Abstract—Rho-like small GTPases, with their main representatives (Rho, Rac, and Cdc42), have been recognized in the past decade as key regulators of the F-actin cytoskeleton. Rho-like small GTPases are now known to play a major role in vascular processes caused by changes in the actin cytoskeleton, such as smooth muscle cell contraction, endothelial permeability, platelet activation, and leukocyte migration. Data are now accumulating regarding the involvement of Rho GTPases in vascular disorders associated with vascular remodeling, altered cell contractility, and cell migration. The unraveling of signal transduction pathways used by the Rho-like GTPases revealed many upstream regulators and downstream effector molecules, and their number is still growing. An important action of Rho, Rac, and Cdc42 is their ability to regulate the phosphorylation status of the myosin light chain, a major regulator of actin-myosin interaction. Present knowledge of the Rho-like small GTPases has resulted in the development of promising new strategies for the treatment of many vascular disorders, including hypertension, vasospasms, and vascular leakage. (Arterioscler Thromb Vasc Biol. 2001;21:300-311.)

Key Words: small GTPases ■ vascular disorders

After the discovery of the central role of the Rho family of small or low molecular weight GTPases as regulators of the actin cytoskeleton in the early 1990s, it was found that these small GTPases were also involved in gene regulation and cell cycle progression. Subsequently, a large body of evidence has been obtained regarding the important functions of Rho GTPases in many processes in the vasculature, as diverse as the regulation of (elevated) blood pressure, platelet activation, wound healing, and leukocyte extravasation. The aim of the present review is to give an overview of the evidence relating to processes in which Rho GTP–binding proteins (G proteins) are involved in the vascular system, with emphasis on their effects on the F-actin cytoskeleton. We summarize the general regulatory patterns that these small GTPases use to examine what determines specificity in each process and to determine how these processes can be modulated pharmacologically. A review of all possible modulators of GT-Pase activity, of all target molecules of Rho GTPases and a description of their molecular structures, and of their involvement in gene regulation and cell cycle progression is beyond the scope of this survey. The reader is referred to other reviews on these topics (Rho as a mediator of G protein–coupled receptor [GPCR] signaling,3,4 Rho signaling pathways,5-9 Rho GTPases and the cytoskeleton,10-14 Rho and integrin function,15-17 Rho and gene regulation,18 and Rho and development19).

The Rho GTPase Family

The Rho proteins belong to the widespread Ras superfamily of small G proteins20 from which they got their name as Ras homologues. Prototypes of the Rho protein family are Rho, Rac, and Cdc42. Rho GTPases are key regulators of the actin cytoskeleton. By their action on the actin cytoskeleton, they play a major role in fundamental processes, such as cell contraction, cell motility, cell adhesion, and cell shape. Therefore, it is not surprising that knockout mice of Rho GTPases often are not viable,21 inasmuch as these proteins fulfill many essential functions. The Rac2−/− mouse is the only published Rho GTPase knockout mouse thus far, but Rac2 seems to be exceptional, inasmuch as Rac2 expression is restricted to hematopoietic cells.22

With the identification of more members and isoforms, a confusing nomenclature has developed. Members of the Rho protein family can be divided into 6 different classes consisting of the following members: Rho (RhoA, RhoB, and RhoC), Rac (Rac1, Rac2, and Rac3, which is also known as Rac1B [RhoG]), Cdc42 (Cdc42Hs, Cdh, G25K, and TC10), Rnd (RhoE/Rnd3, Rnd1/Rho6, and Rnd2/Rho7), Rhod, and TTF.23-24 In this list, RhoE is the same as Rnd3, RhoF does not exist, and Cdc42, TC10, and TTF lack the R in their name to identify them as members of the Ras superfamily of proteins.

Rho, Rac, and Cdc42 are the 3 classes for which the most is known. Each has its own specific effects on the actin...
cytoskeleton, likely resulting from the activation of different protein subsets involved in actin polymerization. A striking feature of the activation of Rho is the formation of cytoplasmic stress fibers (SFs) in cultured cells that can form SFs and an increase in actomyosin-based contractility in cells that cannot form SFs (such as neuronal cells). SFs are long cytoskeletal cables or bundles of actin and myosin II/nonmuscle myosin filaments that can contract and exert tension (see below under SF Formation) and are linked to the plasma membrane at focal adhesions (FAs). Rac and Cdc42 regulate peripheral F-actin assemblies. Rac is involved in the formation of membrane ruffles and lamellipodia, whereas Cdc42 induces the formation of radial unipolar bundles termed microspikes or filopodia.

All 5 protein classes can also regulate the assembly of integrin-containing FA complexes and thus regulate cell-matrix interactions and cell adhesion. Rho induces the formation of the classical FAs. These integrin-containing complexes are connected to bundles of SFs and are clustered over the basal surface of the cell, maintaining their firm attachment to the underlying substratum. Rac and Cdc42 induce the formation of the smaller focal contact sites at the cell periphery, associated with lamellipodia and filopodia.

Recently, new members of the Rho family of small GTPases, Rnd1, Rnd2, and Rnd3/RhoE, have been identified. Rnd proteins have a close homology to RhoA, but they display a very distinct biochemical behavior. Rnd proteins lack GTPase activity and are constitutively active in the active state. Therefore, expression levels of Rnd proteins primarily determine their involvement in signaling. Expression of these proteins in fibroblasts causes cell rounding (Rnd indicates round) and inhibits the formation of SFs, lamellipodia, and FAs and therefore appears to have an antagonistic effect on Rho and Rac.

**General Outline of Rho-Like Small GTPase Action**

**Rho GTPase Signaling**

Rho GTPases can be activated principally in 2 different ways: by soluble factors via heterotrimeric GPCRs, tyrosine kinase receptors, and cytokine receptors and by cell adhesion and integrin clustering. One example of the former is cellular activation by lysophosphatidic acid (LPA), which was the first agonist identified to activate Rho. LPA is present in serum and activates Rho via several 7-transmembrane GPCRs. Many other GPCR agonists have been described to activate Rho-family GTPases, including thrombin, endothelin, carbachol, prostaglandin E2, bombesin, formyl-Met-Leu-Phe (fMLP), angiotensin, α-adrenergic agonists, sphingolipids, and purinergic receptor agonists (see review 3,4). The activity of low molecular weight G proteins is not directly regulated by agonist binding to GPCRs, as is the case with the α subunit of the heterotrimeric G proteins, but is indirectly regulated (see below). Similar to other G proteins, low molecular weight G proteins, with the exception of the Rnd proteins, are molecular switches, which can bind either GDP or GTP, which results in a change in conformation. They are active in their GDP-bound status and inactive in the GDP-bound form. In the GTP-bound form, Rho GTPases interact with and activate their target molecules. The capacity to cycle between 2 conformations enables these molecules to amplify or to temporize upstream signals. For example, it has recently been shown in neuronal cells, by fishing for GTP-bound RhoA with the Rho-binding domain of Rho kinase, that LPA induces an increase in GTP-bound Rho in a protein tyrosine kinase–sensitive way via Gα12/13, concomitant with growth cone collapse. Activation of Rho is accompanied by an increase of membrane-associated Rho and decrease of cytosolic Rho.

The activity of small G proteins is under the direct control of a large set of other regulatory proteins: specific factors that activate GTPases, specific factors that turn them off, and finally, specific factors that keep them in their inactive state (see Figure 1). For each of these regulating factors, several different molecular entities have been identified, and their number still is growing. Guanine nucleotide exchange factors (GEFs) enhance or catalyze the exchange of GDP for GTP. The slow intrinsic rate of GTP hydrolysis of the small GTPases is enhanced by GTPase-activating proteins, whereas guanine dissociation inhibitors (GDIs) slow the rate of GDP dissociation from the GTPases, thereby locking the G proteins into the inactive state. These GDIs bind to the carboxyl terminus of Rho. In this way, they prevent the translocation of the GTPases from the cytosol to the plasma membrane when activated. Ezrin, radixin, and moesin (ERM) proteins also have been implicated in membrane recruitment. In quiescent fibroblasts, Rho, Rac, and ERM proteins, but not Rho GDIs, are enriched in caveolar membranes compared with plasma membranes. Stimulation with growth factors results in a further recruitment of Rho, Rac, and ERM proteins. In endothelial cells (ECs), RhoA colocalizes with ERM proteins.

Upstream signaling events, which result in the activation of the small GTPases, are poorly understood. Very recently, 2 possible mechanisms were identified by which GPCR stimulation results in the activation of small GTPases. The first was the identification of a direct link between Gα and p115RhoGEF. The second is a ligand-independent activation of the epidermal growth factor receptor acting upstream from Rho. In fibroblasts, Cdc42, Rac, and Rho were initially found to act in a hierarchical cascade, which also seems to be true in some cases in ECs. Later on, it turned out that this cannot be taken as a general rule, inasmuch as more recent reports indicate that Rac and Rho also have some mutually antagonistic effects. This becomes immediately clear as one compares the cell extension–promoting effects of Rac and Cdc42 with the cell contraction–promoting effects of Rho.
MLC Phosphorylation

Rho GTPases regulate cytoskeletal changes involved in cell motility, shape, and contraction. The dominant regulatory system of the nonmuscle and smooth muscle F-actin cytoskeleton involves activation of myosin by phosphorylation of the myosin (regulatory) light chains (MLCs). Activated myosins bundle F-actin, resulting in the formation of F-actin filaments, of which the stress fibers are the most prominent group. Evidence is now accumulating that Rho GTPases are important regulators of MLC phosphorylation and F-actin organization.

MLC Kinases

The myosin II molecule is composed of 2 heavy and 2 distinct light chains, an essential and a regulatory one. Phosphorylation of the regulatory MLC increases ATPase activity of the myosin molecule and regulates myosin motor function of the actin/myosin system as its primary function. In the nonphosphorylated folded form, myosin cannot assemble into filaments. Phosphorylation also promotes myosin filament assembly by a conformation change in the myosin molecule. MLC phosphorylation is accomplished by a set of specific kinases, the classic Ca\(^2+\)/calmodulin-dependent MLC kinases (MLCKs). Several MLCK isoforms, in the range of 130 to 150 kDa, have been identified. Ser19 is the major site of phosphorylation induced by agonists that stimulate MLC phosphorylation. Under conditions of maximal stimulation, Thr18 also becomes phosphorylated.

In search of other MLCKs, Gallagher et al isolated a developmentally regulated MLCK, which was called embryonic MLCK. Embryonic MLCK seems to be an unique kinase because it is immunologically distinct from the above-mentioned 210-kDa MLCK and is also regulated by Ca\(^2+\). ECs significantly express embryonic MLCK. Besides embryonic MLCK, ECs express an endothelium-specific MLCK with a molecular mass of 214 kDa, which seems to have a role in endothelial permeability. Recently, evidence was obtained for a Ca\(^2+\)-independent MLCK, distinct from MLCK, involved in Ca\(^2+\) sensitization of smooth muscle cell contraction. This kinase phosphorylates MLC on Ser19 or Thr18, but its molecular identity remains to be elucidated.

Rho GTPases and MLC Phosphorylation

Several mechanisms by which the small G proteins regulate MLC phosphorylation have been elucidated (see Figure 2). The first Rho GTPase target shown to be involved in MLC phosphorylation was Rho kinase. Rho kinase acts itself as an MLCK, at least in vitro. Although the apparent \(K_m\) value (0.91 \(\mu\)mol/L) of Rho kinase for the MLC is lower than that (52 \(\mu\)mol/L) for MLCK, the molecular activity of Rho kinase for MLC is \(\approx 3\) times lower than that of MLCK. This may be the result of the lower amount of Rho kinase present in cells compared with the amount of MLCK, as in blood platelets, or this may be the result of a different localization within the cell. Therefore, the Ca\(^2+\)/calmodulin-dependent MLCK is thought to be the primary regulator of MLC phosphorylation at Ser19.

Rac and Cdc42 activate another family of kinases (the p21-activated kinases [Paks]) that is involved in MLC phosphorylation. Three isoforms of Pak have been identified: Pak1 (\(\alpha\)Pak), Pak2 (\(\gamma\)Pak), and Pak3 (\(\beta\)Pak). In HeLa cells, overexpression of Pak reduces MLCK activity and MLC phosphorylation, and cell spreading was inhibited. In vitro, it has been shown that Pak1 phosphorylates MLCK and inhibits the MLCK activity. This might in part explain the antagonistic effects of Rac and Rho. In addition to inhibiting MLCK activity, active Pak2 itself was shown to increase MLCK phosphorylation in ECs. Pak2 phosphorylates the MLCK on Ser19, in contrast to MLCK and Rho kinase, which phosphorylate MLCK on Ser19 and Thr18. ECs contract on exposure to Cdc42 or activated Pak2. Pak is required to initiate Ca\(^2+\)/calmodulin-independent cell retraction. These apparently contradictory findings probably do not reflect Pak isoform differences, inasmuch as other investigators have demonstrated that Pak1 increases MLC phosphorylation in ECs by use of microinjection of active Pak1. A physiological role of Pak remains to be elucidated, but Pak seems to be involved in cell migration.

Besides phosphorylating MLC directly, Rho GTPase–dependent kinases regulate MLC phosphate levels by inhibition of the MLC dephosphorylation. This reaction is as important as the phosphorylation reaction is for controlling the extent of MLC phosphorylation. MLC dephosphorylation is accomplished by a specific myosin phosphatase, myosin phosphatase type 1 (PP1M). Initially, it was assumed that the phosphatase activity was a steady one, but now it is known that phosphatase activity is regulated by other factors, including small GTPases. Activity of PP1M is inhibited by phosphorylation of the myosin binding subunit of PP1M by Rho kinase. In many cases, inhibition of PP1M accounts for the major contribution of Rho kinase to the elevation of MLC phosphorylation.
that SFs develop during EC adaptation to unfavorable or pathological situations, including wound healing, atherosclerosis, and hypertension.\textsuperscript{64,67–71} Remarkably, for each of these conditions, evidence has been obtained for the involvement of Rho-like small GTPases (see below in Functional Studies).

As pointed out above, Rho plays an eminent role in the formation of SFs, but how does Rho induce SFs? Many targets of Rho have been identified, including rhoteckin, rhophilin, PRK2, and citron.\textsuperscript{7} The target that has received major attention, however, is Rho kinase. Activation of Rho kinase results in an increased MLC phosphorylation. This MLCK phosphorylation promotes myosin filament assembly and actin-activated myosin ATPase activity, resulting in the bundling of actin filaments and the generation of tension.\textsuperscript{17}

However, other targets of Rho kinase are also involved in the regulation of SF formation (see Figure 3).\textsuperscript{72} Rho kinase phosphorylates proteins of the ERM family, promoting their interaction with actin and transmembrane receptors. Phosphorylation of adducin\textsuperscript{73} and of the Na-H exchanger\textsuperscript{74} by Rho kinase is also involved in SF formation in an unknown way.

Rho kinase also phosphorylates LIM kinase.\textsuperscript{75,76} LIM kinase is a cofillin-inactivating kinase.\textsuperscript{77} Cofillin exhibits actin-depolymerizing activity. Thus, activation of LIM kinase inhibits depolymerization of F-actin and in this way can promote the formation of SFs. Recently, the picture became even more complicated, inasmuch as LIM kinase was found to be activated also by Pak kinases.\textsuperscript{78} This means that LIM kinase could also be involved in the formation of actin structures induced by Rac and Cdc42.

Furthermore, Rho kinase activity alone is not sufficient for proper SF formation. Expression of a dominant active mutant of Rho kinase induces the formation of stellate SFs, which differ from the parallel SFs found in normal cells or induced by the activation of Rho.\textsuperscript{79} Recently, it has been shown that an appropriate balance of Rho kinase activity and Dia (another target of Rho) activity can induce SF formation, which is indistinguishable from Rho-induced SF formation. The expression of a dominant active mutant of Dia alone results in weak formation of parallel SFs.\textsuperscript{72,79,80} Dia is a profilin-binding protein and probably contributes to SF formation by localizing profilin-bound actin to sites where Rho is active. Phosphatidylinositol 4,5-biphosphate (PIP\textsubscript{2}) is also likely to be involved in SF formation, inasmuch as intracellular levels of PIP\textsubscript{2} can be increased by phosphatidylinositol 4-phosphate 5-kinase after the activation of Rho\textsuperscript{81}, and PIP\textsubscript{2} is known to promote actin polymerization.\textsuperscript{82}

**Figure 3.** Signal transduction pathways involved in RhoA-induced SF formation. RhoA induces SF formation by an increased actin polymerization and by an increased interaction between actin and actin-binding proteins, which are required for proper SF formation.\textsuperscript{72} Actin polymerization is increased by activation of Dia by Rho A.\textsuperscript{79} Activation of LIM kinase enforces this process by inhibition of the depolymerization of actin.\textsuperscript{75} The major target of activated RhoA in SF formation is Rho kinase. Rho kinase phosphorylates and activates many downstream targets involved in SF formation. PIP\textsubscript{2}-kinase indicates phosphorylation promotes myosin filament assembly and actin–myosin contractility. Furthermore, Rho kinase also phosphorylates LIM kinase.\textsuperscript{75,76} LIM kinase is a cofillin-inactivating kinase.\textsuperscript{77} Cofillin exhibits actin-depolymerizing activity. Thus, activation of LIM kinase inhibits depolymerization of F-actin and in this way can promote the formation of SFs. Recently, the picture became even more complicated, inasmuch as LIM kinase was found to be activated also by Pak kinases.\textsuperscript{78} This means that LIM kinase could also be involved in the formation of actin structures induced by Rac and Cdc42.

### Functional Studies

**Involvement of Rho GTPases in Cell Contraction and Cell Shape**

**Smooth Muscle Cell Contraction**

Many agents that activate Rho in vascular smooth muscle cells (VSMCs) in vitro are vasoconstrictors, such as LPA, thrombin, endothelin, and bombesin. In smooth muscle, there is strong evidence for the involvement of small GTPases in the contractile mechanism, of which RhoA plays the most prominent role, although other small G proteins, such as Rnd, have also been implicated. In VSMCs, it is known that the activation of RhoA results in Ca\textsuperscript{2+} sensitization,\textsuperscript{83} which is associated with the tonic component of VSMC contraction; ie, independent of a change in Ca\textsuperscript{2+}, MLC phosphorylation levels and contraction increase on the activation of Rho by vasoactive agents.\textsuperscript{84} Rho-mediated Ca\textsuperscript{2+} sensitization has been demonstrated to be responsible for hypertension in several animal models\textsuperscript{84} and to result in coronary artery vasospasm.\textsuperscript{85} The role of Rho GTPases in the process of Ca\textsuperscript{2+} sensitization in VSMCs will be described in some detail, as it was first described in VSMCs. The same process occurs not only in VSMC contraction but also in several other vascular processes, including platelet activation and endothelial permeability regulation, which have many aspects in common with VSMC contraction.

The dominant regulatory mechanism of nonmuscle and smooth muscle contraction is a Ca\textsuperscript{2+}/calmodulin-dependent MLC phosphorylation.\textsuperscript{86} However, the [Ca\textsuperscript{2+} ], is not always paralleled by the MLC phosphorylation level. So, other mechanisms in addition to a Ca\textsuperscript{2+}-dependent MLC phosphorylation must exist. It is firmly established now that Rho plays an important role in Ca\textsuperscript{2+} sensitization. A decade ago, the importance of G proteins in Ca\textsuperscript{2+} sensitization was suggested from experiments with permeabilized blood vessels. At a constant [Ca\textsuperscript{2+}], nonhydrolyzable GTP analogues or GTP plus α-adrenergic agonists induced a contraction.\textsuperscript{87,88} The involvement of Rho in Ca\textsuperscript{2+} sensitization was demonstrated by use of the specific Rho inhibitor C3-transferase.\textsuperscript{89} The Ca\textsuperscript{2+} sensitization is accompanied by a translocation of Rho from the cytoplasm to the cell membrane.\textsuperscript{90}

The first evidence that Rho kinase mediates the effects of Rho in VSMC contraction came from a study of Uehata et al.,\textsuperscript{84} who developed a specific inhibitor of Rho kinase, Y-27632, and showed that Rho kinase is involved in Ca\textsuperscript{2+} sensitization induced by a variety of agonists, including...
phenylephrine, thrombin, serotonin, endothelin-1, and the thromboxane agonist U-46619. This finding was confirmed by other studies.91,92 RhoA and Rho kinase were shown to be present in a variety of VSMCs.93 An interesting observation was that MLCK activity is not necessarily required for Rho kinase–induced contraction.94 Inhibition of Rho kinase did not affect the basal blood pressure, vessel tone, or heart rate.95 In vitro studies indicated that Rho kinase can increase MLC phosphorylation by inhibition of PP1M and by direct MLC phosphorylation.51,60 In a swine model of coronary artery spasm, it has recently been demonstrated in vivo with the use of hydroxyfasudil (another new inhibitor of Rho kinase) that Rho kinase was involved in agonist-induced hyperphosphorylation of MLC at Ser19 and Thr18.85

Rho kinase is also involved in VSMC migration and proliferation. These processes are essential for the remodeling of the vessel wall, which also contributes to the development of hypertension, (re)stenosis, and atherosclerosis (see below).95,96 Interestingly, the Rho-related protein Rnd1 inhibits Ca2⁺ sensitization of rat smooth muscle and acts as a natural antagonist of Rho in Ca2⁺ sensitization.90 The expression of Rnd1 in VSMCs was increased by the sex hormones estradiol and progesterone. These hormones are known to reduce vascular contractility.97

**Endothelial Permeability**
The endothelium is the main barrier that regulates the extravasation of blood constituents to the surrounding tissues. The most prevalent type of dysfunction of this barrier, which can result in vascular leakage, involves actin-myosin interaction at the margins of ECs via a Ca2⁺/calmodulin-dependent activation of the MLCK (see reviews98,99). This process has many characteristics in common with smooth muscle cell contraction. Rho proteins have been implicated in increased endothelial permeability. Comparable to the involvement of Rho in the tonic component of VSMC contraction, Rho is involved in increased endothelial permeability.100,101

Initial evidence of the involvement of Rho proteins in cell barrier (dys)function came from studies involving epithelial cell monolayers.102 The first experiments in ECs using the nonselective *Clostridium difficile* toxin B, which inhibits Rho, Rac, and Cdc42, showed that Rho proteins are essential for a proper endothelial barrier function.103 Additional experiments showed that the specific Rho inhibitor C3 exoenzyme inhibited thrombin- but not histamine-induced endothelial hyperpermeability and MLC phosphorylation in human umbilical vein ECs.100,101 Furthermore, it has been demonstrated that thrombin directly activates Rhoa, but not Rac1, in ECs.104 Other Rho-like small G proteins may be involved.105 However, it is less likely that small GTPases such as Rac and Cdc42 are involved in endothelial barrier hyperpermeability, inasmuch as they stimulate cell extension instead of cell contraction. A model was hypothesized in which the transient Ca2⁺-dependent increase in endothelial permeability can be prolonged or sensitized by activation of Rhoa and Rho kinase, similar to Ca2⁺ sensitization in VSMCs. Earlier studies have indicated the importance of inactivation of myosin phosphatase by thrombin,106,107 Essler et al100 have shown that transient inhibition of PP1M by thrombin is Rho dependent. Rho-mediated endothelial retraction is not restricted to thrombin-induced endothelial permeability but seems to be involved in many more cases of increased endothelial permeability. *Pasteurella multocida* toxin, endotoxin, and minimally oxidized LDL also induce an endothelial barrier dysfunction via Rho/Rho kinase, even without an increase in [Ca2⁺].108,109 Leukocytes probably use the same mechanism to transmigrate through endothelial monolayers (see Transmigration of Circulating Cells). Interestingly, Siess et al108 have shown that LPA, a well-known Rho activator, is probably a major active component of oxidized LDL with respect to endothelial activation and accumulates in atherosclerotic plaques, and LPA has been shown to increase endothelial permeability.111 Endothelial barrier dysfunction is a hallmark of the early atherosclerotic lesion development. This suggests an important contribution of the Rho-mediated endothelial permeability increase to the development of atherosclerosis.

In addition to the inhibition of the myosin phosphatase by Rho, other mechanisms of Rho action may be involved in endothelial barrier dysfunction. In the case of peroxynadate-induced endothelial hyperpermeability, a Rho-mediated activation of the (endothelial) MLCK by tyrosine phosphorylation of the MLCK has been reported.112 It remains to be investigated whether vasoactive compounds, such as thrombin and LPA, induce such an activation of MLCK by tyrosine phosphorylation and whether Rho kinase is involved in MLCK activation.

Another function of Rho proteins, which may be involved in the regulation of endothelial barrier function, is the regulation of cell-cell interactions. Such a mechanism has been demonstrated in epithelial cells.113,114 However, Braga et al115 have shown that ECs are exceptional in this sense. They have demonstrated that in contrast to activity in other cell types, Rho activity is not necessary for cadherin-based endothelial cell-cell interaction and that vascular endothelial cadherin localization was insensitive to the inhibition of either Rho or Rac. Furthermore, Essler et al100 have shown that inhibition of Rho by C3-transferase does not prevent the thrombin-induced dissociation of catenins from the cytoskeleton. Wojciak-Stothard et al44 have shown that the Cdc42-, Rac-, and Rho-dependent tumor necrosis factor-α–induced stress fiber formation is also accompanied by an at least partly Cdc42-, Rac-, and Rho-independent dispersion of vascular endothelial cadherin from intercellular junctions. Thus, at the moment, there is no firm support for the role of Rho proteins in the direct regulation of adherens junction organization in ECs.

Future studies will be necessary to verify whether a similar Rho-induced Ca2⁺-sensitization mechanism underlies the increased permeability that is enhanced by leukocytes and humoral factors circulating in patients with prolonged edema as well as in the increased permeability in arterial segments after stent implantation.116

**Platelet Activation**
Similar to VSMC contraction, Rho/Rho kinase signaling has been implicated in MLC phosphorylation in activated blood platelets.52,79,117,118 In fact, Rho kinase was first isolated from platelets.119 Phosphorylation at Ser19 of platelet MLCK increases an actomyosin contractile response that is involved in platelet shape change and secretion. Inhibition of Rho kinase prevented ATP secretion.52 Several reports indicated that the agonist-induced Ca2⁺ rise is not required for platelet shape change.120,121 Rho signaling is also involved in platelet adhesion to fibrinogen.122 Activation of Rho kinase is accom-
panied by a translocation of Rho kinase to the actin cytoskeleton. There is evidence that Rho kinase contributes to a great extent to the platelet secretion induced by agonists and at low concentrations of thrombin (a strong agonist) but not at high concentrations of thrombin. The shape changes induced by weak agonists are fully prevented by the inhibition of Rho kinase.

**Cardiac Myocyte Hypertrophy**

Several reports have indicated that the Rho/Rho kinase pathway is important for hypertrophic signaling in cultured cardiac myocytes induced by adrenergic agonists, angiotensin II, and endothelin-1 (see review). A role for the Rho GTPases in myocyte hypertrophy is supported by several recent studies demonstrating the effects of active and inactive forms of RhoA on hypertrophic target gene expression. Similarly, expression of active Rac1 stimulated the hypertrophic program, whereas expression of inactive Rac was inhibitory. The effects of RhoA on the morphological and cytoskeletal aspects of the hypertrophy were less clear, with recent reports giving conflicting results. Cardiac-specific overexpression of RhoA in mice could not unequivocally confirm a major role of RhoA in cardiac hypertrophy. Overexpression of RhoA resulted in atrial, but not ventricular, enlargement and was accompanied by contractile failure. Interestingly, it has been suggested that RhoA regulates cardiac sinus and atrioventricular nodal function. Cardiac-specific overexpression of active Rac1 resulted in 2 different phenotypes: a (lethal) dilated cardiomyopathy and a resolving transient cardiac hypertrophy in juvenile mice.

**Involvement of Rho GTPases in Cell Motility**

**Vascular Smooth Muscle Migration**

Many vascular remodeling processes depend on the motility of vascular cells, which requires a coordinated rearrangement of the actin cytoskeleton and cell-matrix interactions. In cardiovascular diseases, such as hypertension, atherosclerosis, and restenosis after angioplasty, vascular remodeling requires changes in the VSMC cytoskeleton.

The migratory response can be induced by signals from chemotactic factors and growth factors as well as by mechanical wounding. In a large variety of cell types, an essential role of Rac in cell migration has been established. On the one hand, Rac is important for the formation of protrusion of lamellipodia at the leading edge of the cells and forward movement. On the other hand, Rac seems to be involved in cell retraction at the trailing edge via Pak. Cdc42 has been shown to be involved in chemotactant gradient sensing; ie, Cdc42 regulates cell polarity and the direction of migration. Data concerning the role of Rho in cell migration are still conflicting and may depend on the extent of Rho activation. A low degree of Rho activity is necessary for the generation of adhesive forces and probably for cell retraction. A high degree of Rho activity seems to inhibit migration in some cases through the formation of strong FAs, but these studies were performed in nonvascular cells. In VSMCs, Rho/Rho kinase signaling is involved in cell migration in wound healing assays. Recent preliminary data indicate that the inhibition of Rho kinase reduces neointimal formation in several animal models and underscores the importance of RhoA/Rho kinase signaling in VSMC migration. How the responses of the different GTPases are coordinated remains an interesting area for future research.

**Endothelial Migration and Angiogenesis**

Comparable to VSMC migration, RhoA/Rho kinase signaling has been implicated in endothelial migration. Angiogenesis, the formation of new blood vessels from existing ones, is a process that depends not only on proliferation but also on the migration and invasion of ECs. Lee et al recently identified sphingosine-1-phosphate as a new angiogenic factor and showed that sphingosine-1-phosphate–induced angiogenesis was completely blocked by the inhibition of Rho with C3-transferase. Similarly, the inhibition of RhoA/Rho kinase signaling reduced tube formation in the Matrigel assay and angiogenesis in the chick embryo. Interestingly, Rho is involved in activation of the vascular endothelial growth factor receptor VEGFR2. The involvement of similar signal transduction cascades seems appropriate for the mutual interaction between angiogenesis and endothelial permeability. Vascular leakage can be an early manifestation of angiogenesis and results in the extravasation of a fibrinous exudate, providing a provisional matrix for the ingrowth of ECs. It now seems that Rho activation is an ongoing process in angiogenesis and that initial alterations in actin–nonmuscle myosin interaction prepare the ECs for migration and ingrowth. When the importance of Rac/Pak signaling in EC migration is considered, it is likely that Rac activation is also involved in angiogenesis.

**Transmigration of Circulating Cells**

For lymphocyte transmigration, a multistep model of lymphocyte-EC recognition and recruitment of lymphocytes from the blood has been proposed in which the activation of Rho GTPases plays a central role. This model involves (1) contact through microvillous receptors and rolling of lymphocytes and (2) activation of lymphocytes through G protein–linked receptors, which trigger (3) integrin adhesion to vascular ligands in seconds through an intracellular pathway involving the small GTP-binding protein Rho, followed by (4) diapedesis. This general model implicates changes in Rho GTPase activity in the migrating and in the barrier-forming (endothelial) cell. This model also seems to be applicable to leukocyte transmigration and tumor cell invasion.

Rho, Rac, and Cdc42 regulate the actin cytoskeleton dynamics necessary for chemotaxis of circulating cells. Stimulation of leukocytes with FMLP or interleukin-8 induces a rapid activation of RhoA. In human neutrophils (and eosinophils), FMLP also induces a very rapid and transient activation of Rac. Inhibition of Rho by C3 exoenzyme blocks the adhesion of neutrophils to fibrinogen, and inhibition of Rho kinase completely inhibits chemotactic peptide–induced MLC phosphorylation and neutrophil migration. Whereas activation of Rho GTPases is clearly critical for the adhesion and migration of circulating cells, the regulation of their activity and the relative individual contribution of each of the distinct GTPases is far from being resolved. Comparable to transmigration of leukocytes and lymphocytes, tumor cell invasion involves RhoA/Rho kinase signaling.
Evidence that adhesion of circulatory cells to the endothelium directly activates Rho signaling in the endothelium, without the involvement of an intermediate inflammatory mediator, is now accumulating. In the multistep model outlined above, lymphocyte integrin clustering and adhesion to the counterreceptors on the endothelium takes a central place. Integrin-mediated adhesion can also activate Rho signaling in the EC and thus cause local endothelial retraction. Activation of Rho in ECs in this way might facilitate the transmigration of these cells across the endothelium,159,160 by creating small pores in the endothelial barrier comparable to those involved in the passage of macromolecules,161 and it is accompanied by an increased MLC phosphorylation162–165 and the formation of SFs159,161,163,166 (see Endothelial Permeability). In contrast to the activation of circulatory cells by chemoattractants, in which Rho, Rac, and Cdc42 are involved, activation of the endothelium by circulatory cells probably involves the activation of Rho but not of Rac and Cdc42.166

Pharmacological Modulation of Rho-Like Small GTPase Signaling

Studies involving the function and signaling of Rho-like small GTPases have resulted in the identification of a range of new targets for pharmaceutical intervention.167 However, progress in the field of Rho-like GTPase research is still hampered by the lack of suitable inhibitors and activators even for in vitro work. One of the most important tools currently used in in vitro experiments is the expression of constitutive active or dominant-negative mutants, which are available for Rho,1 Rac,1 Cdc42,26 and their targets Rho kinase,168 Pak,58,169,170 and MRCK.171 However, their (therapeutic) application is limited because the expression of these mutants is prolonged and therefore interferes with all of the functions of the particular protein.

Rho proteins are targets for covalent modification by toxins of many pathogenic bacteria (see reviews10,172,173). This suggests an important role for Rho in vivo. Among the bacterial toxins, several specific activators and inhibitors of Rho function are currently known. Toxin B from Clostridium difficile is a general inhibitor of Rho, Rac, and Cdc42. C3-transferase from Clostridium botulinum has a high specificity toward Rho.172,173 A new development is the in vivo application of C3 transferase in mice via an osmotic minipump.174 Cytotoxic necrotizing factor-1 from Escherichia coli and Pasteurella multocida toxin are specific activators of Rho.174,175

The strategies indicated above are based on the inhibition of all of the functions of either one or more Rho-like small GTPases. Specificity might be achieved by interfering with the function of specific downstream effector or upstream regulator molecules. The latter principle has been applied in the development of peptides based on Rac GTPase-activating protein molecules, which are effective in the inhibition of Rac-mediated oxidant production.167,176 The former principle was used in the development of several inhibitors with high specificity for Rho kinase compared with MLCK and protein kinase C: Y-27632 and related compounds184,177 and fasudil and its hydroxyl derivative.185 Both compounds can be used in vivo without major effects on basal heart rate and blood pressure, and no changes in blood and urine chemistry have been reported so far.184,185,151 Y-27632 reduced elevated blood pressure in several animal models of hypertension.184 Hydroxyfasudil reduced coronary artery vasospasm in a swine model.185 SCH51344 has been shown to inhibit certain downstream activities of Rac, including regulation of the cytoskeleton and transformation, but it also interferes with Ras signaling.178,179

Posttranslational lipid modifications are important for interactions with GEFs and for downstream functions of Rho and are subject to regulation.13 An exciting new development with therapeutic consequences is the use of statins as inhibitors of Rho function. Statins are inhibitors of the enzyme β-hydroxy-β-methylglutaryl coenzyme A reductase and are used in lipid-lowering therapy. Statins prevent isoprenylation of Rho proteins as a side effect of inhibition of the β-hydroxy-β-methylglutaryl coenzyme A reductase. Isoprenylation is necessary for targeting of RhoA to the plasma membrane.37,180,181 This may contribute to the non–lipid-related beneficial effects of statins, including reduced smooth muscle cell proliferation,182 reduced inflammation and endothelial permeability,183,184 stroke protection,185 and increased fibrinolytic activity.186 However, relatively high concentrations of statins in the micromolar range are necessary to prevent isoprenylation of Rho in vitro.37,180,187,188 The reduction of stroke size by statins in an experimental mouse model via this mechanism underscores the importance of this recognition.174,185 Specificity could be obtained by the use of inhibitors of protein isoprenylation, ie, inhibitors of farnesyltransferase and geranylgeranyltransferases, as was successful in blocking the oncogenic properties of Ras.189 Whether this alternative really will be an advantage in the case of inhibition of Rho-like small GTPases remains to be seen, because the statin drugs themselves are generally regarded as safe.

Perspective

Our understanding of the mechanisms by which Rho GTPase activity is regulated and of the downstream signaling pathways involved has been enormously expanded during the past few years. The involvement of Rho GTPases in many vascular pathologies has now been established. By regulating the actin cytoskeleton, Rho proteins control critical processes, including cell migration, contraction, cell shape, and adhesion. Each of these processes is involved in several vascular phenomena: Rho-regulated cell motility is involved in vascular processes, including angiogenesis, wound healing, leukocyte transmigration, and tumor cell invasion; Rho-regulated contractility is involved in the sensitization of Ca2+-induced MLCK activity, resulting in a prolonged cell contraction in processes including VSMC contraction related to hypertension, endothelial retraction in vascular hyperpermeability, and platelet activation; Rho-regulated cell shape changes are involved in the shear stress–induced changes in ECs that are involved in atherosclerosis and restenosis, in cardiac hypertrophy, and in platelet activation; and Rho-regulated adhesion is involved in platelet aggregation and leukocyte transmigration (Figure 4). It is notable that less information is available regarding the role of Cdc42 compared with Rho and Rac in vascular disorders.

However, our knowledge regarding the precise role of Rho GTPases in these disorders remains fragmentary, and we are in an early stage of learning how these processes are
integrated and what the initial triggers are. Furthermore, one has to be aware that similar processes can be regulated differently in distinct cell types. This is demonstrated by the following examples: thrombin-induced Ca^{2+} mobilization is blocked by the inhibition of Rho in fibroblasts but not in ECs. Rho is involved in cadherin function in epithelial cells but not in ECs. and phosphorylation of MLC is induced by Pak in ECs, whereas MLC kinase activity is reduced by Pak in HeLa cells.

Studies involving the function of Rho-like GTPases have resulted in the identification of new targets for pharmacological intervention. The detailed present knowledge of the structure of these proteins will facilitate the development of additional drugs with higher specificity. The discovery that statin drugs inhibit Rho function will obtain clinical application. It is a future challenge to apply the present knowledge of Rho-like GTPase function in the specific treatment(s) of vascular disorders.

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References


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155. Walsh AB, Dhanasekaran M, Bar-Sagi D, Kumar CC. SCH 51342-induced reversal of RAS-transformation is accompanied by the specific


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