the endothelial barrier after approximately 2 hours by initiating cell junction reassembly.²⁰ In contrast, the

combined activity changes of Rac1, Cdc42 and RhoA induced by VEGF cause cell protrusion and migration.⁵ Unlike thrombin, VEGF induces rapid activation of Rac1 and the therewith-coupled generation of reactive oxygen species (ROS), leading to endothelial barrier dysfunction.²⁵⁻²⁷ VEGF-mediated activation of Cdc42 and RhoA is involved in SF formation.²⁸ These data thus show ligand-dependent regulation of the Rho GTPases, and indicate that Rho GTPases under different conditions can have distinct biological effects. This points to contextual activation of downstream targets.

Figure 1: Time profiles of RhoA, Rac1 and Cdc42 GTPase activities after stimulation of endothelial cells by thrombin (left) and VEGF (right panel)



Solid lines represent reported Rho GTPase activities, dotted lines represent extrapolated GTPase activities.^{17, 19-23}

1.2 Regulation of Rho GTPase activities

To appreciate the role of Rho GTPases in health and disease it is necessary to understand how they are regulated. GTPases cycle between an active, GTP-bound state and an inactive, GDP-bound state (figure 2). The activation of Rho GTPases requires guanine nucleotide exchange factors (GEFs) that catalyze the exchange of GDP for GTP in the switch regions of the Rho GTPase. Inactivation of Rho GTPases is mediated by GTPase activating (GAP) factors. The type of nucleotide (GDP or GTP) that is bound at the switch region of the GTPase determines the conformation of the switch region as well as the GTPase activity. Furthermore, guanine

dissociation inhibitors (GDIs) subsequently keep Rho GTPases in their inactive state (see below). For more detailed information about small GTPase signaling and their domain functions we refer to Siderovski & Willard²⁹ and Rossman *et al.*,³⁰ respectively. Here, we highlight some characteristics that are important to understand the function of RhoA, Rac1 and Cdc42 in the regulation of permeability.

1.2.1 Activation of Rho GTPases by guanine nucleotide exchange factors (GEFs)

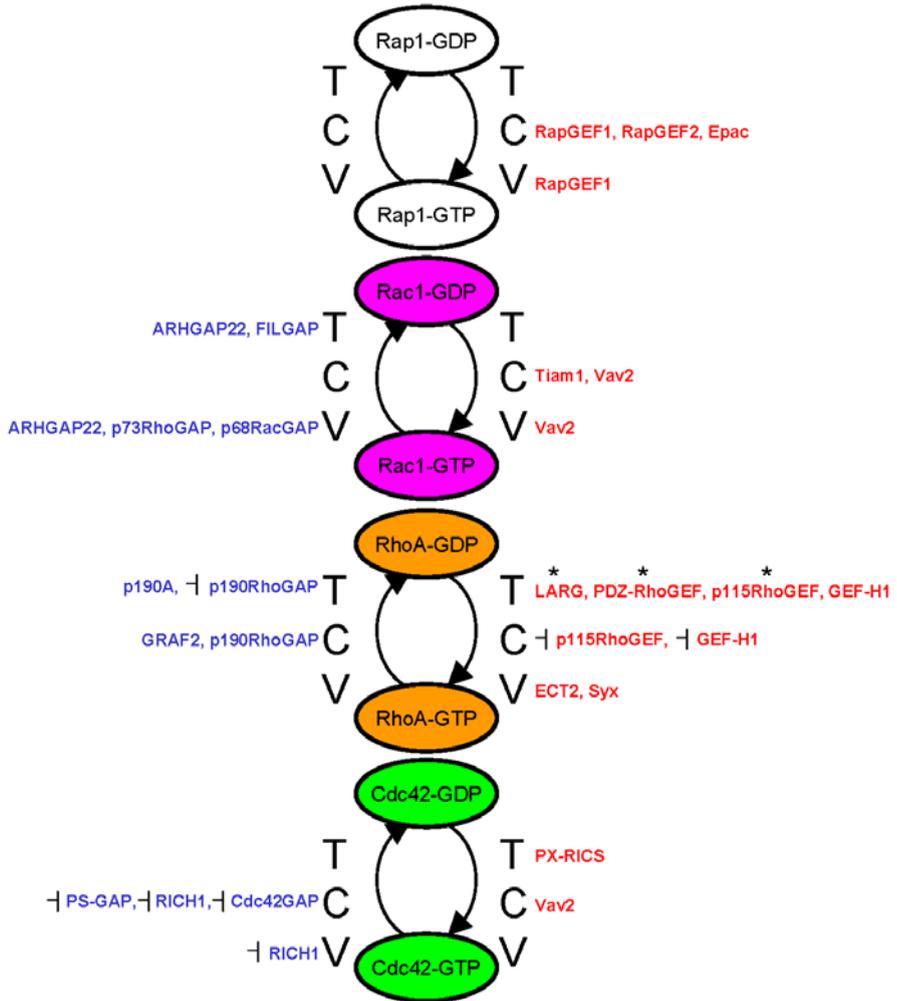
The human genome contains at least 83 GEFs that fall into two distinctive families.³¹ The first is the diffuse B-cell lymphoma (Dbl)-family. Members of this family contain a Dbl homology (DH) domain followed by a pleckstrin homology (PH) domain. The DH domain mediates RhoGEF activity, while the PH domain has an auto-inhibitory role.³⁰⁻³³ The PH domains of these typical RhoGEFs interact with phospholipids and may therefore localize the GEF and subsequent GTPase activation to the membrane. A specific subgroup of these RhoGEFs provides a structural motif known as regulator of G-protein signaling (RGS) domain, by which they can form molecular complexes with $G\alpha_{12/13}$ units of GPCRs. This interaction accelerates the GTPase activity of $G\alpha_{12/13}$ 5- to 10-fold, thus enhancing the signal-to-noise ratio of RhoA activation by $G\alpha$ proteins. Known members of this sub-group (* in figure 2) are leukemia-associated RhoGEF (LARG, also known as ARHGEF12), p115RhoGEF (also known as GEF1 and ARHGEF1) and PDZ-RhoGEF (also known as GTRAP48 and ARHGEF11).^{29, 34-36}

The second typical RhoGEF family is the DOCK-family. This family lacks DH and PH domains. RhoGEF activity is instead mediated by DOCK homology region (DHR) 1 and 2 domains.³⁹ DOCK-family members activate either Rac1 or Cdc42.³⁹⁻⁴¹ To date no member of this family has been associated with RhoA activation.

There are several mechanisms for GEF activation. The first is by phosphorylation. It has e.g. been shown that protein kinase C α (PKC α) activates p115RhoGEF via a serine phosphorylation,^{25, 42} while tyrosine phosphorylation of PDZ-RhoGEF and LARG was shown by focal adhesion kinase (FAK).^{42, 43} More importantly, GEFs can be activated by RTKs via Src and adaptor proteins. Of the 58 human RTKs, more than half are known to activate at least one of 16 RhoGEFs in addition to activating their common signal transduction pathways via phosphatidylinositol 3-kinase (PI3K),

phospholipase C (PLC) and the mitogen-activated protein kinase (MAPK) pathways.⁴⁴

Figure 2: Regulation of GTPases Rap1, Rac1, RhoA and Cdc42 by currently known GAPs (blue) and GEFs (red) during barrier maintenance (C) and thrombin (T)- or VEGF (V)-mediated endothelial permeability



Finally, activation of GEFs may be dependent on their subcellular localization. This mechanism is known to regulate the spatio-temporal

activation of RhoA and Rac1. Examples are ECT2 and Net1 (also known as ARHGEF8 and NET1A). These are nuclear RhoGEFs, but their translocation to the cleavage furrow and the plasma membrane, respectively, has been associated with RhoA activation.^{45, 46} In addition, the activation of Rac1 was associated with plasma membrane localization of the cytosolic RhoGEF Tiam1.^{30, 47} The RhoGEF GEF-H1 (also known as ARHGEF2, GEF, p40, LFP40) interacts with MTs and is involved in the cross-talk between the MT- and actin-network.^{48, 49} MT interaction inhibits its GEF activity for RhoA at the plasma membrane, but alternatively may switch its GEF specificity towards Rac1.^{30, 50}

1.2.2 Inactivation of Rho GTPases by GTPase activating proteins (GAPs)

The second group of molecules to regulate Rho GTPase activity are the GAPs. In contrast to what their name suggests, Rho GTPases have a relatively slow intrinsic GTPase activity. GAPs enhance the GTP hydrolysis of the active GTPases thereby causing inactivation of these proteins (figure 2). To date, more than 70 RhoGAPs have been identified. Interestingly, at least one of them, p73RhoGAP, has a preferential endothelial expression,^{51, 52} but expression of most GAPs is ubiquitous. Similar to RhoGEFs, RhoGAPs are also regulated by extracellular cues, such as growth factors.

Several mammalian RhoGAPs have been implicated in specific Rho GTPase-mediated biological functions such as angiogenesis, migration, cell blebbing and endocytosis. As nicely surveyed by Tcherkezian and Lamarche-Vane,⁵³ studies of the past decade have shed some light on the question why the RhoGAPs outnumber Rho GTPases by 2- to 3-fold.^{53, 54} Probably more important than tissue-specificity, is that GAPs, but also GEFs, may selectively regulate a specific downstream signaling pathway by activating Rho GTPase activities when they are in complex with other proteins, thereby leaving other GTPases and their pathways unaltered. Some RhoGAPs seem to specifically act on a single Rho GTPase, whereas others display activity towards multiple Rho proteins, explaining why Rho activities simultaneously rise or fall. Furthermore, RhoGAPs may act as scaffold molecules independent of their GAP activity, mediating cross-talk between Rho GTPases and other signaling pathways. In addition to GAP and GEF domains, these Rho GTPase regulatory proteins often also have

docking sites for other proteins so that they integrate signals from different pathways thereby directing specific downstream signaling.

1.2.3 Rho GTPase regulations by guanine nucleotide dissociation inhibitors (GDIs)

During nucleotide exchange GTP is preferentially bound to Rho GTPases because intracellular levels of GTP are substantially higher than those of GDP. GDIs interact with the switch domains of Rho GTPases to prevent activation by GTP and lock Rho GTPases in their inactive state. Furthermore, GDIs retain inactive GTPases in the cytosol by shielding off their membrane tag.³⁰ There are currently three RhoGDIs known. RhoGDI α (also known as RhoGDI-1, Rho-GDI α , RABGD1A and RABGDIA) is ubiquitously expressed, whereas RhoGDI β (also known as D4, Gdid4, Ly-GDI, ArhGDI β) is expressed in haematopoietic cells and RhoGDI γ (also known as Rho-GDI3 and ARHDIAG) is preferentially expressed in organs and the brain, where it is anchored to the membrane.^{55,56} Although the mechanisms are still largely unknown, RhoGDIs seem to regulate the fine-tuning of Rho GTPase activities via their association with multiprotein complexes in particular membrane microenvironments.^{55,56}

2. Basal maintenance of the endothelial barrier

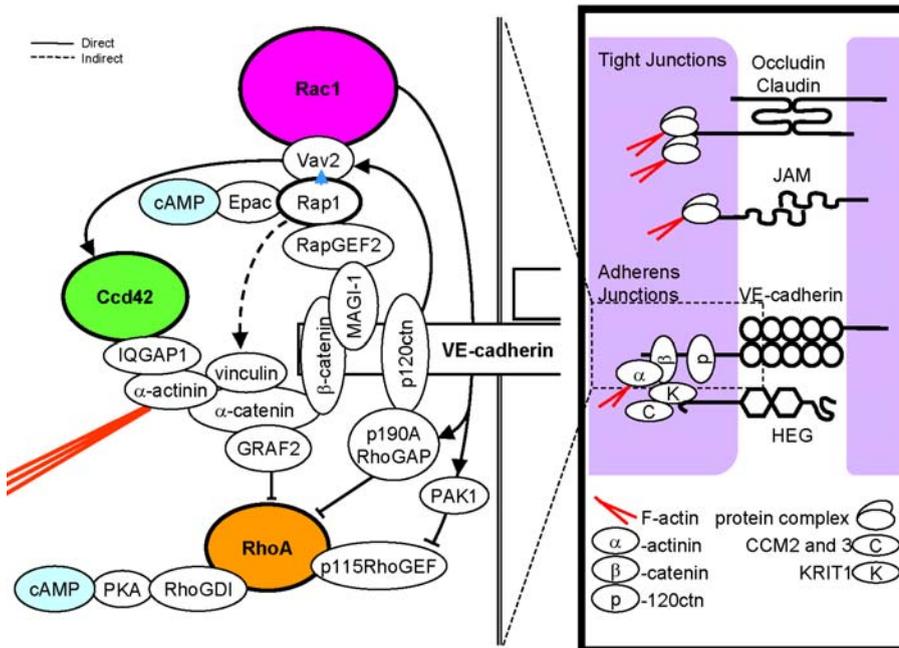
Initial attention for Rho GTPases was drawn to RhoA activation and its role in cell contraction and endothelial hyperpermeability (see section 3). During the last years the focus has moved to the role of Rac and Cdc42 in the assembly and stability of inter-endothelial junctions.^{20,57} Furthermore, an additional role for RhoA and its effector Rho kinase in barrier maintenance was recognized.²⁰

Both tight junctions (TJs) and adherens junction (AJs) contribute to the intercellular sealing of the endothelial monolayer and the resulting endothelial barrier function. In contrast to blood-brain ECs, in which the TJs form a sealing belt, TJs in most other ECs have a mosaic structure leaving space for the passage of macromolecules. The AJs contribute largely to the barrier properties of these latter endothelia in interaction with the actin cytoskeleton (figure 3).

2.1 Proteins and assembly of AJs

Much attention has been given to the regulation of AJs and their pivotal protein VE-cadherin, a membrane spanning protein that forms homotypic interactions between adjacent ECs²⁵ (figure 3, right panel). The intracellular part of VE-cadherin is connected to the F-actin cytoskeleton via both α - and β -catenin.²⁵ In addition, a third catenin, p120catenin (p120ctn), binds to VE-cadherin.⁵⁸ P120ctn binding to VE-cadherin is an important step in the regulation of AJ stabilization as well as in Rho GTPase regulation.

Figure 3. Rho GTPase activity regulates barrier integrity



The major TJ and AJ protein interactions are indicated in the right box. On the left side part of an AJ is enlarged. Here, cadherin engagement with the catenins via Vav2 activates Cdc42 and Rac1. VE-cadherin bound p120ctn and Rac1 inhibit RhoA by activation of p190RhoGAP. Via PAK1-mediated inhibition of p115RhoGEF Rac1 activity further suppresses RhoA. Linking of the junctional complex to the actin cytoskeleton stabilizes the AJs and further inhibits RhoA by activation of GRAF2. Enhanced levels of cAMP induce Rap1-mediated Rac1 activation as well as RhoGDI-mediated inhibition of RhoA. For further details see text.

Rac1 and Cdc42 are activated at sites where junctional complexes are formed,^{59,60} while RhoA activity is downregulated when the monolayer reaches confluence.⁶¹ Indeed, Rac1-deficient ECs are unable to form lamellipodial structures, focal adhesions and cell-cell contacts.⁶² Furthermore, many GTPase regulator activities are directly initiated by cadherin engagement (see below). Thus, a precise spatial and temporal fine-tuning of the activity of Rho family GTPases is critically important in the establishment and maintenance of junctions.

Another AJ protein is the endothelial-specific HEG (heart of glass) transmembrane receptor (figure 3, right panel), which interacts with an as yet unknown extracellular ligand to activate a coordinated signaling response through inhibiting the activity of RhoA. This response requires the cerebral cavernous malformations proteins CCM1 (or KRIT1), CCM2 and CCM3⁶³ and leads to a cellular response that may include induction of endothelial vacuolization and homotypic endothelial cell-to-cell junction formation. These cellular events are required for normal vascular development and maintenance of vascular integrity. Defects in these steps result in enhanced activation of RhoA and may lead to the pathological changes associated with hereditary cerebral cavernous malformations in humans.^{64,65} Loss of RhoA degradation, which is achieved by the ubiquitin ligase (E3) Smurf-1, after interaction with CCM2 is accompanied by increased endothelial permeability.^{66,67} Deletion of CCM2 did not affect the Rac1 concentration in ECs.⁶⁶

2.2 Interaction of junctional proteins with Rho GTPases

Concerning basal endothelial barrier regulation, much attention has been given to the relationship between VE-cadherin, the catenins and Rho GTPases. After cadherin engagement p120ctn binding activates Vav2, a GEF for Cdc42 and Rac1^{68,69} (figure 3, left panel). This GEF positively regulates Cdc42 and Rac1 activity. Both GTPases bind IQGAP (IQ motif containing GTPase-activating protein 1). Unlike the name suggests IQGAP1 does not have GAP activity, meaning that it does not enhance the GTP hydrolytic activity of the GTPases. Instead its binding to Cdc42 and Rac1 inhibits their intrinsic GTPase activity, thus keeping them in their active, GTP-bound state.⁷⁰⁻⁷² By recruiting IQGAP1, Rac1 and Cdc42 inhibit the interaction of IQGAP1 with β -catenin thereby initiating the binding of α - and β -catenin.^{20,73} This cross-links the complex with the actin cytoskeleton and stabilizes the AJ.⁷⁴ In contrast, inactivation of Rac1 and Cdc42 causes

dissociation from IQGAP1 that then binds to β -catenin at the AJ complex. As a result α - and β -catenin dissociate, leading to weaker adhesion and inter-endothelial gap formation.⁷⁵

It is likely that similar mechanisms also occur at the TJ level. Recently, data revealed that in epithelial cells RICH1 (also known as ARHGAP17), which has GAP activity for Cdc42 is required for the stability of epithelial TJs. *In vivo* the TJ component and scaffolding protein angiominin binds RICH1. This suppresses its GTPase activity, thus allowing Cdc42-mediated barrier protection.⁷⁶

2.3 RhoA activity and gap closure

Consistent with previous data in other cells,⁷⁷ we recently found that in non-stimulated EC monolayers RhoA activity, as measured by Raichu-FRET, occurs prior to gap closure (unpublished data). The inactivation of RhoA upon gap closure was explained by binding of p120ctn to the newly formed AJ complex, which causes p120ctn-mediated activation of p190RhoGAP and thus inactivation of RhoA^{58, 78} (figure 3, left panel).

Another molecular mechanism has been described that reduces RhoA activity upon establishment of AJs. ARHGAP10 (also known as GRAF2) *in vitro* inactivates RhoA and Cdc42.⁵³ *In vivo* human GRAF2 inhibits RhoA and recruits α -catenin to the newly formed cell-cell contacts⁷⁹ (figure 3), thereby stabilizing the AJs.

2.4 Inactivation of RhoA by Rac1

Under confluent conditions, cyclic adenosine monophosphate (cAMP) stimulates Epac which has GEF activity for Rap1.¹⁵ Rap1 initiates the activation of Vav2 resulting in active Rac1.⁸⁰ Activated Rac1 further inactivates RhoA by several downstream targets (figure 3). Rac1 subsequently activates p190RhoGAP thereby inhibiting RhoA.⁵⁸ Furthermore, Rac1 activates its downstream target PAK1, which inhibits p115RhoGEF, thus further repressing the activity of RhoA.^{24, 81} In addition, enhanced cAMP levels activate protein kinase A (PKA), which stimulate RhoGDI binding – and thus further inactivation – of RhoA.⁸²

An additional pathway for Rap1 activation was shown upon VE-cadherin engagement with β -catenin (figure 3). The membrane-associated guanylate kinase with inverted domain structure-1 (MAGI-1), which links via β -catenin to VE-cadherin,^{83, 84} binds RapGEF2 (also known as PDZ-GEF1, RA-GEF,

NRAPGEP, Rap-GEP and CNrasGEF).⁸⁴ The subsequently activated Rap1 recruits vinculin from cell-matrix to cell-cell contacts.⁸³ Vinculin supports the linking of α -catenin to α -actinin, thereby further stabilizing VE-cadherin-based cell adhesion.⁸⁵ Rap1 thus not only mediates the activation of Rac1 but also promotes the stabilization of VE-cadherin based junctions. Finally, it has been suggested that activation of Rac1, which is accompanied by the release of RhoGDI α , induces the inhibition of RhoA activity by increasing the interaction of RhoA with the newly released RhoGDI α .⁸⁶

2.5 Summing up

In conclusion, the maintenance of the endothelial barrier depends on RhoA, Cdc42 and Rac1 activation. RhoA activity decreases upon gap closure due to VE-cadherin and p120ctn engagement. The binding of the AJs to the actin cytoskeleton involves Cdc42, which is kept in its active state by binding to IQGAP1. The linking of the AJs to the actin cytoskeleton also activates GRAF2, which inhibits RhoA. The rise in cAMP levels, via PKA, activates RhoGDI α thereby inhibiting RhoA. In addition, rising cAMP levels and cadherin engagement induce the activation of Epac and RapGEF2, respectively. These GEFs stimulate Rap1, which via Vav2 activates Rac1. Rac1 activation results in the inhibition of p115RhoGEF and thus a suppression of RhoA. Vav2 activity is further enhanced by p120ctn binding to the junctional complexes. The latter also directly activates p190RhoGAP thereby further inhibiting RhoA activity.

3. Receptor-mediated cell activation by thrombin

One of the best-studied activators of RhoA in the context of endothelial permeability regulation is thrombin. Thrombin mediates its effect on RhoA directly via its receptor PAR-1.^{87, 88} Thrombin-mediated cleavage of PAR-1 activates several heterotrimeric G-proteins and their subsequent signaling pathways. The release of the α - subunits of these G-proteins ($G\alpha_{11/Q}$, $G\alpha_{12/13}$ and $G\alpha_i$) regulates the activity of RhoA^{35, 89-91} and Rac1,^{15, 80} either directly,^{35, 91} or via intracellular Ca^{2+} ^{35, 89, 91} or cAMP elevation.^{15, 80, 82, 90} As recently shown, knockdown of $G\alpha_{11/Q}$ or $G\alpha_{12/13}$ in human ECs prevents RhoA activation by thrombin,^{92, 93} indicating that both pathways are highly interrelated at the level of RhoA activation and together control downstream signaling leading to EC contraction and permeability.

3.1 Direct and indirect (in) activation of Rho GTPases by thrombin

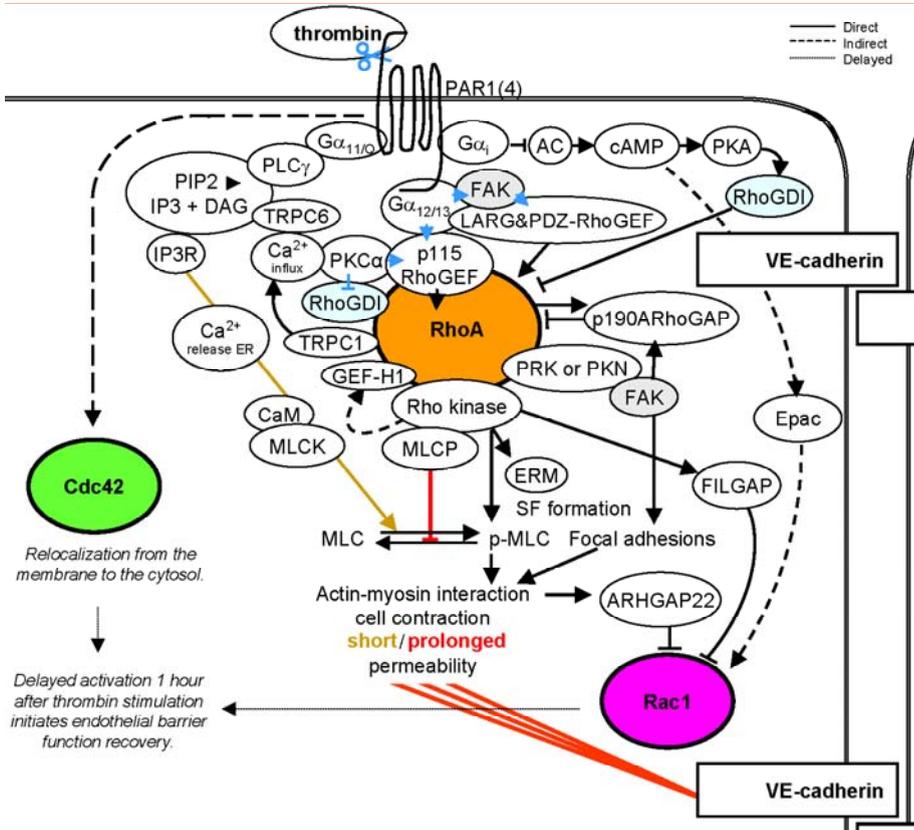
In the following chapter we survey the thrombin-mediated activation of RhoA, which involves several pathways and is associated with SF formation and cell contraction. Next, we discuss how thrombin may prolong RhoA activity. Furthermore, it is explained how thrombin initially causes an inhibition of Rac1, which is implicated in weakening of the junctional complexes, while later, a delayed recovery of both Rac1 and Cdc42 activities results in endothelial barrier recovery.

3.1.1 G-protein-mediated activation of RhoA by thrombin

Thrombin-mediated cell contraction requires two pathways that are interrelated at the level of RhoA activation (figure 4). The first pathway involves thrombin-mediated activation of $G\alpha_{11/Q}$, which causes coupling to phospholipase $C\gamma$ (PLC γ) that in turn generates inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 via its receptor on the endoplasmic reticulum (ER) liberates Ca^{2+} from the ER. This Ca^{2+} release activates calmodulin (CaM), which phosphorylates myosin light chain kinase (MLCK) resulting in enhanced myosin light chain (MLC) phosphorylation (Thr18 and Ser19).⁹⁴⁻⁹⁶ Increased MLC phosphorylation enhances acto-myosin interactions,⁹⁷ cell contraction and permeability.⁹⁸ Meanwhile DAG activates the transient receptor potential channel (TRPC)-6 at the plasma membrane, which allows for Ca^{2+} influx from the extra-cellular matrix and activation of PKC α .⁹⁹ PKC α has a dual role. It directly mediates the inhibitory phosphorylation (Ser96) of RhoGDI α ^{100, 101} and the stimulatory phosphorylation of p115RhoGEF.^{91, 101} This causes RhoA liberation and activation, respectively.

The second pathway involves the thrombin-mediated activation of $G\alpha_{12/13}$, which binds and activates p115RhoGEF, thus resulting in activated RhoA.^{34, 102} Via its downstream target Rho kinase, RhoA directly enhances SF formation and cell contraction. Rho kinase induces the phosphorylation of the regulatory subunit MYPT1 (Thr691 and Thr855) of the myosin light chain phosphatase (MLCP).¹⁰³ This decreases the phosphatase activity of MLCP, thereby reducing the de-phosphorylation of MLC.^{13, 104} Rho kinase by itself also can phosphorylate MLC (Ser19).¹⁰⁵ Thereby further enhancing MLC phosphorylation, which leads to acto-myosin interactions as described above.

Figure 4. Thrombin-mediated regulation of Rho GTPases involves several G-protein-activated pathways



For details see text. The inhibitory effects of Rac1 on RhoA activity are discussed in section 3.1.3 and depicted in figure 3.

3.1.2 Feedback mechanisms prolonging RhoA activation

Several positive feedback mechanisms can prolong the activation of RhoA and the subsequent cell contraction (figure 4). Via the membrane localized TRPC-1, RhoA further increases the Ca²⁺ influx²⁵ that prolongs the Gα_{11/Q}-mediated activation of MLCK, PKCα and RhoA. Three additional feedback pathways involve Rho kinase signaling. First, Rho kinase phosphorylates ezrin (Thr567), radixin (Thr564) and moesin (Thr558), which are collectively known as the ERM-proteins.¹⁰⁶ ERM phosphorylation enhances SF formation thereby stimulating cell contraction and endothelial permeability.

Second, it was shown for endothelin-1-stimulated vascular smooth muscle cells that Rho kinase causes an inhibitory phosphorylation (Ser1150) of p190RhoGAP (also known as ARHGAP5, GRLF1, GRF-1)¹⁰⁷ of which 2 isoforms, p190A and p190B are known. The first is associated with prolonging RhoA activation.¹⁰⁷ As prolonged RhoA activation is also seen during thrombin stimulation, it is likely that Rho kinase-mediated p190A inhibition also occurs during thrombin stimulation of ECs. Third, thrombin/p115RhoGEF-mediated activation of Rho kinase induces MT disassembly. This activates GEF-H1, a RhoA specific GEF that is known to localize at the MTs.^{48,49} In its MT-bound state, the guanosine-exchange activity of GEF-H1 is suppressed, whereas GEF-H1 released from MTs stimulates GEF activity and may thus induce further activation of RhoA.⁴⁸

In addition to Rho kinase, RhoA also activates protein kinase C-related kinase (PRK or PKN), which can regulate FAK-mediated focal adhesions. Rho kinase and PRK-mediated activities are both required for full thrombin-induced EC hyperpermeability.⁹² Accumulating evidence suggests a dual, time-dependent role for FAK in the regulation of thrombin-mediated RhoA activity: Thrombin-mediated FAK phosphorylation occurs after 5 minutes and peaks after 10 minutes.⁴³ Approximately 3-5 minutes after thrombin stimulation $G\alpha_{12/13}$ -mediated FAK activation results in an activating phosphorylation of PDZ-RhoGEF and LARG, which causes prolonged RhoA activity.⁴³ Subsequently, 10 minutes after thrombin stimulation FAK activates p190RhoGAP,¹⁰⁸ which *in vivo* preferentially inhibits RhoA activity.¹⁰⁸⁻¹¹⁰ This time course of p190RhoGAP phosphorylation coincides with the decrease in RhoA activity both in human pulmonary artery and umbilical vein ECs.^{21, 108} Thus, thrombin-mediated FAK activation in ECs first extends and then down-regulates the activation of RhoA (see to Suzuki *et al.*¹¹¹ for more detail about interactions between $G\alpha_{12/13}$ proteins and their RhoGEFs).

Finally, thrombin stimulates RhoA activity by activating $G\alpha_i$. The $G\alpha_i$ inhibits adenylyl cyclase (AC), which reduces cAMP levels in vascular smooth muscle cells¹¹² and in ECs.^{90, 113} Via attenuation of PKA this causes a reduced RhoGDI α activity thereby allowing further activation of RhoA.⁸²

3.1.3 Modulation of Rac1 activity by thrombin

As indicated before, thrombin-mediated endothelial permeability *in vitro* also involves a 1-hour lasting inactivation of Rac1.⁹³ This is thought to weaken the junctional complexes, which in combination with cell contraction, results in enhanced endothelial permeability. Several thrombin-mediated pathways

may be involved in the inhibition of Rac1 (figure 4). First, it is known that the PAR-1/ $G\alpha_i$ -mediated inhibition of AC, which decreases cAMP levels^{90, 113} causes the inhibition of Rac1,⁹³ a process that involves the inhibition of the RapGEF Epac and Rap1⁸⁰ (see section 2.4). Second, in tumor cells the RhoA/Rho kinase-mediated acto-myosin interaction inhibits Rac1 via the activation of the usually vascular specific ARHGAP22 (also known as RhoGAP2).³⁷ Third, Rho kinase activates the vascular specific FILGAP, a longer isoform of p73RhoGAP (also known as ARHGAP24 and RC-GAP72), which also antagonizes Rac1.⁵¹ Although no data is available on thrombin-mediated ARHGAP22 or FILGAP regulation in ECs, it has been shown that this Rho-mediated inhibition of Rac1 regulates amoeboid movement, which depends on Rho/Rho kinase signaling.^{37, 51} It seems likely that thrombin signaling also causes ARHGAP22 and FILGAP activation. It is of interest to note that suppression of Rac1, indirectly enhances p115RhoGEF activity thus further stimulating RhoA activation.²⁴

Thrombin not only inhibits Rac1, but occasionally may also cause a delayed Rac1 activation by indirect mechanisms. In mouse pulmonary ECs thrombin-mediated activation of PKC α leads to the activation of sphingosine kinase that catalyzes the formation of S1P from sphingosine, thereby causing paracrine activation of the S1P receptor.¹⁷ Subsequently, $G\alpha_i$ -activated PKC ϵ induces phospholipase D2 activation and enhanced Rac1 activity in vascular smooth muscle cells.¹¹⁴ Delayed thrombin-mediated activation of Rac1 stabilizes newly formed junctional complexes and enhances the endothelial barrier integrity.^{20, 73, 93} Rac1 activity increases during junction formation.^{10, 60} In contrast to other authors, Diebold *et al.*¹¹⁵ reported a rapid increase in active Rac1 15 min. after thrombin stimulation. This increase, which was accompanied by activation of NADPH oxidase, occurred indirectly via phosphodiesterase 2.

For more details about the role of Rac1 in maintaining the endothelial barrier integrity see section 2.2.

3.1.4 Cdc42 modulation by thrombin

Besides Rac1 Cdc42 participates in recovery of the endothelial barrier integrity. Thrombin stimulation of ECs quickly causes the re-localization, but not inactivation¹¹⁶, of Cdc42 from the plasma membrane to the cytosol.⁹³ This basal Cdc42 activity seems to contribute to the correct thrombin-induced acto-myosin contraction and SF formation.¹¹⁷ Delayed Cdc42 activation coincides in time with the activation of Rac1 (1 hour after thrombin

Driving Rho GTPases in endothelial permeability

stimulation).⁹³ Cdc42 additionally, is involved in Golgi-derived membrane trafficking.^{118, 119} The cycling of Cdc42 between the active and inactive form seems essential for its function in protein transport and polarity. Cdc42 at the Golgi occurs via PX-RICS, a splicing variant of RICS (also known as p250RhoGAP, p200RhoGAP and Grit), which is expressed in a wide variety of cells and has GAP activity for Cdc42. In HeLa cells PX-RICS interacts with β -catenin and regulates the ER-to-Golgi transport of the N-cadherin/ β -catenin complex.¹²⁰ In epithelial cells the E-cadherin recycling to the membrane is regulated by both Rac1 and Cdc42 and takes place prior to p120catenin binding to the junctional complexes (see chapter 4).¹²¹

3.1.5 Summing up

By activation of its receptor PAR-1 thrombin mediates activation of RhoA via $G\alpha_{11/Q}$, $G\alpha_{12/13}$ and $G\alpha_i$ -mediated pathways. The availability of RhoA for activation is enhanced by $G\alpha_{11/Q}/Ca^{2+}/PKC\alpha/-$ and $G\alpha_i/cAMP/PKA$ -mediated RhoGDI α inhibition. The activation of RhoA is induced by $G\alpha_{11/Q}/Ca^{2+}/PKC\alpha$ and $G\alpha_{12/13}$ (FAK)-activated RhoGEFs (p115RhoGEF, LARG and PDZ-RhoGEF). Subsequent inactivation of RhoA is mediated by FAK-activated p190(A)RhoGAP. Thrombin stimulation further inhibits Rac1, partially by decreased cAMP levels and partially due to Rho kinase-mediated activation of FILGAP and acto-myosin interaction-induced activation of ARHGAP22. Finally, thrombin causes a delayed activation of both Rac1 and Cdc42. Cdc42, which at the Golgi is activated by PX-RICS, stimulates the transport of junctional proteins towards the plasma membrane, thereby causing the repair of the junctional complexes.

3.2 Consequences for the regulation of permeability

Infusion of thrombin receptor activating peptide, which mimics the action of thrombin, was shown to induce pulmonary microvascular permeability *in situ*.¹²² Its infusion into the hindleg of a heparinized rat causes a rapid swelling due to increased endothelial permeability (unpublished data). Endothelial permeability induction by thrombin in endothelial cell monolayers *in vitro* has extensively been used to study mechanisms underlying permeability regulation.¹²³ This enhancement occurs in a biphasic way. Initially a rapid drop in transendothelial electrical resistance occurs accompanied by a moderate increase in permeability for macromolecules like albumin and peroxidase. This is accompanied by Rac1 inhibition and

Cdc42 translocation, which facilitate loosening of the AJs. Together with the thrombin-induced activation of tyrosine kinases like Src,^{21, 25} which by phosphorylational changes reduces the homotypic interaction between junctional proteins,^{98, 124} this causes the disruption of the AJs.¹²⁵ At the same time RhoA/Rho kinase activity together with Ca²⁺/MLCK activity enhance actin-myosin interaction, induce SFs and cause a general cell contraction. The latter mechanism causes a second phase of a further increased hyperpermeability, which starts 10-15 minutes after thrombin exposure and ceases after 60 minutes, when Rac1 and Cdc42 activities also normalize again. Overall this second phase leads to inter-endothelial gap formation and cell rounding. While cell contraction was already implied in early studies on endothelial permeability *in vivo*,¹²⁶ the extent of contraction and leakage *in vitro* seems disproportional as compared to the *in vivo* situation. *In situ* thrombin stimulation of arteriolar and venular ECs indeed leads to cell contraction and cell rounding, but the inter-endothelial gaps are clearly smaller.¹²⁷ In ECs *in vivo* RhoA/Rho kinase activation only leads to a contraction at the cell margins, which is nonetheless enough to cause vascular leakage.¹²⁸ An increasing amount of evidence suggests that this apparent difference in degree of cell contraction is at least partially due to the flexibility of the underlying tissue.

Interestingly RhoA/Rho kinase activity, which is present at the cell margins, disappears after thrombin stimulation, while then, Rho kinase activity at the SFs becomes visible.¹²⁷ This local decrease at the cell margins, together with altered local activities of Rac1 and Cdc42 mentioned above, point to potential roles of Rho GTPases in the formation and maintenance of junctional complexes and endothelial barrier function.

Finally, when primed e.g. during the late phase of sepsis or upon activation with activated protein C, PAR1 switches from being a vascular-disruptive receptor to a vascular-protective receptor, a phenomenon that might involve transactivation of PAR2 and S1P receptor-1.¹²⁹⁻¹³¹ This 'role reversal' of PAR-1 is accompanied by a dampening of RhoA activation and recovery of Rac-GTP levels.

4. VEGFR-mediated activation involved in permeability

VEGF-mediated endothelial permeability is complex and involves the formation of transendothelial pores through the fusion of endothelial vesicles and disruption of endothelial junctions. Though the precise mechanisms are still unclear, Rho GTPases are also involved in this latter aspect of VEGF-

mediated permeability.¹¹ Compared to thrombin, VEGF-mediated Rho GTPase activation appears more restricted to a direct effect on the disruption of the AJs.

ECs express two VEGF receptors that recognize VEGF-A, known as VEGFR1 (also known as Flt-1) and VEGFR2 (known as Flk1 and KDR). They belong to the family of receptor tyrosine kinases (RTKs). Though during embryogenesis both receptors are involved, in adult ECs VEGFR2 is in particular involved in activating ECs during angiogenic processes. Upon binding to VEGF, VEGFR2 undergoes dimerization and oligomerization, which activates its intrinsic tyrosine kinase activity resulting in auto- and trans-phosphorylation on specific tyrosine residues in the cytoplasmic domain.¹³² These tyrosine residues, when phosphorylated, are docking sites for Src homology 2 (SH2) binding domain containing downstream effectors. For more detailed information on VEGFR2 phosphorylation see Olsson *et al.*¹³³.

Under confluent conditions *in vitro* and *in vivo*, VE-cadherin interacts with VEGFR2^{10, 134} in an IQGAP1-mediated manner.¹³⁵ While the number of these complexes increase upon VEGF stimulation,¹³⁵ VEGF, in a Rac1-dependent manner, leads to vascular permeability.^{26, 136} VEGF-mediated RhoA and Cdc42 activation are involved in cytoskeleton rearrangements needed for cell migration,²² a process that requires binding of activated VEGFR2 to the $\alpha\beta 3$ integrin.¹³⁷

4.1 VEGF/RTK-mediated Rac1 activation and ROS generation

During activation by VEGF, the VEGFR2 is phosphorylated on multiple tyrosine residues, including Tyr951, Tyr1175, Tyr1212 and Tyr1214. While Tyr1175 is essential for PLC γ -mediated ERK1/2 activation and DNA synthesis,¹³⁸ and Shb-mediated focal adhesion formation,¹³³ Tyr1212, Tyr1214, and probably Tyr951, are involved in activation of Rho GTPases.

As a result of VEGF-induced phosphorylation of VEGFR2 (Tyr1212) Src is activated.¹³⁹ Within 3 minutes this stimulates Vav2, Rac1 and its downstream target PAK1.^{23, 140} PAK1 phosphorylates VE-cadherin (Ser665) inducing the recruitment of β -arrestin2 and the internalization of VE-cadherin into clathrin-coated vesicles¹⁴¹ (figure 5). Deletion of Rac1 *in vitro* in primary ECs indeed decreased VEGF-mediated endothelial permeability.⁶² This observation contrasts other observations that Rac is required for the generation of the endothelial barrier under basal conditions.^{59, 60, 62} This

suggests different modes of action of Rac1 in regulating endothelial permeability.

VEGF-activated Rac1 can also activate NADPH oxidase at the EC membrane. Indeed, in human pulmonary microvascular ECs it was demonstrated that VEGF-mediated activation of Rac1 caused ROS generation that was inhibited by a NADPH oxidase inhibitor.²⁶ This activation was accompanied by an increase in endothelial permeability, which was based on the phosphorylation of Tyr658 and Tyr731 of VE-cadherin, which cause detachment of p120ctn and β -catenin, respectively, and phosphorylation of Tyr654 on β -catenin. Inhibition of either Rac1 or NADPH oxidase activity prevented the increase in endothelial permeability and the accompanying phosphorylations of VE-cadherin. Whether ROS act directly on the kinase activity, or, more likely, inhibit VE-cadherin associated tyrosine phosphatases, has still to be clarified. Furthermore, the phosphorylations of VE-cadherin may occur contextually. It is unknown whether phosphorylation of VE-cadherin at Tyr658 and Tyr731 will occur simultaneously with that of Ser665 or that they exclude each other mutually. Similarly, the phosphorylation of Tyr685 of VE-cadherin may affect the other phosphorylations.¹⁴²

ROS production is apparently an essential component in signaling cascades that mediate Rac1/p190RhoGAP-induced down-regulation of RhoA.²⁷ ROS production causes p190RhoGAP translocation to the AJs, where it binds p120ctn, and subsequently inhibits local RhoA activity.⁵⁸ It thus plays a role in the stabilization of cell-cell contacts (figure 5). A regulatory role in this process was shown for IQGAP1, which upon VEGF stimulation is rapidly (<5 min) recruited to VEGFR2. This is associated with tyrosine phosphorylation of IQGAP1 and subsequent binding and activation of Rac1. In addition, it causes further VEGFR2 association with the VE-cadherin/ β -catenin complex.¹³⁵ Deletion of IQGAP1 indeed decreases Rac1-mediated ROS production and prevents cell migration and proliferation.^{135, 143}

4.2 Additional effects of VEGF/RTK on RhoGTPases

In addition to the Tyr1212 phosphorylation, phosphorylation of the adjacent Tyr1214 on VEGFR2 causes binding and activation of heat-shock protein 90 (HSP90). HSP90 indirectly mediates the activation of RhoA and subsequently Rho kinase, which, as described above, leads to acto-myosin interaction and cell contraction.¹⁴⁴ Rho kinase also phosphorylates FAK (Ser732). This specific phosphorylation is a crucial step for subsequent

additional phosphorylations of FAK by Src¹⁴⁴ and Shb,^{144, 145} which are crucial for paxillin and vinculin recruitment to the focal adhesion sites.¹⁴⁴

The transient Tyr1214 phosphorylation of VEGFR2 furthermore recruits and activates the adapter protein Nck, which triggers the Src family kinase Fyn, indirectly (maybe via PAK2) resulting in Cdc42 activation.¹⁴⁴ Cdc42 targets PAK1 which turns on SAPK2 (stress-activated protein kinase 2) belonging to the family of p38 MAPK.^{144, 146} Via SAPK2 signaling the actin-polymerization factor HSP27 is activated, thereby further increasing SF formation^{28, 144} (figure 5). HSP90/RhoA-dependent phosphorylation of FAK is required for this Cdc42/SAPK2-mediated SF formation.¹⁴⁴ The overexpression of Cdc42GAP indeed inhibited endothelial migration.¹⁴⁷

Interestingly, in tumor ECs lacking associated pericytes VEGF-mediated Tyr951 phosphorylation of VEGFR2 binds TSAd (T-cell-specific adapter molecule) which is required for SF formation and motility¹³³, suggesting that this pathway is involved in RhoA activation. Although the cross-linking of RTK and GPCR signaling lies outside the scope of this review, we like to mention that VEGF-induced phosphorylation of VEGFR2 at Tyr951 also mediates activation of RhoA and Rac1 via the $G\alpha_{11/Q}$ and $G_{\beta\gamma}$ subunits¹⁹ (figure 5). Additionally, VEGF-induced G-protein coupled PLC β 3 activation regulates migration of ECs and vascular sprouting by activation of Cdc42.¹⁴⁸ $G\alpha_{12/13}$ seems not to be involved in the cross-linking of RTK and GPCR signaling, which may explain why activated RhoA levels are much lower in VEGF-activated ECs than in thrombin-stimulated ECs.²² The different levels of RhoA activation may underlie functional differences between thrombin and VEGF signaling. Thrombin stimulates a generalized contraction of ECs, whereas VEGF-mediated migration involves retraction of the trailing edges of the cells, which nonetheless can lead to inter-endothelial gap formation.²²

4.3 VEGF-induced Rho GEFs and GAPS

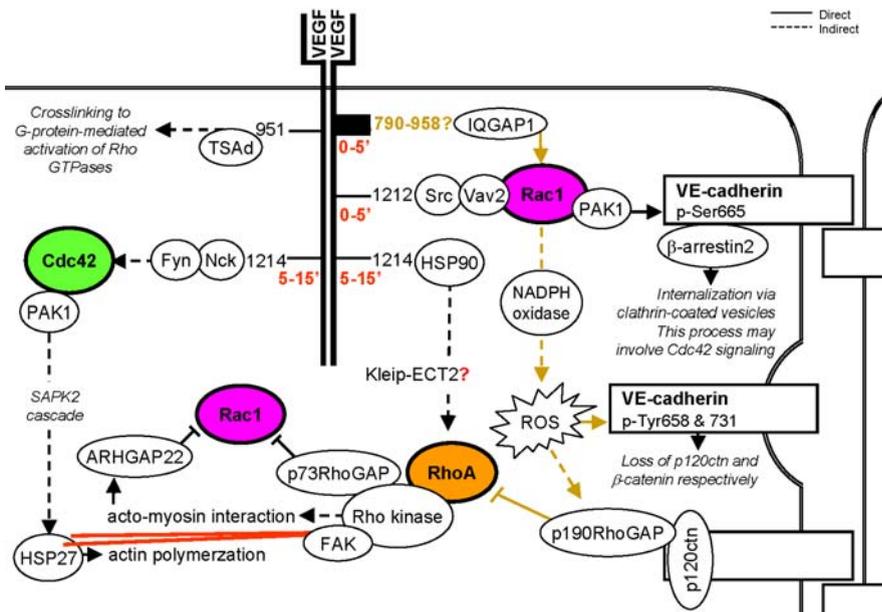
Several Rho GTPase regulating proteins have been identified that are involved in VEGF-induced vascular responses in animal models. VEGF was shown to activate the RhoA-specific GEF ECT2 via the actin binding protein Kleip, a process that is further stimulated under hypoxia due to up-regulation of Kleip.¹⁴⁹ Since Kleip/ECT2 is required for cell migration,¹⁴⁹ it seems reasonable to hypothesize that Kleip/ECT2 forms part of the HSP90-mediated RhoA activation pathway (figure 5). In addition, the angiomin/RICh1 complex, which stabilizes the TJs by maintaining Cdc42

activity⁷⁶, upon VEGF stimulation recruits the RhoGEF Syx, which mediates local RhoA activity.^{150, 151} Syx has been encountered in ECs *in vivo*.^{150, 151}

Rho kinase activity and the RhoA/Rho kinase-mediated acto-myosin interaction cause activation of FILGAP (p73RhoGAP) and ARHGAP22, respectively. In HUVECs overexpression of p68RacGAP (the mouse equivalent of human ARHGAP22) indeed inhibited Rac1-dependent lamellipodia formation and cell migration.¹⁵² Although not much is known about it, contraction-mediated inhibition via p73RhoGAP and ARHGAP22 may explain the dip in the VEGF-mediated Rac1 activation after five minutes (see figure 1).

One may anticipate that these endothelial GAPs and GEFs also play a role in the regulation of VEGF-mediated permeability.

Figure 5. VEGF-mediated GTPase regulation induces endothelial permeability



VEGFR2 can be phosphorylated at multiple tyrosine sites of which 3 are indicated. Tyr1212 phosphorylation of VEGFR2 induces Rac1-mediated VE-cadherin internalization and endothelial permeability. VEGF further induces Rac1-mediated ROS production, which via VE-cadherin phosphorylation at Tyr658 and Tyr731 induces the disassembly of p120ctn and β -catenin respectively. ROS-mediated p190RhoGAP recruitment to the AJs locally inhibits RhoA. Rac1 thus dually mediates permeability and adhesion. Phosphorylation of Tyr1214 mediates RhoA and Cdc42 activation needed for acto-myosin interactions and SF formation. Finally, Tyr951 phosphorylation induces crosslinking to G-protein-mediated activation of Rho GTPases. It is likely that TSAd is involved in this process. For further detail see text.

4.4 Summing up

VEGF/VEGFR2-mediated activation of Rac1 involves Vav2 and mediates VE-cadherin phosphorylation and internalization. Meanwhile, Rac1 is also activated in a VEGFR2/IQGAP1-mediated manner, leading to ROS production. This results in tyrosine phosphorylation of VE-cadherin, subsequent detachment of β -catenin and p120ctn, and increased permeability. ROS also induces a p190RhoGAP/p120ctn-mediated inactivation of RhoA. Cdc42 is further activated by VEGF-mediated inhibition of Cdc42GAP and VEGFR2-phosphorylation. In the latter pathway PAK2 may be involved as a GEF for Cdc42. VEGF/VEGFR2/HSP90-mediated activation of RhoA occurs possibly via activation of Kliep and ECT2 and is involved in cell migration. In addition, RhoA activation by Syx occurs at the cell margins and is involved in protrusion formation. VEGF-mediated Rho/Rho kinase-activated p73RhoGAP, supported by the acto-myosin interaction-activated ARHGAP22, subsequently inhibits Rac1. And finally, VEGF-mediated activation of RhoA, Rac and Cdc42 was shown in a G-protein-mediated manner.

5. Rho GTPases in permeability regulation by other agents

Many other vascular mediators, such as platelet activating factor¹⁵³, hydrogen peroxide¹⁵⁴, oxidized LDL¹⁵⁵, bacterial toxins¹⁵⁶, TNF- α ,² lysophosphatidic acid (LPA)¹⁵⁷, angiopoietins^{158, 159} and also leukocytes can modulate Rho GTPase-mediated inter-endothelial gap formation. GTPase regulation by TNF α and LPA involves known but also new GEFs and GAPs. Little is known about the Rho GTPase regulation by LPA in the endothelium. From other cell types it is known that, like thrombin, LPA mediates a FAK-dependent activation of RhoA via PDZ-RhoGEF.^{160, 161} In addition, LPA enhances the expression of Net1, which positively effects RhoA activity and cell migration.¹⁶² The RhoA effector mDia was furthermore shown to associate with LARG thereby forming a positive feedback mechanism for LPA-mediated RhoA activity.¹⁶³ It is unknown whether these pathways also play a role in thrombin-mediated RhoA activation. The same is true for LPA-mediated activation of p116-Rho-interacting protein (p116RIP), a RhoA specific GAP¹⁶⁴ that simultaneously binds RhoA and MYPT1, the regulatory subunit of MLCP. This way LPA activates MLCP and simultaneously inhibits RhoA, thus dually suppressing acto-myosin interaction and cell contraction.¹⁶⁵

Like thrombin, TNF α induces RhoA-mediated SF formation, cell contraction and endothelial permeability.² Unlike thrombin, TNF-induced GEF-H1-mediated RhoA activation, was shown downstream of TNF α -induced ERK signaling.¹⁶⁶ Furthermore, TNF α induces the expression of interleukin (IL)-6 and IL-8. This involves TNF α -mediated Rac1 activation, which modulates p68, a subunit of the transcription factor NF- κ B.^{167, 168} To date however, it is not known how this Rac1 activity is regulated. TNF α signaling involves factor-associated-with-nSMase (FAN), an adaptor protein that links the plasma membrane to the actin cytoskeleton. FAN is a key mediator of TNF α -induced Cdc42 activation and filopodia formation.¹⁶⁹ The RhoGEF that links FAN to Cdc42 activation has not yet been identified.

Angiopoietins recently have drawn a lot of attention as modifiers of endothelial barrier function through modulation of Rho GTPase activity^{158, 159}, as was reviewed by van der Heijden *et al.*¹⁵⁹ Angiopoietins are growth factors known to function as ligands for the RTK Tie2. Angiopoietin (Ang)-2 provokes inflammation and vascular hyperpermeability, while Ang-1 has a protective effect. The ratio of the circulating levels of Ang-1 and Ang-2 strongly associates with pulmonary microvascular permeability in diseases such as ARDS.¹⁵⁹ Ang-2 inhibits phosphorylation of the Tie2 receptor, which increases RhoA and Rho-kinase activity.¹⁵⁸ In contrast, Ang-1-induced Tie2 receptor phosphorylation signals via PI3-kinase and Rac1 to activate p190RhoGAP.¹⁵⁹ Alternatively, Ang-1 might act by promoting the activation of mDia through RhoA, resulting in the association of mDia with Src. This deprives VEGFRs of an essential molecule required for the disruption of endothelial cell-cell contacts and paracellular permeability.¹⁴ This barrier-protective effect of RhoA through mDia is in apparent contrast to the response of e.g. thrombin and LPS, which enhance vascular permeability through a robust and persistent RhoA activation and the consequent Rho kinase-dependent assembly of SFs and cell contraction. The strength and location of RhoA activation may therefore affect the choice of the RhoA downstream target, thereby determining whether RhoA-initiated signals protect the barrier function of ECs or promote vascular leakage.

Finally, leukocyte activation of the ECs induces clustering of ICAM-1. This stimulates the Rho specific SGEF, which activates the small GTPase RhoG.¹⁷⁰ The dorsal ruffles that are subsequently formed assemble a docking structure around the leukocyte and enable leukocyte migration through the EC monolayer. In addition to SGEF, the Rho/Rac GEF Trio may also be activated downstream of ICAM-1 clustering, since it associates to filamin.

6. Conclusion and perspectives

Rho GTPases, which were discovered as regulatory proteins that regulate specific actin cytoskeletal structures – SF by RhoA, lamellipodia by Rac1 and filopodia by Cdc42 – are now known as complex integrators of cytoskeletal structures that can have multiple functions depending on the cellular context. Fundamental to these functions are three-dimensional complexes between the individual Rho GTPases, their specific GEFs and GAPs, which largely outnumber the Rho GTPases themselves, and additional regulatory proteins. By this complexity of regulation different vasoactive mediators can induce various cytoskeletal structures that enable the ECs to respond adequately. In this review we have focused on the consequences of Rho GTPase regulation for endothelial barrier function.

From the foregoing discussion it has become clear that GPCR- and RTK-mediated activations of Rho GTPases have markedly different effects. The G-protein coupled thrombin receptor PAR-1 induces mainly RhoA-mediated cell rounding, whereas RTK VEGFR2, by means of VEGF, induces protrusion, cell migration and angiogenesis. Thus the initial idea to investigate Rho GTPase signaling in order to find a therapeutic target to counteract permeability seems logical. However, we have seen here that many different and even independent pathways lead to thrombin-mediated activation of RhoA. Moreover, there are several pathways that independently result in the stimulation of different Rho GEFs and GAPs, suggesting that targeting just one of them is not going to efficiently inhibit RhoA activation and permeability.

In contrast, VEGF-mediated Rho GTPase regulation shows that the fine-tuning of RhoA, Rac1 and Cdc42 activities drives different processes at the same time. The spatio-temporal regulation is crucial here and may thus form an interesting opportunity to interfere with one process while stimulating another.

An interesting feature of Rho GTPases in endothelial barrier regulation is that they often have both barrier protecting and barrier disturbing functions. While Rac1 in most conditions enforces the junctions that form the endothelial barrier, it becomes part of a barrier disturbing mechanism as activator of NADPH oxidase, which generates ROS. Similarly RhoA has a dual role in barrier regulation, being protective in basal conditions, but strongly involved in barrier dysfunction after activation of ECs by thrombin.

At present most information on Rho GTPases has been acquired from studies in cultured cells. Cultured ECs display certain characteristics more

pronounced than their *in vivo* counterparts, e.g. the extent of SF formation, while other aspects are more dominant *in vivo*. Future studies have to clarify the importance of various parts of the signaling pathways in pathological conditions *in vivo*. Both studies in transgenic animals and evaluation of clinical samples are needed to assemble the insight required for pharmacological intervention in man. In this context, location-specific GTPase regulators, preferably EC-specific GEFs and GAPs, may be the most interesting to modulate the formation of cell protrusions and migration or endothelial permeability. The challenge today is to unfold their regulation, thereby not only looking at the GTPase for which they have the highest GEF or GAP affinity, but also determining their effect on the other GTPases since it is this cross-talk, or the lack of it, that makes them an interesting therapeutic target.

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Driving Rho GTPases in endothelial permeability

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