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Review

Multifaceted role of Rho, Rac, Cdc42 and Ras in intercellular junctions, lessons from toxins

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ABSTRACT

Tight junctions (TJs) and adherens junctions (AJs) are dynamic structures linked to the actin cytoskeleton, which control the paracellular permeability of epithelial and endothelial barriers. TJs and AJs are strictly regulated in a spatio-temporal manner by a complex signaling network, including Rho/Ras-GTPases, which have a pivotal role. Rho preferentially regulates TJs by controlling the contraction of apical acto-myosin filaments, whereas Rac/Cdc42 mainly coordinate the assembly–disassembly of AJ components. However, a subtle balance of Rho/Ras-GTPase activity and interplay between these molecules is required to maintain an optimal organization and function of TJs and AJs. Conversely, integrity of intercellular junctions generates signals through Rho-GTPases, which are involved in the regulation of multiple cellular processes. Rho/Ras-GTPases and the control of intercellular junctions are the target of various bacterial toxins responsible for severe diseases in man and animals, and are part of their mechanism of action. This review focuses on the regulation of TJs and AJs by Rho/Ras-GTPases through molecular approaches and bacterial toxins.

Contents

1. Introduction

In multicellular organisms, cells come into contact and interact specifically with other cells, and are organized in tissues and organs. Epithelial and endothelial tissues are of a particular importance, since they constitute barriers controlling the passage of water, solutes, and cells from one compartment to another. In addition, epithelia such as those of the respiratory or gastro-intestinal tract, form the interface between the external and internal environments of the organism, and thereby constitute the first line of defense against invasive pathogens or toxins. For example, the intestinal epithelium represents the largest area of the body in contact with the environment. Its main function is the absorption of nutrients, but it also regulates water and ion homeostasis, and assures a protective barrier against the resident microflora and pathogens. Epithelial and endothelial cells are connected by two main types of intercellular junctions, tight junctions (TJ) and adherens (AJ)

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junctions, which control the paracellular permeability through the intercellular spaces. In epithelial cell barriers, TJs and AJs are well defined and are differentially distributed along the intercellular cleft. TJs form an apical rim, whereas AJs are localized basolaterally below TJs. In contrast, in endothelia, TJs and AJs have no distinct spatial localization, but are intermingled [1]. It is noteworthy that TJs and AJs are intracellularly linked to the actin cytoskeleton. TJs and AJs are highly regulated dynamic structures, whose pivotal regulatory partners, among many other regulators, include Rho family GTPases, which control both the actin cytoskeleton and the integrity of intercellular junctions, and Ras involved in cell proliferation and differentiation. Moreover, intercellular junction components and regulatory molecules such as Rho- and Ras-GTPases are a target for various pathogens and toxins, as well as a target for mutations or dysregulation leading to tumor initiation. Indeed, mutations of Ras proteins or dysregulation of Rho-GTPase signaling contribute to the initiation and progression of numerous types of cancer [2–5]. Rho-GTPases have an effect on the invasion and migration of metastatic cells through remodeling of the actin cytoskeleton and subsequent disassembly of intercellular junctions [32]. Loss of cell–cell contacts is a major step in cancer progression.

After a succinct description of TJ and AJ organization (for more details see reviews: [1, 6–15]), and Rho/Ras-GTPases, this review focuses on the role of Rho-GTPases and Ras in the regulation of mature junctions and barrier function, based on works using genetic mutants and bacterial toxins.

2. Tight junctions

TJs are localized at the most apical part of epithelial cells, and form a network of close contacts (TJ strands) between membranes of adjacent cells. TJs control the paracellular transport of ions, water, solutes and cells, and in addition they constitute a fence separating apical and basolateral membrane proteins. TJs are formed of two types of membrane proteins, occludin and claudins, which are associated with cytoplasmic proteins, linking TJs to the actin cytoskeleton (Table 1).

Occludin (65 kDa, two isoforms) contain four transmembrane domains and two extracellular loops. The C-terminal domain, localized in the cytoplasm, directly binds to ZO-1 (zonula occludens), which in turn associates with the apical actin rim. This region is rich in phosphorylation sites (tyrosine, serine, and threonine) which can be modified by kinases (c-Yes, protein kinase C) or phosphatases (PP2A). Non-phosphorylated occludin is distributed on basolateral membrane and in cytoplasmic vesicles, whereas phosphorylated occludin is localized in TJs, leading to a decreased paracellular permeability [12,13,16].

Claudins (18–27 kDa; 24 isoforms) like occludin, are tetraspan proteins with two extracellular loops and a C-terminal cytoplasmic domain, which binds through PDZ (Post synaptic density protein, Drosophila disc large tumor suppressor, Zonula occludens protein) binding motifs to ZO proteins, to PATJ (PALS1 associated tight junction protein) and also to MUPP1 (multi-PDZ domain protein-1). Claudins however, share no sequence homology with occludin. Claudins are the major components of TJ strands and have a determinant role in the barrier function. They form paracellular channels, which are selective for ions through their first extracellular loop from neighboring cells [6,7,9].

ZO (Zonula occludens) proteins (ZO-1, 220 kDa; ZO-2, 160 kDa; ZO-3, 130 kDa) contain protein binding domains (PDZ, SH3, guanylate kinase homologous domains) and are scaffolding proteins linking TJ proteins to the actin cytoskeleton, and possibly also linking TJs to AJs. ZO-1 and ZO-2 bind to occludin and α-catenin, while ZO-3 binds to p120-catenin *in vitro*. Phosphorylation of occludin reduces the

Table 1
Main tight junction (TJ) and adherens (AJ) junction components, binding partners and potential roles.

Protein	Protein type	Binding partners	Potential role	References
Tight junctions				
Occludin	Transmembrane protein	ZO-1, -2, -3, JAM-A, cingulin	Cell–cell adhesion Regulation of paracellular permeability	[6, 189]
Claudins	Transmembrane proteins	ZO-1, -2, -3, PATJ, MUPP-1	Cell–cell adhesion Regulation of paracellular permeability	[16]
JAMs, CAR, ESAM	Transmembrane proteins	Occludin, ZO-1, cingulin, PAR-3, MUPP-1, AF-6/afadin	Cell–cell adhesion Regulation of junction assembly	[18]
Tricellulin	Transmembrane protein		Cell–cell adhesion	[189]
ZO-1	Cytoplasmic adaptor protein	Occludin, claudins, JAMs, ZO-2, -3, cingulin, F-actin, α-catenin	Regulation of junction assembly	[6,189]
ZO-2, ZO-3	Cytoplasmic adaptor protein	Occludin, claudins, ZO-1, F-actin, cingulin, α-catenin	Regulation of junction assembly	[6, 189]
MUPP-1	Cytoplasmic adaptor protein	Claudins, JAMs, CAR	Regulation of junction assembly	[6,189]
PATJ	Cytoplasmic adaptor protein		Regulation of junction assembly	[6,189]
Cingulin	Cytoplasmic adaptor protein	ZO-1, -2, -3, JAM, F-actin, myosin	Regulation of junction assembly	[6,189]
PAR-6	Cytoplasmic adaptor protein	PAR-3, PKC, Cdc42,	Regulation of junction assembly and polarization	[190]
AF-6/afadin	Cytoplasmic adaptor protein	ZO-1, Ras, F-actin	Regulation of junction assembly	[1]
PILT, JEAP	Cytoplasmic adaptor protein			[1]
Adherens junctions				
Cadherins	Transmembrane protein	Cadherin, β-catenin, p120-catenin, plakoglobin	Cell–cell adhesion	[1,24]
α-catenin	Cytoplasmic adaptor protein	β-catenin, F-actin, ZO-1, Ena-VASP, formin-1, afadin, Zyxin, Spectrin, α-actinin, vinculin	Regulation of junction assembly	[1,191]
β-catenin	Cytoplasmic adaptor protein	α-catenin, p120-catenin, Lin-7, IQGAP, fascin, PI3-kinase	Regulation of junction assembly	[1,191,192]
p120-catenin	Cytoplasmic adaptor protein	E-cadherin, β-catenin, VAV-2 (Rho-GEF)	Regulation of junction assembly	[20]
Plakoglobin	Cytoplasmic adaptor protein	α-catenin, cadherin, desmocollin, desmoglein, desmoplakin, keratin5	Regulation of junction assembly	[192]
Nectin	Transmembrane protein	Nectin, afadin, PAR-3	Cell–cell adhesion	[33,193]
Afadin	Cytoplasmic adaptor protein	Nectin, α-catenin, F-actin, ZO-1, Ponsin, profilin, Bcr, c-Src	Regulation of junction assembly	[33,35,191]
Vezatin	Transmembrane protein	Myosin VIIA, cadherin–catenin complex	Regulation of junction assembly	[194]
EPLIN	Cytoplasmic adaptor protein	F-actin, α-catenin	Regulation of junction assembly	[32]

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association of ZO proteins to occludin. ZO-1 and ZO-3 bind to actin filaments through their proline rich C-terminal region, whereas ZO-2 associates with the actin binding protein 4.1R. In addition, the three ZO isoforms associate to form complexes [1,17].

Junctional adhesion molecules (JAM) (32 kDa glycoprotein, 3 isoforms) contain a transmembrane segment, an extracellular domain encompassing two immunoglobulin-like subdomains, and a short cytoplasmic tail. JAMs function as cell–cell adhesion molecules through their extracellular domains, which are capable of homophilic interaction and can form heterophilic associations with various ligands such as integrins. In addition, JAMs associate with intracellular partners such as ZO-1 and the protease-activated receptor PAR-3 [6,7,9].

Numerous other proteins are involved in TJs: tricellulin, a tetraspan protein; coxsackie and adenovirus receptor (CAR); endothelial cell-selective adhesion molecule (ESAM); JAM4; AF-6/afadin; PAR-3; MUPP-1; cingulin; PILT (protein incorporated later into TJ); and JEAP (junction-enriched and -associated protein). This results in a complex TJ organization. The assembly of junctional adhesion proteins through homophilic and heterophilic interactions is mediated by specific domain-binding motifs. For example, JAM-A, JAM-B and JAM-C contain a class II PDZ domain-binding motif and directly interact with ZO-1 and PAR-3, whereas CAR, has a class I PDZ domain-binding motif at its C-terminus and associates with Ligand-of-Numb protein X1. Epithelial CAR mediates heterophilic interaction with JAM-like molecules, thereby modulating neutrophil migration as well as the attachment and entry of group B coxsackie viruses [7,18].

3. Adherens junctions

AJs result from the complex association of multiple components (Table 1) and play a pivotal role in the initiation of intercellular contacts between neighboring cells and in stabilizing adhesion. Moreover, AJs are multifunctional structures involved in the control of the actin cytoskeleton, focal adhesion, intracellular signaling, and transcription regulation.

Cadherins (120 kDa) contain only one transmembrane segment and five extracellular repeat domains, which mediate Ca^{++}-dependent cell adhesion via homophilic interactions between adjacent cells. Among the isoforms E- (epithelial), VE- (vascular endothelia) cadherin, N- (neural), P- (placental), and R- (retina) cadherins, E-cadherin is the major type distributed in epithelial tissues and VE-cadherin is specifically expressed in endothelial cells [1,8,17,19].

The C-terminal cytoplasmic domain of E-cadherin interacts with proteins, which controls E-cadherin regulation (endocytosis, recycling and degradation) as well as E-cadherin-mediated intracellular signaling, gene transcription, and local actin cytoskeleton organization. It contains a juxtamembrane domain (JMD) that interacts with p120-catenin and Hakai, an E_3-ubiquitin ligase, and a catenin binding domain (CBD) for β-catenin [20,24].

E-cadherin has an important role in cell–cell adhesion as well as in the stabilization of epithelial cell phenotype. Decreased cadherin function induces disassembly of cell–cell adhesion and disturbs cadherin-mediated signaling, while leading to de-differentiation from an epithelial to a mesenchymal phenotype, as well as to an increased cell migration characteristic of invasive tumor cells [25].

Catenins mediate the interplay between a cadherin complex and the actin cytoskeleton, and also govern several signaling pathways that control morphogenesis and tissue homeostasis.

β-Catenin (88 kDa) is characterized by 13 repeats of the armadillo domain, which consists in a triple α-helix of 42 amino acids. β-Catenin binds to the C-terminal part of E-cadherin when phosphorylated on three serine residues (Ser684, ser686, Ser692) by CKII (casein kinase) or GSK-3β (glycogen synthase kinase). In contrast, tyrosine phosphorylation (Tyr489 or Tyr654) of β-catenin by Src, Abl, or EGF receptor prevents the binding to E-cadherin [9,11]. In addition, phosphorylation of β-catenin at Tyr142 by Fer/Fyn, tyrosine kinases constitutively bound to p120-catenin and activated by Yes kinase, decreases the association between α- and β-catenin [26,27]. β-Catenin also binds to α-catenin in a mutual exclusive manner (see below). Plakoglobin (or γ-catenin), which is homologous to β-catenin, is also able to bind to α-catenin and cadherin, but its role in AJs is still not fully understood [1,28].

α-Catenin (102 kDa) is homologous to vinculin, an actin-binding protein. α-Catenin contains three vinculin homology domains and differs considerably in sequence from the other catenins [11]. α-Catenin exists both as monomers and dimers. Recently, it has been shown that monomeric α-catenin binds to β-catenin and not to actin, and inversely dimeric α-catenin associates with actin filaments but not with β-catenin (reviewed in [17,29]). In addition actin filaments bound to α-catenin can no longer associate with the Arp2–3 complex. Thus, α-catenin dimerization, which occurs at high concentrations of α-catenin probably in membrane at cell–cell contacts, where cadherin–catenin complexes are clustered, inhibits Arp2–3 and in so doing switches the actin polymerization from a branched structure such as is characteristic of lamellipodia, to bundled actin cables [17,30]. Moreover, α-catenin binds to other actin-binding proteins: ZO-1, a linker between AJ and TJ structures; Ena-VASP (enabled, vasodilatation stimulated phosphoprotein) which promotes actin polymerization; formin-1, an actin nucleator of unbranched actin filaments, afadin (see below); zyxin; spectrin [11,31]; and EPLIN (epithelial protein lost in neoplasm) [32]. But, the exact role of each protein in the control of actin polymerization at AJs remains to be determined.

p120-catenin (120 kDa, 4 isoforms) from the armadillo related proteins, was first described as Src substrate. p120 binds to the juxtamembrane domain of E-cadherin and regulates the transport of E-cadherin to membrane as well as the stability of AJs [31]. Phosphorylated p120 binds to E-cadherin with high affinity and thus prevents its endocytosis and degradation, or it increases the recycling of internalized E-cadherin to membranes [17,31]. In addition, p120 modulates the actin cytoskeleton through inactivation of RhoA and activation of Rac and Cdc42 (see below) [20]. The role of p120 is complex and differs according to the cell types, leading to an increase or a decrease in adhesion activity [1,23].

The nectin–afadin complex is involved in Ca^{++}-independent intercellular adhesion and plays a role in the organization of AJs and TJs.

Nectin (4 isoforms and several variants) contains a single transmembrane domain, an extracellular region containing three immunoglobulin-like domains, and a cytoplasmic extension. Nectin binds to the cytoplasmic C-terminus of afadin through a PDZ domain, and afadin binds along the side of actin filaments, but not to filament extremities. Nectin–afadin form dimers and nectin molecules from neighboring cells associate with each other through at least the first immunoglobulin-like domain. In epithelial cells, nectin–afadin are the first complexes, which assemble at the primary cell–cell contacts. Subsequently they recruit E-cadherin. Independently of nectin–afadin complexes, E-cadherin–catenin complexes linked to actin filaments form microclusters and rapidly recruit nectin–afadin molecules. These primordial junctions fuse and mature in AJs along the basolateral side of neighboring cells. At the apical side, they recruit JAMs, occludin, and claudins to form TJs [33,34]. Nectin–afadin and E-cadherin–catenin complexes are physically and functionally associated by a mechanism that remains to be elucidated [35].

4. Rho- and Ras-GTPases

Rho proteins belong to the Ras superfamily. They are small (21–25 kDa) molecules that share structural homology and become activated only when bound to GTP. They are molecular switches that regulate various cellular processes including actin dynamics, endocytosis, gene transcription, cell-cycle progression, and differentiation. So far twenty members have been identified in the Rho-GTPase family

Table 2
Role of Rho-GTPases in the control of tight junctions (TJ) and adherens junctions (AJ) and functional consequences in cell barrier integrity.

GTPase	Mode of GTPase alteration	Cell model	Major effects on TJ/AJ	Functional consequences	References
RhoA	D. active	MDCK	Increased actin stress fibers Alteration of ZO-1 distribution	Increased paracellular permeability	[66]
			Redistribution of occludin, ZO-1, claudin-1 and -2, JAM-1 Increased detergent solubility of ZO-1 and claudin-2 No alteration of AJ protein localization		[60]
			Disorganization of TJ ultrastructure Redistribution of ZO-1 and occludin Increased detergent solubility of ZO-1 No change in detergent solubility of ZO-2 and occludin		[59]
			Increased actin filaments at basal and apical sides No change in E-cadherin and β-catenin localization	ND	[63]
			Accumulation of ZO-1 and occludin at TJs Increased occludin phosphorylation No alteration of ZO-1 phosphorylation		[61]
	D. inactive	MDCK	Reduction of apical and basolateral actin filaments No change in TJ ultrastructure No change in ZO-1 and occludin localization Decreased detergent solubility of ZO-1 No change in detergent solubility of ZO-2 and occludin	Increased paracellular permeability	[60] [59]
			Reduction of actin filaments Inhibition of LPA-induced actin filaments Inhibition of LPA-induced increase of paracellular permeability	No change in paracellular permeability	[72]
			No change in E-cadherin and β-catenin localization	ND	[63]
	D. inactive, C3		Decreased localization of ZO-1 and occludin at TJs Decreased occludin phosphorylation No change in E-cadherin distribution		[61]
	C3 (incativation)		Disappearance of ZO-1 and E-cadherin staining		[63]
Rho	C3 (inactivation)	T84	Depolymerization of actin filaments at the apical side, but not at the basal side Redistribution of ZO-1 No change in E-cadherin distribution	Increased paracellular permeability	[81]
	D. inactive C3	HUVEC	Disappearance of actin stress fibers	Decreased paracellular permeability	[64]
	C3 (inactivation)		Disappearance of actin stress fibers No change in E-cadherin distribution	ND	[113]
		EC	Disassembly of actin stress fiber with focal adhesion Reduction of actin filaments Reorganization of actin filaments and tyrosine-phosphorylated proteins to cell–cell junctions, increased cell surface area, and increased cell–cell apposition	Enhanced endothelial barrier function	[107]
		SCLC	Actin cytoskeleton reorganization	Increased E-cadherin mediated cell–cell adhesion	[195]
	EDIN, C3, RhoA RNAi	HUVEC HMEC	Disruption of actin stress fibers Formation of microapertures	Increased endothelial permeability	[115]
	CNF_y (activation)	MyEnd, MesEnd, HDMEC, PAEC	Increased stress fiber formation Redistribution of VE-cadherin Formation of intercellular gaps (except in MyEnd)	Increased endothelial permeability (except in MyEnd)	[177]
RhoA	D. active D. inactive	S180 cells		No change in E-cadherin cell–cell adhesion	[121]
Rac1	D. active	MDCK	Increased of actin filaments at cell–cell contacts	No change in barrier permeability	[66]
			Disorganization of TJ ultrastructure Alteration of ZO-1 and occludin localization No change in detergent solubility of ZO-1, ZO-2 and occludin	Increased paracellular permeability	[59]
			Redistribution of claudin-1 and -2 Increased detergent solubility of claudin-1 and -2 Reduction of actin stress fibers		[60]
	D. inactive		Redistribution of claudin-1 and -2, JAM-1 Reduction of E-cadherin detergent solubility		[60]
			No change in TJ ultrastructure No change in ZO-1 and occludin localization No change in detergent solubility of ZO-1, ZO-2 and occludin		[59]
	D. active		Increased actin filaments at cell–cell adhesion sites Increased E-cadherin and β-catenin at cell adhesions Decreased detergent solubility of E-cadherin Thicker cell–cell contacts on the lateral side (EM)	ND	[63]
	D. inactive		No change in actin filaments at cell–cell contacts Decreased E-cadherin and β-catenin at cell adhesions Increased detergent solubility of E-cadherin Loose cell–cell contacts on the lateral side (EM)		
	D. active		Recruitment of WAVE2 at cell–cell contact	Formation, stabilization, maturation of E-cadherin-mediated adhesion	[134]
	D. inactive		Actin reorganization at cell–cell contacts		

Table 2 (*continued*)

GTPase	Mode of GTPase alteration	Cell model	Major effects on TJ/AJ	Functional consequences	References
Rac1	D. active	Keratinocytes		Disassembly of E-cadherin cell–cell adhesion	[120]
Rac1	D. active	Keratinocytes		Establishment of E-cadherin cell–cell adhesion	[103,104]
	D. inactive	HUVEC			
Rho	D. active C3 (inactivation)				
Rac1	D. active	HUVEC	No change in ZO-1 distribution	Increased paracellular permeability	[64]
	D. inactive		Redistribution of E-cadherin, β-catenin, ZO-1 Disassembly of TJ and AJ ultrastructure		
Rac, Ras	LT82 (inactivation)		Redistribution of E-cadherin	ND	[113]
Rac	LT 82 (inactivation)	MyEnd intact vessels	Depolymerization of actin filaments Redistribution of E-cadherin and β-catenin Reduction of E-cadherin adhesion	Increased endothelial permeability	[174,175]
Rac1	D. active	S180 cells		Reduced E-cadherin-mediated cell–cell adhesion	[121]
	D. inactive				
Cdc42	D. active				
	D. inactive				
Cdc42	D. active	MDCK	Decreased localization of occludin, ZO-1, claudin-1 and -2, JAM-1 at TJs Increased detergent solubility of claudin-1 and -2	Increased paracellular permeability	[60]
	D. inactive		Redistribution of claudin-2		
	D. active		Thickening and disorganization of cortical actin Irregular distribution of ZO-1 and occludin		[74]
	D. inactive		Thickening and disorganization of cortical actin No change in ZO-1 and occludin distribution		
	D. active		No change in actin filaments at cell–cell contacts	ND	[63]
	D. inactive		No change in E-cadherin and β-catenin localization		
	D. active	HMEC1, HPAEC	Inhibition of interaction between α- and β-catenin	Increased endothelial permeability	[126]
	D. inactive	HUVEC	No alteration of actin filaments and AJ proteins	No change in paracellular permeability	[64]
Rac, Cdc42	Rho-GDI	MDCK	Rho-GDI a negative regulator of Rho, Rac, and Cdc42 alters E-cadherin cell–cell adhesion Reversion by co-transfection of Rho-GDI with D. active Rac or Cdc42, but not Rho	Maintenance of E-cadherin cell–cell adhesion	[118]
Rho, Rac, Cdc42	ToxA, ToxB (inactivation)	T84	Depolymerization of actin filaments at the apical side Alteration of apical and basal actin filaments Redistribution of ZO-1, ZO-2, occludin No change in E-cadherin distribution Alteration of TJ ultrastructure Reduced ZO-1-actin association	Increased paracellular permeability	[92–94] [96]
	ToxB (inactivation)		Increased detergent solubility of ZO-1 and occludin		
	ToxA (inactivation)		Redistribution of ZO-1, occludin, claudin-1 Increased detergent solubility of ZO-1, no change in ZO-2, occludin, E-cadherin, β-catenin detergent solubility		[95]
	ToxB (inactivation)	MCCD	Depolymerization of apical and basal actin filaments Increased detergent solubility of E-cadherin		[172]
	CNF1 (activation)	T84	Redistribution of ZO-1, occludin, JAM-1 Internalization of occludin No change in E-cadherin, β-catenin distribution Slight increase in apical actin filament ring		[102]
	CNF1 (activation)	HUVEC	Increased stress fiber formation No change in E-cadherin distribution	ND	[113]
	CNF1 (activation)	MesEnd, HDMEC, PAEC	Increased stress fiber formation Redistribution of VE-cadherin in PAEC Formation of intercellular gaps in PAEC	Increased endothelial permeability In PAEC	[177]
	ToxB (inactivation)	MyEnd	Reduction of actin filaments (stress fibers and junctional actin) Redistribution of E-cadherin	Increased endothelial permeability	[114,175]
Rac, Cdc42	CNF1 (activation)	MyEnd	Increased junction-associated actin filaments Translocation of cortactin and VASP to cell junctions	Enhanced endothelial barrier function	[177,180]
Rac (Cdc42) Ras	LT82 LT9048 (inactivation)	MCCD	Reduction of actin filaments at basal side but not at apical side Redistribution of E-cadherin, α- β-catenin No change in ZO-1, occludin distribution Increased detergent solubility of E-cadherin No change in detergent solubility of ZO-1, occludin	Increased paracellular permeability	[172]

EC, bovine pulmonary endothelial cell; HUVEC, human umbilical venous endothelial cells; SCLC, small cell lung carcinoma cell; HDMEC, human dermal microvascular endothelial cells; HMEC-1 human microvascular endothelial cells, HPAEC, human pulmonary arterial endothelial cells; PAEC, porcine pulmonary artery endothelial cells; MDCK, Madin–Darby canine kidney cell; MCCD, mouse renal collecting duct cell; MesEnd, mouse mesentery endothelial cell; MyEnd, mouse microvascular endothelial cell; EM, electron microscopy; C3 which inactivates RhoA, RhoB, and RhoC; epithelial differentiation inhibitor (EDIN) which inactivates RhoA, RhoB, and RhoC; LT82, lethal toxin from *C. sordellii* IP82 which inactivates Rac, Ras, Rap, Ral; LT9048 from *C. sordellii* VPI9048 which inactivates Rac, Cdc42, Ras and Rap; ToxA and ToxB from *C. difficile* which inactivates Rho, Rac and Cdc42; CNF1, cytotoxic necrotizing factor from *E. coli* which activates Rho, Rac and Cdc42. CNF_y, cytotoxic necrotizing factor from *Yersinia pseudotuberculosis*; VASP, vasodilatator-stimulated phosphoprotein; RNAi, RNA interference; ND, not determined. The horizontal bars of the submitted table have been omitted. Please insert a space line instead of the horizontal bars, for more clarity of the table.

[36]. The best-characterized molecules are Rho, which controls the stress fibers and focal adhesion formation, and Rac and Cdc42, which regulate membrane ruffling, and filopodium formation, respectively. Cycling between active and inactive forms of Rho-GTPases is regulated by at least three protein classes: 1) the guanine-nucleotide-exchange factors (GEFs), which promote exchange of bound GDP for GTP in response to extracellular signal(s); 2) the GTPase-activating proteins (GAPs), which enhance intrinsically low GTPase activity thereby inactivating Rho-GTPases; and 3) the guanine-nucleotide-dissociation inhibitors (GDIs), which stabilize the GDP, forms, of Rho-GTPases – therefore inactive – in the cytosol [37–41]. Despite the evident diversity among GEF, GAP, and GDI molecules, each class shares a conserved mechanism of action [42]. Activated Rho-GTPases bind to membrane lipids via isoprenylated cysteines found in a Cys-Aliphatic residue-Aliphatic residue-X motif within the C-terminus, and then subsequently interact with their effectors. The conformational changes between GDP- and GTP-bound forms of Rho are localized within two surface loops, named switch I and switch II, which play an important role in GTP catalysis. Switch I of Rho-GTPases is the main region for interactions with effector molecules. Most Rac/Cdc42 effectors contain a conserved, GTPase-binding consensus site (Cdc42/Rac Interactive Binding, CRIB), and many Rho effectors possess a N-terminal Rho effector homology domain (REM) composed of three leucine-zipper-like motifs. Rho-GTPases effectors are often Ser/Thr kinase, lipid kinase, lipase, or a scaffold protein [37].

Ras controls cell division and cell differentiation and mutated Ras is involved in about 30% of human cancers of different origins [2]. In difference to Rho-GTPases, Ras is permanently bound to the plasma membrane through farnesyl and palmytoyl residues. Ras proteins also cycle between a GTP active form and a GDP inactive form regulated by specific GEF and GAP proteins. Activated Ras interacts and activates Raf-kinase, which in turn passes the signal on to MAP kinase pathways and then on transcriptional factors [43,44]. It has emerged that extensive cross-talk exist between Ras and Rho GTPase families [37, 38, 44]. A well-characterized cross talk pathway involves activation of phosphatidylinositide 3-kinase (PI3K) by Ras and subsequent stimulation of the Rac-Rho signaling pathway [40].

Rho/Ras protein activation is under the control of extracellular factors via membrane receptors such as receptor tyrosine kinases or G-protein couple receptors. But, Rho-GTPases are also regulated through the integrity of cell–cell adhesion. Intercellular junctions are dynamic structures controlled by a complex network of regulation including Rho-GTPases, which also generate signals involved in regulation of multiple cellular processes such as cell polarization, cell segregation, morphogenesis, and tumor invasion. Formation of E-cadherin or nectin cell–cell contacts activates Rho-GTPases, mainly Rac and Cdc42, as well as downregulation of Rap1, which play a role in the organization of AJs and TJs [45–51] (and see below). Inversely, disassembly of epithelial cell–cell contacts induces Rho activation leading to an increased actin polymerization through the Rho-kinase (ROK) pathway [52,53]. Concomitant activation of Rac contributes to actin polymerization via p21(Rac/Cdc42) activated kinase (PAK) and gene activation possibly by p38 MAP kinase activation [54,55]. In addition, disassembly of E-cadherin junctions accompanied by E-cadherin endocytosis triggers Rap1 activation, and integrin-dependent focal adhesion [51]. This illustrates cross-talk between cadherin- and integrin-dependent adhesions.

In addition, Rho and Ras proteins are target of bacterial toxins, which modulate the actin cytoskeleton and intercellular junctions ([56–58] and see below).

5. Rho and TJs

In epithelial cells, TJs are closely associated with the apical actin cytoskeleton, which plays a crucial role in the regulation of these junctions by mechanisms including cortical actin contraction and control of interactions between the actin cytoskeleton and certain TJ components such as occludin and ZO proteins. Rho is a major regulator of actin polymerization and has been also recognized as an important regulator of TJ function.

Transfection of dominant negative or constitutively active RhoA in epithelial cells such as MDCK cells results in alteration of TJ function consisting in an increase in cell barrier permeability (decrease in transepithelial resistance (TER) and increase in passage of fluorescent tracers) (Table 2). Constitutively active RhoA induces a disorganization of TJs, resulting in loss and chaotic arrangement of TJ strands (as observed by freeze fracture electron microscopy) as well as in discontinuous distribution of occludin, ZO-1, claudin-1 and -2, and JAM-1. The latter leads in turn to submembranous diffusion of these proteins away from the membrane at the cell apical side. In contrast, dominant negative RhoA does not significantly modify TJ organization [59,60]. However, a partial loss of occludin and ZO-1 was observed at cell–cell contacts of cells transfected with dominant negative Rho by Gopalakrishnan et al. [61]. In cells transfected with constitutively active RhoA, actin filaments are mainly localized in stress fibers at the base of the cell and form a broader and more diffuse cortical belt compared to controls [62]. Similar results on actin distribution were obtained by Takaishi et al. using MDCK cell lines stably expressing dominant active RhoA mutant [63]. In contrast, dominant negative RhoA does not significantly perturb actin filament organization [62], or reduces the actin filament content at the apical side and the basal stress fibers [60]. Thus, RhoA controls TJ function even in the absence of gross abnormalities in TJ organization. RhoA might regulate subtly actin filament contraction and, subsequently, the forces pushing TJ strands between neighboring cells. Alteration of traction and distance between TJ strands are probably sufficient to modulate the fence function [59].

RhoA also controls the permeability of endothelial cells. Dominant negative RhoA prevents thrombin- and histamine-dependent increase in permeability and decrease in TER. In addition, inhibition of Rho prevents the effects induced by thrombin and histamine including assembly of actin stress fibers and disorganization of TJ components [64].

Various extracellular factors can modulate the activation of Rho. Indeed, lysophosphatidic acid (LPA) is a well-known effector which activates signaling cascades through RhoGTPases. LPA binds to a seven transmembrane G-protein-coupled receptor on endothelial cells. LPA increases TJ permeability, probably via dissociation of actin filament from TJ components [65].

Prostaglandin E2 receptor (EP3β) has been evidenced to regulate epithelial barrier through RhoA. Ligand activation of this receptor leads to a RhoA-dependent increase in TER and also, paradoxically, to a selective increase in paracellular permeability, such as the passage of mannitol [66]. The signaling pathway leading to Rho activation by EP3β has not been fully identified, but might involve an activation of Rho-GEF. Indeed, a specific Rho-specific GEF was found to be associated to TJs. In MDCK cells, cGEF-H1 from the Dbl family, an homologue of human GEF-H1, is concentrated at TJs. Over-expression of cGEF-H1 results in a specific activation of RhoA and in an increase in paracellular permeability without apparent change in distribution of actin filaments as well as of TJ components such as occludin, ZO-1, and claudins [67]. Thereby, cGEF-H1 modulates paracellular permeability through local activation of RhoA without gross disturbance of TJ and actin filament structures. GEF-H1 directly binds to cingulin, an adaptor protein present in TJs, and when bound to cingulin, it no longer activates RhoA. When TJs are nascent between neighboring cells, cingulin accumulates at the forming TJs while sequestering GEF-H1 and inhibiting RhoA signaling at cell confluence [6]. This process contributes to Rho regulation in epithelial cells.

How does Rho control TJ function? Rho is known to interact with several effectors and to stimulate several signaling pathways. A main Rho downstream signaling pathway includes Rho-kinase (or ROK,

p160ROK, ROK-α) a serine–threonine kinase which directly phosphorylates myosin light chain as well as phosphorylates and inactivates myosin phosphatases, resulting in an increase in bundle formation and contraction of acto-myosin filaments (Fig. 1) [36]. Indeed, myosin light chain phosphorylation correlates with increased TJ permeability induced, for example, by activation of Na^+-glucose cotransport in intestinal CaCo-2 cells, while inhibition of myosin light chain phosphorylation by specific inhibitors of myosin light chain kinase results in the prevention of increased TJ permeability [68]. The perijunctional actin ring at the apical side of epithelial cells is capable of generating contractile forces, and thus, controls TJ permeability [69]. Inhibition of ROK by specific inhibitor or transfection of a dominant negative mutant in intestinal epithelial cells increases the paracellular permeability in parallel with a loss of actin filament-rich brush border and a reduced intensity of the perijunctional actin ring, but without redistribution or disassembly of the TJ complexes. This further supports the fact that ROK is involved in the regulation of the apical actin cytoskeleton, which in turn controls TJ function [70]. In addition, ROK directly phosphorylates LIM-kinase (protein kinase containing two zing finger domains separated by two hydrophobic residues), which is activated and subsequently phosphorylates cofilin, an actin-severing factor that is inactive when phosphorylated. Thus ROK via LIM-kinase-cofilin contributes to the organization of actin filaments by preventing actin depolymerization (Fig. 1). Moreover, moesin from the ERM (*e*zrin, *r*adixin, and *m*oesin) family, which is involved in microvilli formation and indirectly in regulation of acto-myosin contraction, is also a substrate of ROK. (review in [40,71]. However, Rho also controls TJ function by ROK-independent mechanisms including phosphorylation of occludin [61,72]. Indeed, dominant active Rho increases the level of occludin phosphorylation in MDCK cells [61].

6. Rac/Cdc42 and TJs

Rac and Cdc42 also regulate TJ function in epithelial cells. In fact, active and negative mutants of Rac increase the permeability of MDCK cell barrier. Nevertheless, an active mutant of Rac alters the TJ organization as monitored by freeze fracture electron microscopy, whereas a dominant negative mutant has no effect [62]. Similar observations of disruption of MDCK barrier function by active and negative mutants of Rac and Cdc42 have been done by Bruewer et al., but with differential effects on TJ organization [60] (Table 2). Active mutant of Cdc42 causes a redistribution of TJ proteins (ZO-1, claudin-1, claudin-2, JAM-1) as visualized by a decreased immunofluorescence staining at cell–cell contacts, albeit to a lesser extent than a negative mutant of Rho, whereas an active mutant of Rac partially alters the localization of only claudin-1 and -2. A dominant negative mutant of Rac also causes a redistribution of claudin-1 and -2 in addition to JAM-1, while a dominant negative mutant of Cdc42 partially alters the organization of claudin-2 [60].

Since Rho-GTPases are part of a complex interconnected network of signaling pathways, their effect on intercellular junctions might reflect an imbalance in their activation state rather than an individual mechanism of each GTPase acting on intercellular junction components. Thus, activation of Cdc42 in fibroblasts could promote the stimulation of Rac and subsequently Rho [73] which, as discussed above, is an important regulator of TJs. Rac and Cdc42 participate in TJ function through the control of the actin cytoskeleton. Thickening and disorganization of cortical actin is indeed observed in epithelial cells expressing active or negative mutant of Cdc42, respectively [74]. In addition, RhoGTPases are involved in the formation and maintenance of epithelial cell polarity, and this might influence the control of TJs, which are crucial for the maintenance of cell polarity, by

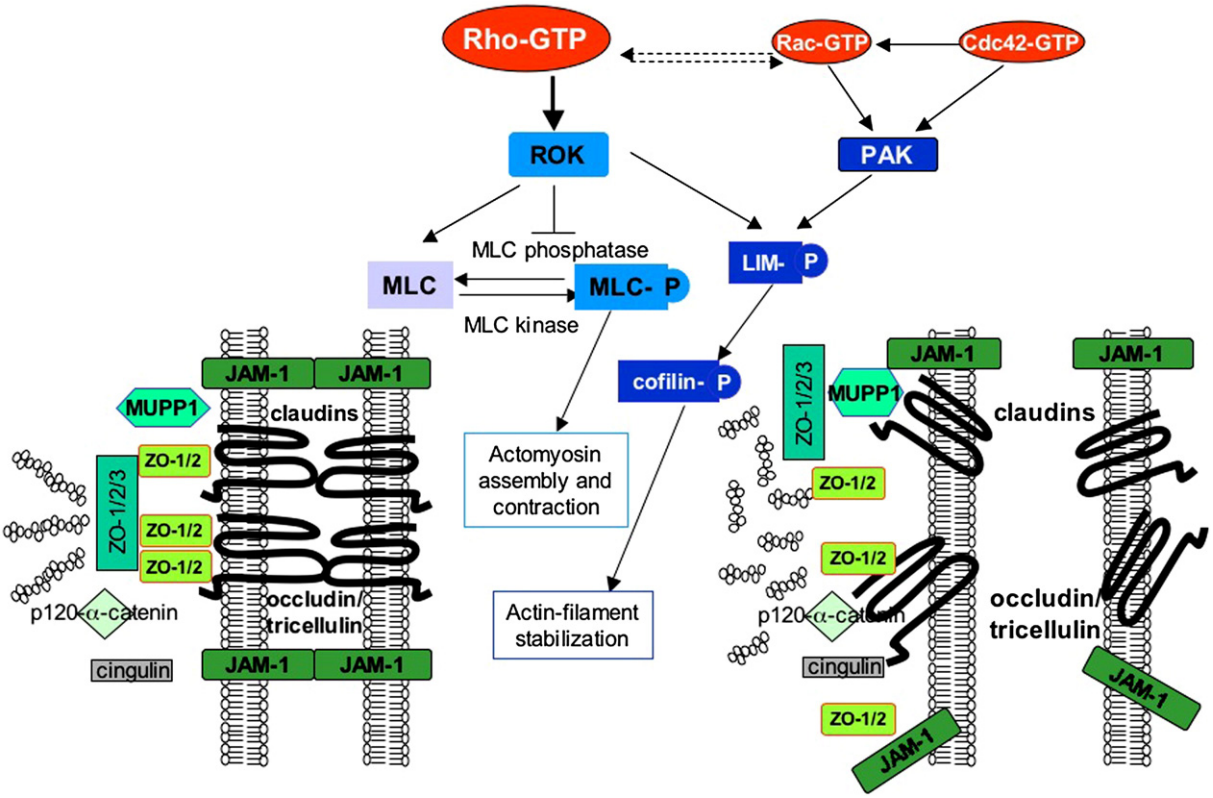

Fig. 1. Regulation of tight junctions by Rho GTPases. Protein complexes of tight junctions (green) are controlled by small GTPases of the Rho family through different pathways (blue). The main pathway (sky blue) involves activated Rho, which activates ROK leading to actomyosin assembly and contraction. Rac and Cdc42 also modify tight junctions by activating PAK, LIMK thus leading to cofilin inactivation by phosphorylation (navy blue pathway). This pathway induces actin filament stabilization

impeding the diffusion of lipids and proteins between apical and baso-lateral domains. Cdc42 has thereby been found to modulate TJ and polarized membrane protein trafficking by multiple cellular pathways [74,75].

7. Rho-GTPases and TJs, lessons from toxins

C3 enzyme is commonly used as a specific inhibitor of Rho. C3 was first identified from *Clostridium botulinum* types C and D as an ADP-ribosyltransferase specific for Rho protein [76,77]. C3 related proteins are also produced by other Gram-positive bacteria such as *Clostridium limosum*, *Bacillus cereus* and *Staphylococcus aureus* [78]. The C3 related enzyme produced by *S. aureus* is called epithelial differentiation inhibitor (EDIN). The EDIN-producing strains of *S. aureus* (~7.8% of all clinical isolates) are preferentially obtained from skin suppurations as well as other infection sites, versus only 3.7% of *S. aureus* isolated from healthy nasal carriers, thus suggesting that EDIN may be a virulence factor involved in epithelium disorganization [79].

C3 induces cell rounding and destruction of actin filaments in epithelial and fibroblast cells, but only a limited disorganization of cortical actin [80]. In polarized epithelial intestinal T84 cells, C3 profoundly alters the apical actin cytoskeleton and the distribution of occludin, ZO-1 and claudin-1, while it increases cell barrier permeability [70,81]. C3 microinjected into MDCK cells causes the disappearance of actin filaments at cell–cell adhesion sites, as well as altering formation of TJs [61,63]. In addition, C3 inhibits association of the ERM family of proteins and vinculin with the plasma membrane, which are necessary for developing the basal edge and focal adhesions of cells [82]. Overall, this information indicates that Rho plays a central role in forming and maintaining TJs. It is noteworthy that inhibition of Rho by C3 induces a more drastic effect on TJ organization than the specific inhibitor of ROK, indicating that Rho controls TJ and apical actin via other downstream effectors. Indeed, ROK does not alter the distribution of AJ molecules, it mainly regulates TJs by modifying their interaction with actin filaments [70].

The glucosylating toxins produced by various clostridial species, also known as the large clostridial toxins, are 250–300 kDa single-chain proteins that include *Clostridium difficile* toxins A (ToxA) and B (ToxB), *Clostridium sordellii* lethal toxin (LT) and hemorrhagic toxin (HT), as well as *Clostridium novyi* α toxin (α-novyi) [83]. Glucosylating toxins represent the main virulence factors of these *Clostridium* species, commonly involved in intestinal diseases and/or myonecrosis. *C. difficile* can cause pseudomembranous colitis and enteritis that often follow antibiotic treatment and which represent the most prevalent nosocomial form of diarrheal disease in hospitals. *C. sordellii* and *C. novyi* both appear as common causative agents of gas gangrene in humans and animals and *C. sordellii* is also responsible for dramatic and non-traumatic toxic shock syndrome subsequently to medical abortion [84–87]. Additionally, *C. sordellii* is involved in hemorrhagic enteritis of cattle as well as enterotoxemia in sheep, and *C. novyi* can induce bovine hepatotoxemia [88].

Large clostridial toxins catalyze the glucosylation of Rho and Ras family proteins using UDP-glucose, with an exception being α-novyi which utilizes UDP-*N*-acetylglucosamine as co-substrate. These enzymes transfer one molecule of glucose or *N*-acetylglucosamine to the hydroxyl group of an acceptor amino acid, such as Thr37 of Rho or Thr35 of Rac, Cdc42, and Ras proteins [89,90]. ToxA and ToxB glucosylate Rho, Rac, and Cdc42, whereas LT modifies Rac, Cdc42, and several Ras proteins (i.e., Ras, Rap, Ral). ToxB induces loss of actin stress fibers and reorganization of focal adhesions accompanied by cytoplasmic retraction and cell rounding, whereas long protrusions radiating around the cell yield a particular "actinomorphic" morphology [91]. ToxA and α-novyi cause similar cell alterations, but LT produces cell rounding without branched, membrane protrusions. In contrast to C3, large clostridial toxins modify more than one GTPase that renders more difficult the interpretation of cellular effects. Such effects could be linked to the simultaneous inhibition of several GTPase pathways and/or activities other than GTPase glucosylation attributed to the large clostridial toxins.

ToxA and ToxB alter the barrier function of polarized intestinal cells such as CaCo-2 and T84 cells by increasing paracellular permeability [92–94]. Concomitant with the permeability increase, ToxA and ToxB disrupt apical and basal actin filaments with a subsequent disorganization of the ultrastructure and component distribution (i.e., ZO-1, ZO-2, occludin, claudin) within TJs. ToxB decreases actin-ZO-1 association and disperses ZO-1 and occludin from lipid-rich membrane microdomains, without changing the occludin phosphorylation status [95,96]. Since Rho plays an important role in TJ assembly, the effects of ToxB and ToxA on TJs presumably result from Rho glucosylation [59]. However, ToxA induces protein kinase C (PKC) activation independently of GTPase modification, which could account for altered TJs and increased paracellular permeability [95]. In addition, ToxA induces pro-inflammatory cytokine release, caspase activation, and apoptosis in T84 cells in a Rho-dependent manner, which taken together likely contribute to mucosal damage [97].

The cytotoxic necrotizing factor (CNF) is produced by some pathogenic strains of *Escherichia coli*. CNF1 (110 kDa) is synthesized by human strains mainly isolated from urinary tract infections and neonatal meningitis [98]. CNF1 preferentially targets Rho and catalyzes deamidation of Gln63 to Glu, but Rac and Cdc42 are also modified at an equivalent residue (Gln61). Gln63 (Gln61) is located within switch II of the Rho-GTPases and has a pivotal role in intrinsic and GAP-stimulated GTPase activity. Deamidation of Gln63 (Gln61) into Glu impairs binding of a water molecule required for GTP hydrolysis [99,100]. Therefore, CNF1 locks Rho-GTPases into a biologically active form linked to GTP.

In cultured cells, CNF1 converts Rho-GTPases into active forms that stimulate downstream signaling pathways leading to multinucleation, increased actin polymerization, reorganization of stress fibers, membrane ruffles, increased phagocytic activity among human epithelial cells, as well as modification of intercellular junctions. The Rho-Glu63 and Rac-Glu61 forms activate phosphatidylinositol 1-4 phosphate 5-kinase (PI4P5K), a subsequent increase of PIP_2 levels enhances actin polymerizaton, and activated ROK stimulates bundling of the actomyosin filaments. One of the primary CNF-induced disturbances involves modification of intestinal or urinary tract cell barriers. In intestinal cell monolayers, CNF1 decreases TER and enhances paracellular permeability from the basal to apical surface. In T84 cell monolayers, CNF1 induces profound alterations of TJ components. Occludin, ZO-1, claudin-1, and JAMs are highly redistributed from the apical ring to the cytoplasm, leading to a complete disruption of the continuity of TJ integrity between neighboring cells. Total amounts of occludin and ZO-1 are unchanged following CNF1 treatment. TJ proteins are not degraded through the late endosome/lysosome pathway but are probably recycled via membranous structures resembling caveolae/endosomes, early and recycling endosomes. Caveolae are considered as special lipid microdomains containing caveolin-1 in plasma membrane or in endocytic organelles, termed caveosomes [101]. At least a pool of occludin is recycled through the Rab11 recycling endosomes. In addition, microvillus F-actin and its binding protein, villin, disappear. In this way, an excessive stimulation of Rho-GTPases induces alteration of TJ structure and function. CNF1 effects on TJs could be mainly mediated by Rho activation. However, inhibitors of Rho or myosin light chain kinase do not prevent the CNF1 effects, indicating that Rho and subsequent acto-myosin filament reorganization are not the only pathways involved in CNF1 treated cells. TJ alteration by CNF1 through simultaneous activation of Rho, Rac and Cdc42 illustrates the cooperation of these GTPases and the complexity of the regulation network of intercellular junctions [102].

8. Rho and AJs

Rho plays a role in the formation of AJs. Indeed, Rho, in addition to Rac, is required for the establishment of cadherin-mediated cell–cell adhesion [103]. In keratinocytes, Rho signaling modulates the organization/clustering of E-cadherin receptors, since inhibition of Rho removes E-cadherin from AJs. However, in endothelial cells, VE-cadherin localization is insensitive to Rho [104]. Rho might be involved in the clustering of cadherin and/or the recruitment of cytoplasmic proteins to sites of cell–cell contacts. Furthermore it could regulate the formation of acto-myosin filaments which stabilize adhesion between neighboring cells, and/or it could participate in the development of polarized epithelial phenotype [105]. However, in confluent MDCK cells, constitutively active and dominant negative forms of Rho do not affect the distribution of AJ proteins [60].

The role of Rho in the regulation of the endothelial barrier is controversial. In cultured endothelial cells, RhoA has been implicated in the control of barrier permeability. Inhibitors of RhoA or ROK have been shown to prevent the increase in permeability induced by thrombin [64,106–110]. LPA [111], or histamin [64]. In contrast, activation or inhibition of Rho do not modify the distribution of VE-cadherin and do not prevent the permeability induced by thrombin in HUVEC cells despite a reorganization of actin filaments [112,113]. Similarly, inhibition of ROK in intact microvessels does not modify the increased permeability in response to bradykinin or platelet activating factor [114]. The actin/myosin contractile structure under the control of Rho/ROK pathway seems to be more important for the barrier maintenance of cultured endothelial cells than for that of intact microvessels, which are exposed to distending forces of blood pressure and in which tethering mechanisms at cell–cell contacts may be more critical [114]. But differences might exist in signaling pathways between the different types of microvessel. Indeed, the prolonged hyperpermeability induced by thrombin in rat kidney arterioles was found to be mainly mediated by Rho/ROK-dependent contractile response [109].

The level of Rho inactivation is critical for its effect on the endothelial barrier function, protection or alteration, and might explain the opposite results from the literature (Table 2). A higher level of Rho inactivation than that required to prevent thrombin-dependent hyperpermeability in HUVEC by EDIN or Rho RNA interference induces transient macroapertures and increased permeability. This process is associated with a loss of actin stress fibers and ROK inactivation, and constitutes an additional mechanism of alteration of cell barrier integrity, in this case involving tunnel formation through endothelial cells instead of intercellular gaps [115,116].

The mechanism of Rho-dependent control of AJs seems to involve two opposing pathways. The ROK pathway, which generates contractile forces, leads to AJ disruption, whereas the Dia (a Rho effector) signaling promotes actin polymerization and stabilization of E-cadherin–catenin complexes, probably through mediating their association to the cortical actin cytoskeleton. These two mechanisms might be coordinated by the levels of active Rho, with low levels of active Rho favoring signaling through Dia and thus stabilizing AJs, and high levels leading to ROK signaling and AJ disruption [117].

9. Rac/Cdc42 and AJs

Studies with active and negative mutants of Rac and Cdc42 indicate that these RhoGTPases control the formation and maintenance of cadherin-mediated intercellular junctions (Table 2). Microinjection of negative mutant of Rac inhibits the accumulation of E-cadherin at cell–cell contacts in keratinocytes [104], as well as in MDCK cells [63], whereas overexpression of active Rac induces the accumulation of E-cadherin, β-catenin and actin filaments at sites of MDCK cell–cell contacts [63].

Rac and Cdc42 are required for maintaining cell–cell adhesion [118,119]. However, Rac seems to have a major role in controlling AJs, since Rac activation is sufficient to disassemble cadherin-dependent intercellular junctions in keratinocytes [120]. In confluent MDCK cells with mature cell–cell junctions, dominant negative Rac increases the E-cadherin distribution from detergent-insoluble to detergent-soluble fractions, whereas the partition of TJ proteins (occludin, ZO-1) is unaffected [60], further supporting the specific role of Rac in AJ control versus TJ.

Strength of E-cadherin-dependent cell–cell adhesion has been quantified in a cell–cell assay. The actin cytoskeleton and Rho-GTPases are not involved in the initial phase (up to 30 s) of homophilic interaction of E-cadherin extracellular domains. However, a subsequent activation of Rac and Cdc42, but not Rho, is required for the development and strengthening of E-cadherin-mediated adhesion. This clearly demonstrates the major role of Rac and Cdc42, but not Rho, in the control of E-cadherin-dependent junctions [121,122].

Rac is also an important regulator of endothelial permeability. Dominant negative or active mutants of Rac increase the permeability in HUVEC, although the two mutants have opposing effects on stress fibers. The two mutants induce alterations of AJs and TJs resulting in intercellular gaps. In addition, dominant negative Rac prevents actin filament reorganization and VE-cadherin redistribution from membrane to cytoplasm induced by thrombin and histamin in endothelial cells [64]. Increased permeability and alteration of endothelial junctions in response to thrombin stimulation seem to involve down regulation of Rac as well as Rho activation [14,112]. However, the role of Rac in thrombin-stimulated cells was not evidenced by van Nieuw Amerongen et al., who show that the thrombin-dependent hyperpermeability in HUVEC is associated with an acto-myosin contractile response mediated by activation of the Rho/ROK pathway [110]. In contrast, dominant negative Cdc42 does not increase permeability of HUVEC endothelial cells, although this mutant reduces thrombin-induced stress fiber formation and actomyosin contractility [64,123]. Cdc42 might contribute to the increased cell contractility subsequent to thrombin treatment through Rho activation or/and in a Rho-independent manner via activation of PAK and myotonic-dystrophy related kinases (MRCKα), two common effectors of Rac and Cdc42 [64]. Thereby, control of both actomyosin filaments and of the organization of structural intercellular junction components is critical for endothelial permeability.

In contrast to histamin and thrombin which increase endothelial permeability, other compounds such as sphingosine 1-phosphate (S1-P) reinforce the endothelial barrier function. S1-P mainly activates Rac, and weakly in a delayed manner Rho, resulting in a PAK-LIM kinase pathway, subsequent inactivation of cofilin, and accumulation of cortical actin filaments [124,125]. This further supports the critical role of Rac in the control of endothelial barrier function.

Disassembly of AJs, where phosphorylation and subsequent dissociation of VE-cadherin and catenins are usual mechanisms, triggers a specific activation of Cdc42 which plays an essential role in the restoration of endothelial barrier integrity [126–128]. Activated Cdc42, and to a lesser extent Rac1, translocate from the cytosol to the membrane during the period of AJ restoration [127,129]. After loss of homotypic VE-cadherin interactions, the cytoplasmic domain of VE-cadherin is responsible for Cdc42 activation, by a yet unknown pathway, which might involve activation of a Cdc42-specific GEF. Another mechanism could be an activation of a Rho-GAP such as p190RhoGAP, which is recruited to disassemble AJs by its interaction with p120-catenin, leading to local Rho inactivation and subsequent Cdc42 activation [128,130]. Therefore, AJ disassembly regulates Cdc42 which in its turn controls AJ restoration. A model has been proposed for the thrombin-dependent increase and subsequent recovery of endothelial barrier function. Thrombin, through activation of PAR-1 and subsequently of $G_{\alpha q}$ and $G_{\alpha 12/13}$ heterotrimeric G proteins, rapidly (less than 1 h) activates Rho, leading to contraction of acto-

myosin filaments, microtubule disassembly, and increased endothelial permeability. A delayed activation of Cdc42 parallels the endothelial barrier repair [130,131]. The mechanism of Cdc42-dependent reannealing of AJs is still speculative. Cdc42 promotes the association of VE-cadherin with α-catenins, and homotypic adhesion of VE-cadherin, possibly through IQGAP1 (see below) and actin remodeling via WASP (Wiskott–Aldrich syndrome protein) [130].

One mechanism of AJ control by Rac and Cdc42 includes the regulation of local actin cytoskeleton. Active Cdc42 interacts with WASP and stimulates Arp2/3-dependent actin polymerization, which is involved in filopodia formation, whereas Rac associates with WAVEs (Wasp family verprolin homologous proteins) and IRSp53 (protein containing a I-BAR domain, Inverse-bin-Amphiphysins-R) to activate the Arp2/3 complex and to promote membrane protrusions such as lamellipodia and ruffles [132]. Two types of actin population have been identified at cell–cell contacts, a more stable actin population resulting from reorganization of pre-existing actin filaments, termed thin bundles, and a dynamic population termed, junctional actin and consisting in *de novo* actin polymerization [133]. Rac, through the WAVE2-Arp2/3 pathway, which stimulates junctional actin polymerization, was found to have a major role in promoting cell–cell contacts, as well as in stabilization and maturation of E-cadherin-mediated cell–cell adhesion [134]. In addition, a cross talk between Rho-GTPases is involved in the control of AJs. A recent study shows that expression of dominant active Cdc42 in vascular endothelial cells *in vivo* protects the cell barrier integrity by preventing the Rho-dependent hyperpermeability effects [135].

In contrast to Rho, Rac and Cdc42 directly act on the activity and assembly of the cadherin–catenin complex (Fig. 2) [136]. IQGAP1 is a common effector of Rac and Cdc42, and has been shown to interact with β-catenin and the cytoplasmic domain of E-cadherin. A model has been proposed in which Rac and Cdc42 regulate cadherin junctions through IQGAP1. Active Rac and Cdc42 in the GTP-bound form interact with IQGAP1 and thus prevent the association of IQGAP1 with β-catenin, which results in the stabilization of cadherin–catenin complex. In the GDP-bound form, non-active Rac and Cdc42 do not interact with IQGAP1, which associates with β-catenin, thereby displacing α-catenin from its binding to β-catenin. This leads to a dissociation of α-catenin linked to actin filaments from the cadherin–catenin complex, conferring a weak adhesive activity [129,136–139]. Further work with knockdown Rac and/or IQGAP1 supports that Rac positively regulates cell–cell adhesion through IQGAP1 by promoting the accumulation of actin filaments, E-cadherin, and β-catenin at MDCK cell–cell contact sites [140]. In endothelial cells, Cdc42 has been also shown to promote association of α-catenin with VE-cadherin–β-catenin complex, but by a non-defined pathway [126]. However, the exact mode of action of IQGAP1 remains unclear since it has been found that α-catenin cannot form a quaternary complex with β-catenin, E-cadherin, and actin filaments in live cells, and that α-catenin–β-catenin and α-catenin–actin filament interactions are mutually exclusive [141]. α-Catenin probably regulates actin filament assembly by interacting in a monomeric form with E-cadherin–β-catenin complex, and in a dimeric form with actin filaments [142] (and see above). Rac probably contributes through IQGAP1 to the maturation of nascent cell–cell contacts to mature AJs and to maintaining a balance between a dynamic and a more static state [29,143]. In addition, E-cadherin-mediated intercellular junctions activate Rac thus permitting the recruitment of actin to cell–cell

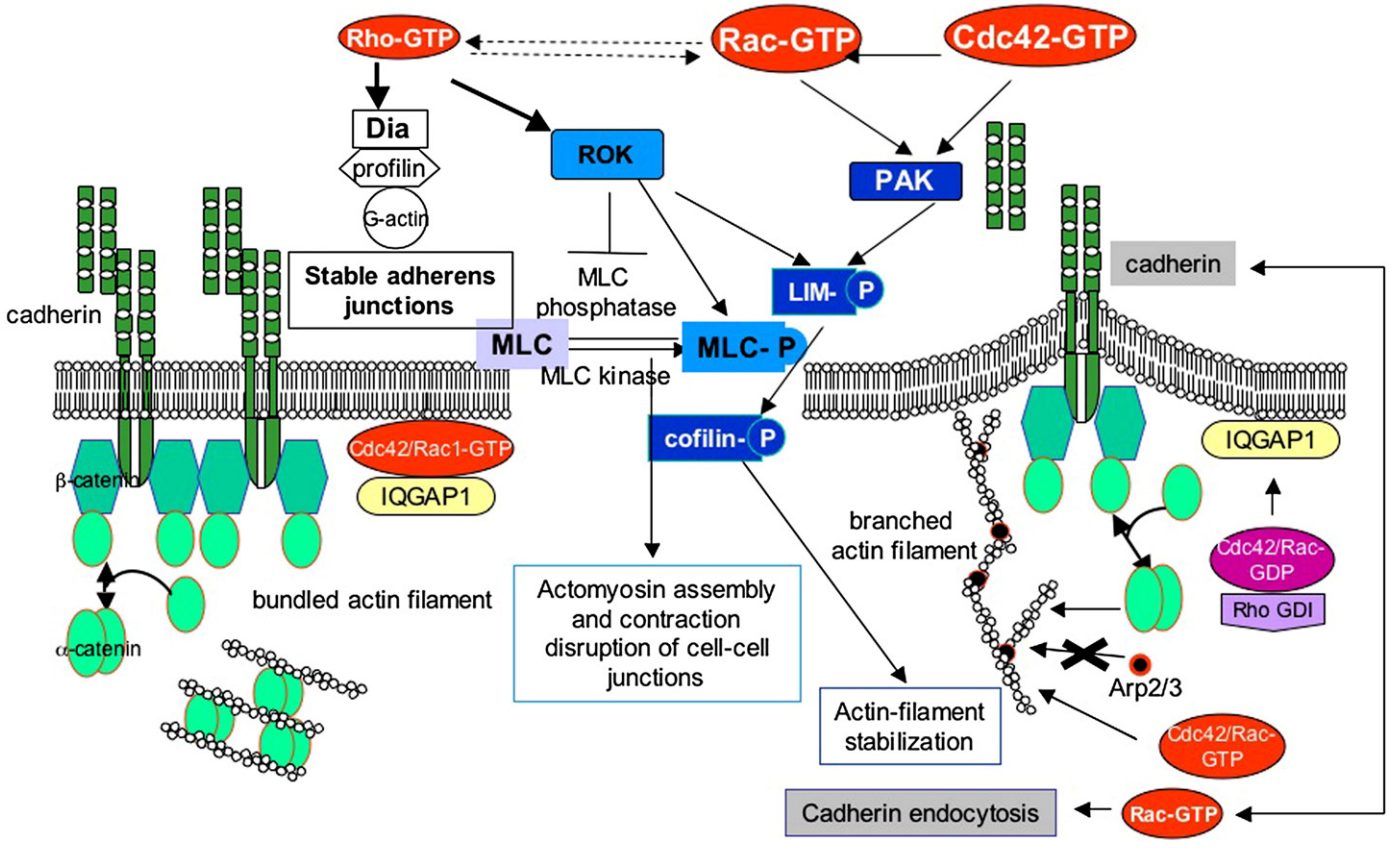

Fig. 2. Regulation of adherens junctions by Rho GTPases. Rac and Cdc42 are the main regulators of adherens junctions. These GTPases activate PAK, LIMK thus leading to cofilin inactivation by its phosphorylation and, thus actin filament stabilization (navy blue pathway), Rac and Cdc42 also regulate adherens junctions via a pathway involving IQGAP1 (yellow, see text). Activated Rho has a dual effect, stabilizing adherens junction via Dia and profilin (white) or leading to contraction and disruption of cell–cell junctions via ROK activation and MLC phosphorylation (sky blue pathway). Activated Rac also contributes to cadherin endocytosis and its recycling. Rac and Cdc42 play a role in organization of actin cytoskeleton through a pathway comprising WASP or WAVE and Arp2/3 complex.

contacts and stabilization of cell–cell adhesion [46,144,145]. The cadherin signaling involves phosphatidylinositol 3-kinase (PI3-kinase) [146,147]. PAK phosphorylation, a Rac effector which stabilizes actin filament by inhibiting the LIM-kinase-cofilin pathway [148], as well as Tiam1, an exchange factor of Rac, which is required for Rac activation and maintenance of cadherin-based adhesions [148,149]. These dynamic processes between intercellular junctions and Rho-GTPase signaling participate in the coordinated assembly and stability of AJs and thus in the regulation of barrier function (Fig. 2) [45].

Focal adhesion proteins have recently been found to be involved in the regulation of endothelial AJs in a Rac/Cdc42-dependent manner. Oxidized phospholipids, which enhance the pulmonary endothelial AJ function, activate Rac/Cdc42 resulting in a redistribution of focal adhesion proteins from cytosol to the cell periphery. Focal adhesion proteins form a novel type of association with AJ complexes via interactions between paxillin and β-catenin, which is strictly mediated by Rac and Cdc42 activation. Concomitantly, an enhancement of peripheral actin cytoskeleton has been observed [150]. The signaling cascade leading to focal adhesion reorganization has not yet been fully determined. Rac might mediate the phosphorylation of paxillin at Ser273 and the G protein-coupled receptor kinase-interacting protein (GIT1) at Ser709 through PAK, thus permitting the relocalization of the paxillin-GIT1-β-PIX(PAK interacting exchange factor)-PAK complex to the cell membrane [151,152].

Another mechanism of AJ regulation includes the endocytosis and protease degradation as well as endocytosis/recycling of cadherin, which are positively or negatively regulated by Rac/Cdc42, according to the cell status [21,153–155]. Tyrosine phosphorylated E-cadherin, possibly by Src or Yes which are enriched in AJs, is ubiquitinated by Hakai, an E3 ubiquitin ligase, leading to the endocytosis of the E-cadherin–catenin complex and lysosomal degradation [21]. In MCF-7 breast cancer cells, Cdc42 has been found to trigger E-cadherin ubiquitination, followed by endocytosis and lysosomal degradation. In this cell model expressing high levels of E-cadherin, Ca^{++} depletion dislocates homophilic interaction resulting in Cdc42 activation. Activated Cdc42 stimulates EGF receptor signaling leading to Src activation, tyrosine phosphorylation of E-cadherin, Hakai-dependent ubiquitination, and lysosomal degradation [156]. A proportion of cell surface E-cadherin is endocytosed and recycled back to the surface, and this dynamic process is influenced by cell–cell contacts. Indeed, in confluent cells with stable intercellular junctions, only a small pool of E-cadherin is recycled. Thus, inhibition of E-cadherin endocytosis by cell–cell contact contributes to AJ stabilization [154]. When cells do not contact other cells and E-cadherin does not trans-interact, E-cadherin is continuously endocytosed [157]. E-cadherin–catenin complex seems to be internalized into non-clathrin vesicles in a Rac-dependent manner and then recycled [158]. However, both clathrin and caveolin-dependent mechanisms of E-cadherin endocytosis have been reported [154,158–161]. Only non-transinteracting, but not trans-interacting E-cadherin is endocytosed. Activation of Rac and Cdc42 by trans-interacting E-cadherin or trans-interacting nectins in stable AJs inhibits E-cadherin endocytosis through IQGAP1, presumably by a cross-linking activity of actin filaments at cell–cell adhesion and thus contributes to strengthening AJs [157]. The effects of Rac on E-cadherin and AJs might depend on the state of intercellular junction maturity. In subconfluent MDCK cells, but not in confluent cells, activation of Ezrin (from the ERM protein family) upon phosphorylation induces Rac activation, E-cadherin internalization, lamellipodia formation and loss of cell–cell contacts [162].

p120-catenin is involved in the control of AJ functions, and it acts, at least in part, through the regulation of RhoGTPases. p120-catenin binding to E-cadherin promotes stabilization of AJ components by an unknown mechanism and also prevents E-cadherin endocytosis [20,22]. Binding of p120-catenin to E-cadherin recruits and activates Rac at nascent cell–cell contacts, which through PAK participates in the reorganization of the actin cytoskeleton and stabilization of AJs [20]. Cytoplasmic p120-catenin activates both Rac and Cdc42 probably by stimulation of the Rho-GEF, Vav2 while in contrast down-regulating Rho [163]. In various cell types, Rho antagonizes Rac and Cdc42 activation, and reciprocally [164,165]. Activated Rac is recruited to AJs by binding to p120-catenin which then recruits and activates p190 GAP, thus inactivating locally Rho [166]. Another possibility is that p120-catenin binds directly to Rho, and as GDI, prevents GDP exchange for GTP [167,168]. Binding of p120-catenin to E-cadherin and its negative regulation of Rho have been shown to be mutually exclusive, only unbound p120-catenin being able to inhibit Rho activation [169]. But p120-catenin and/or α-catenin may recruit Rho to AJs allowing it to be activated by GEF [168]. Cell density possibly regulates the levels of cytoplasmic and cadherin-bound p120-catenin and, subsequently, the balance of activated RhoGTPases that control cell adhesion [170].

10. Rho-GTPases and AJs, lessons from toxins

C. sordellii LT is responsible for myonecrosis and gangrene, which are characterized by an extensive local edema, as well as for enterotoxemia, necrotic and hemorrhagic enteritis in animals, and also for fatal toxic shock syndrome in women. The latter is accompanied by irreversible hypotension, hemoconcentration, pleural effusion, and serosanguinous ascites, indicating that this toxin targets epithelial and endothelial cell barriers [171]. In an epithelial cell monolayer model of mouse cortical collecting duct (MCCD) cells, *C. sordellii* LT82, which inactivates Rac, and LT9048, which inactivates both Rac and Cdc42 among the Rho-GTPases by UDP-glucosylation, induce a specific depolymerization of baso-lateral actin filaments, an increased cell barrier permeability, and a drastic perturbation of AJs (Table 2), whereas apical actin and TJs are preserved. Thereby, LTs cause a dramatic redistribution of E-cadherin, α- and β-catenins from the plasma membrane to the cytoplasm, with a reduced and discontinuous distribution of these molecules at the junctional ring and a diffuse localization into the cytosol. Simultaneously, E-cadherin is reduced at the cell surface and is increased in detergent soluble fraction. LTs remove the whole E-cadherin–catenin complex from the membrane through a mechanism involving the depolymerization of cortical actin, but not the Rac-IQGAP pathway. LTs which inactivate Rac, or Rac/Cdc42, but not Rho, disorganize AJs but not TJs in epithelial cells or only weakly, further supporting the role of Rac in the control of AJs [172,173].

LT, which inactivates Rac, is also responsible for alteration of endothelial cell integrity. In HUVEC endothelial cell monolayers, LT causes a pronounced disorganization of VE-cadherin, the localization of which at cell–cell junctions is discontinuous and interrupted by gaps, and a modest alteration of the actin cytoskeleton [113]. Discontinuous staining of VE-cadherin and β-catenin accompanied by an increased permeability, was also observed in microvascular endothelial cell (MyEnd) monolayers treated with LT [174]. In addition, LT causes a reduced VE-cadherin–cadherin adhesion and a fragmentation of cortical actin in MyEnd cells. In this cell model, it is concluded that LT alters VE-cadherin junctions, via inactivation of Rac and subsequent depolymerization of actin filaments and disconnection of VE-cadherin from the actin cytoskeleton [174]. Similarly, LT increases the permeability in intact venular microvessels. Although LT alters actin filaments including acto-myosin stress fibers and cortical actin, its Rac-mediated effect on increased permeability is not dependent on the acto-myosin contractile mechanism, but rather on the dissociation of VE-cadherin from the actin cytoskeleton. Indeed, inhibitors of myosin contraction do not prevent LT permeability increase in intact microvessels. However, acto-myosin contraction which results from the remodeling of a few thick stress fibers after LT treatment, seems to control permeability in cultured vascular

endothelial cells by generating large intercellular gaps [175]. This supports the idea that in intact microvessels Rac controls AJs and permeability mainly by controlling the anchorage of VE-cadherin to the actin cytoskeleton independently of the acto-myosin contractile mechanism [175]. But the state of actin polymerization, depolymerization or hyperpolymerization, is critical for the stability of endothelial barrier function [176] and Rho-GTPases might have different effects on barrier function according to the endothelial cell types [177]. This further indicates that a subset of actin filaments regulated by Rac is essential for the organization of VE-cadherin junctions.

LT is a highly potent lethal toxin but its mechanism of lethality is still elusive. Intraperitoneal injection of LT in mice induces an increased vascular permeability, mainly at the level of lung vessels, leading to a massive extravasation of blood fluid in the thoracic cavity, dehydration, increase in hematocrit, hypoxia, and finally a cardio-respiratory distress. Alterations of lung endothelial cells were evidenced by electron microscopy showing modifications of AJs that appear fainter and more discontinuous than in control. VE-cadherin immunostaining is markedly altered with an interrupted and locally diffuse staining reflecting a redistribution of VE-cadherin from the junctional ring to the cytoplasm [171]. Thereby, LT, which inactivates Rac among the RhoGTPases, seems to preferentially target endothelial cells in the cardio-respiratory area, to alter the distribution of VE-cadherin, and subsequently to increase the endothelial barrier permeability leading to local edema and hemodynamic perturbations probably responsible for lethality.

ToxA and ToxB modify both TJ and AJ organization as well as apical and basolateral actin cytoskeleton in epithelial cell monolayers, and disrupt the epithelial barrier function (see above) [92,93,172]. Since these toxins simultaneously inactivate Rho, Rac and Cdc42, the toxin effects of each altered GTPase are difficult to show, and a synergetic RhoGTPase modification leading to disruption of all pools of actin cytoskeleton and intercellular junctions is observed, comparable to that induced by toxins, such as cytochalasin and *Clostridium perfringens* Iota toxin, which inhibits the actin filament polymerization by ADP-ribosylating actin monomers [178]. In an *in vivo* zebrafish model, ToxB localizes at the pericardial region and induces a cardiovascular failure including an extensive pericardial edema. But the potential effects of ToxB on endothelial intercellular junctions *in vivo* have not been investigated [179].

CNF1, as mentioned above, activates Rho, Rac and Cdc42, and disturbs the epithelial and endothelial barrier permeability. In HUVEC cells, CNF1 induces a prominent stress fiber formation without significant modification of the VE-cadherin localization [113]. Although, a higher level of Rac and Cdc42 activation than that of RhoA was detected in T84 intestinal cells treated with CNF1, the major CNF1 effects concern TJs, whereas only subtle alterations of AJs containing E-cadherin have been observed. Only, a slight increase in E-cadherin and β-catenin staining at cell–cell contacts has been observed. Since the CNF1-induced alterations are not blocked by Rho inhibitors, a delicate balance of Rho activity and interplay between RhoGTPases must be involved in the maintenance of optimal organization and function of intercellular junctions [102].

CNF has different effects on endothelial cells according to the endothelial cell types (Table 2). CNF1 activates RhoA, Rac and Cdc42 in MesEnd, PAEC and HDMEC cells, but only Rac and Cdc42 in MyEnd cells, whereas CNF_y from *Yersinia pseudotuberculosis* selectively activates RhoA in all four endothelial cell lines. RhoA activation resulting in increased stress fiber formation alters the endothelial barrier integrity only in some endothelial cells such as PAEC. In combination with the results obtained with toxins inhibiting Rho-GTPases (LT, ToxB), it is concluded that Rac is the common regulator of barrier function of the different endothelial cells, even if a complex regulation system involving the other Rho-GTPases is involved [117,180].

11. Ras and intercellular junctions

Contradictory results have been observed in the effects of activated Ras on intercellular junctions [181]. Microinjection of activated Ras in MDCK cells leads to disorganization of AJs, visualized by the loss of E-cadherin and β-catenin from intercellular junctions, but without significant changes in the distribution of TJ components. These Ras effects are mediated by two pathways, MEK1 and PI3K-Rac1, as shown by using specific inhibitors of MEK1 and PI3K, as well as dominant negative mutants of MEK1 or Rac1 (Fig. 3). It is noteworthy that both pathways activated by Ras are required for AJ disassembly [182]. Similar effects on AJ have been observed in epidermal keratinocytes microinjected with activated Ras, which induces the loss of E-cadherin and α-catenin from the cell–cell contacts as well as a redistribution of β-catenin to the cytoplasm and nucleus in a PI3K-dependent manner [183]. In contrast, in MDCK cells transformed with Ras hyperactive mutant, TJ proteins (ZO-1, occludin and claudin-1) are redistributed from the intercellular contacts to the cytoplasm, and E-cadherin expression is decreased. Prevention of Ras effects with MEK1 inhibitor causes reassembly of TJs, phosphorylation of ZO-1 and occludin, as well as the restoration of the cell barrier integrity [184]. The differences in the hyperactive Ras effects on TJs or AJs according to these studies might depend on the different cell models and levels of activated Ras resulting from cell transformation or protein micro-injection. However, these findings show that Ras does not directly control TJs and AJs, but uses complex signaling pathways, some of them involving Rho proteins.

Another Ras-dependent signaling pathway includes afadin (AF-6 for human afadin) (Fig. 3). Afadin contains two Ras-binding domains, in addition to other protein–protein interacting domains like a DIL domain, a PDZ domain, three proline-rich domains and an actin filament-binding domain [33]. It has been shown that afadin associates with ZO-1 through the Ras-binding domains, and that activated Ras prevents the interaction between afadin and ZO-1 *in vitro*. In addition, transformation of Rat1 cells with activated Ras reduces the membrane localization of afadin and ZO-1, and inhibits

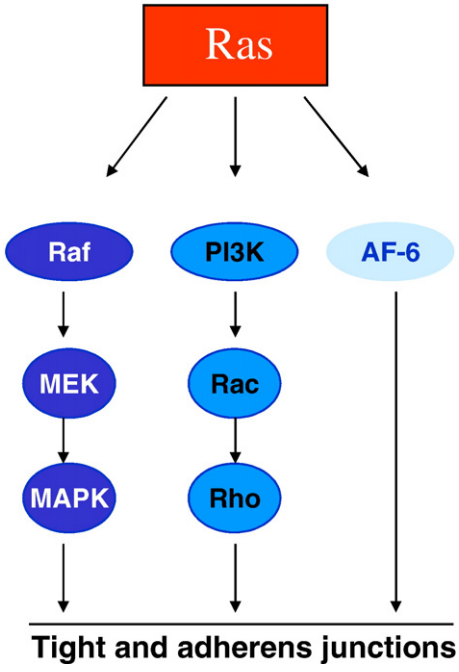

Fig. 3. Ras and the regulation of tight and adherens junctions. Diagram of relationships between Ras its effectors involved in the regulation of tight and adherens junctions. Three main pathways are regulated by Ras. The first one involves Raf, MEK1/2 and the MAPK Erk1/2. The second one acts via activation of PI3K, Rac and Rho. The third one requires activation of AF-6.

the formation of intercellular junctions [185]. Therefore, activated Ras could control intercellular junctions through the afadin target effector. Another possibility could be that afadin, which only binds to GTP-Ras, sequesters activated Ras, preventing its interaction with downstream effectors [181].

Loss of intercellular junctions is a crucial step in tumor progression such as carcinomas. Oncogenic Ras seems to collaborate with Rho-GTPases, at least in part by altering cell–cell association, to induce full malignant activity [3]. For example, oncogenic Ras decreases Rac activity by downregulation of Tiam1 through the Raf/MAP kinase signaling, and increases Rho activity, leading to epithelial–mesenchymal transition [186]. However, in primary epithelial cells overexpression of dominant active Ras results in a decrease in cell–cell adhesion and loss of epithelial morphology through a pathway including activated Rac. Indeed, co-expression of dominant active Rac with dominant active Ras enhances the disassembly of AJs by preventing association of α-catenin with E-cadherin–β-catenin complex [187]. This again highlights the complexity of interplay between Ras and Rho-GTPases.

12. Concluding remarks

Intercellular junctions are dynamic structures, even after cell–cell contacts have been stably formed. Structural proteins of AJs and TJs are continuously recycled between plasma membrane and intracellular compartments. A strict spatio-temporal regulation of intercellular junctions is required for the proper functioning of epithelial and endothelial barriers. Among the complex network of AJ and TJ regulation, Rho/Ras-GTPases play an important role. Cross talk between RhoGTPases and intercellular junctions is complex. RhoGTPases are involved in the formation and maintenance of stable junctions. Conversely, intercellular junctions such as cadherin-dependent junctions activate various signaling pathways including RhoGTPases which participate in cell morphology, growth control, survival, and differentiation [45,46].

Although Rho seems to preferentially regulate TJs, and Rac/Cdc42 mainly control AJs, a fine balance in the activity of all Rho/Ras-GTPases is required for the appropriate epithelial and endothelial barrier function. Up- or down-regulation of one GTpase results in breakdown of barrier function with increased permeability. Thus, a complex cross-talk between Rho/Ras-GTPases is involved in the maintenance of optimal organization and function of epithelial and endothelial barriers. Rho acts mainly by controlling through Rok, the contractility of acto-myosin filaments, which are linked to TJ and AJ components, whereas Rac/Cdc42 preferentially promote assembly/disassembly of AJ components or their association with actin filaments. In endothelial barriers, where TJs and AJs are intermingled, a strict spatio-temporal coordination between Rho-GTPases is required to ensure an optimal barrier function. Such a time-regulated process has been recently evidenced including Rho activation by an external signal, which leads to an increased permeability, and including a delayed activation of cdc42 permitting the restoration of endothelial barrier [150].

Dysfunction of epithelial/endothelial barrier function is a critical step in the pathogenesis of various diseases. For example, localized infection can trigger the release of inflammatory factors, which increase cell barrier permeability. Certain pathogens produce toxins, which impair cell barrier permeability, resulting in alteration of the intestinal cell barrier and/or vascular endothelial barriers, in formation of edema, etc. Interestingly, most of the toxins active on intercellular junctions target Rho/Ras-GTPases, which have a key role in the regulation of TJs and AJs. Modulation of Rho-GTPase activity by toxins, for example CNF1-dependent Rho-GTPase activation followed by inactivation through the ubiquitin–proteasome pathway, contributes to successful epithelium invasion by bacteria [188]. In addition, disruption of endothelial barrier is critical in vascular and lung diseases (atherosclerosis, acute lung injury, etc.). Loss of intercellular junctions is also a determinant in tumor cell progression and malignant behavior. Better understanding of the regulation mechanisms including identification of the partners of the regulation network holds promise for developing new therapeutic strategies against these diseases.

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