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Rho GTPase Signaling in the Development of Colorectal Cancer

Fernanda Leve and José A. Morgado-Díaz*

Grupo de Biologia Estrutural, Divisão de Biologia Celular, Centro de Pesquisas, Instituto Nacional de Câncer–INCa, Rio de Janeiro 2231050, Brazil

ABSTRACT

The involvement of Rho GTPases in major aspects of cancer development, such as cell proliferation, apoptosis, cell polarity, adhesion, migration, and invasion, have recently been attracting increasing attention. In this review, we have summarized the current findings in the literature, and we discuss the participation of the Rho GTPase members RhoA, Rac1, and Cdc42 in the development of colorectal cancer, the second most lethal neoplasia worldwide. First, we present an overview of the mechanisms of Rho GTPase regulation and the impact that regulator proteins exert on GTPase signaling. Second, we focus on the participation of Rho GTPases as modulators of colorectal cancer development. Third, we emphasize the involvement of activation and expression alterations of Rho GTPases in events associated with cancer progression, such as loss of cell–cell adhesion, proliferation, migration, and invasion. Finally, we highlight the potential use of novel anticancer drugs targeting specific components of the Rho GTPase signaling pathway with antineoplastic activity in this cancer type. J. Cell. Biochem. 113: 2549–2559, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: Rho GTPases; COLORECTAL CANCER; ACTIN CYTOSKELETON; TUMOR PROGRESSION; CELL–CELL ADHESION; CELL SIGNALING

Rho GTPases belong to the Ras superfamily of small GTPases, which includes the Ras, Ran, Rab, Arf, Rheb, and Rho families. These proteins were initially described over three decades ago as eukaryotic targets of *Clostridium botulinum* exotoxins. Since that time, a great number of biochemical, cell biological, and physiological studies have demonstrated that Rho GTPases as crucial regulators of cellular homeostasis during both normal and disease conditions [reviewed in Vega and Ridley, 2008]. Mammalian cells express 22 Rho GTPases, including three Rho isoforms (A, B, and C), three Rac isoforms (1, 2, and 3), and Cdc42, which have been the subject of intense investigation. Despite the fact that Rho, Rac, and Cdc42 catalyze identical chemical reactions (see below) and can target the same cellular structures, their functional effects can be vastly different. For example, Rho activation induces the assembly of basal stress fibers in cytoskeleton-driven alterations of cell motility, while increases in Rac and Cdc42 activity result in the formation of lamellipodia and filopodia, respectively. The enormous versatility of the intracellular actions of Rho, Rac, and Cdc42 highlights their ability to control a majority of important cellular processes, including cell proliferation, apoptosis, cell survival, cell polarity, cell adhesion, and membrane trafficking. Therefore, it is not surprising that alteration of either the expression or activation status of different Rho GTPases can lead to different pathologies. This is especially true for tumorigenesis because a large number of recent studies have implicated Rho, Rac, and Cdc42 expression in malignant transformation of mammalian cells as well as the progression and dissemination of various tumors. A majority of these studies focused on breast [Tang et al., 2008], ovarian [Cheng et al., 2009] and hepatocellular [Grise et al., 2009] cancers. In contrast, the involvement of Rho GTPases in colorectal cancer, the second most lethal neoplasia worldwide, is not well understood [Jemal et al., 2006].

Here, we provide an overview of the recent findings concerning the role that the three most studied members of Rho GTPase family, Rho, Rac, and Cdc42, play in the development of colorectal cancer as well as the molecular mechanisms that mediate Rho-dependent cell transformation.

EXPRESSION AND REGULATION OF Rho GTPases

Rho GTPase members are regulated by both protein regulator signaling and cell surface receptors. They also play a pivotal role in gene expression, cell proliferation, apoptosis, and other various

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*Correspondence to: José Andrés Morgado-Díaz, Grupo de Biologia Estrutural, Divisão de Biologia Celular, Centro de Pesquisas, Instituto Nacional de Câncer–INCa, Rio de Janeiro 2231050, Brazil. E-mail: jmorgado@inca.gov.br

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cellular functions. Here, we focus on data regarding variations in the expression levels of Rho, Rac, Cdc42 as well as their primary regulators and the effects of these events during the progression of colorectal cancer.

Rho GTPases are monomeric proteins with an intrinsic capability of cleaving GTP and follow a regulatory mechanism common to almost all Ras GTPases. Cycling between an inactive GDP state and an active GTP state, three classes of proteins regulate Rho: guanine nucleotide-exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide-dissociation inhibitors (GDIs). GEFs activate Rho proteins by catalyzing the exchange of GDP for GTP, while GAPs stimulate the intrinsic GTPase activity and promote the return to the inactive state. The inactive pool of Rho proteins is maintained in the cytosol by association with GDI. In the active GTP-bound conformation, Rho GTPases interact with effectors [Vega and Ridley, 2008]. Interestingly, relatively few studies have addressed the role of GTPase regulators in the development of colorectal cancer.

The GEFs comprise a family of 82 proteins, of which only the Rac specific Tiam1 and the Rho specific Rgnef (p190RhoGEF) are upregulated in colorectal cancer and favor pro-tumoral properties [Malliri et al., 2006; Yu et al., 2011]. As both Tiam and Rgnef GEFs are specific for Rho and Rac GTPases, respectively, they determine which signaling pathway and downstream effectors are activated during cancer progression. Only two members of the GAP family of proteins, which represents a family of 67 members, have been shown to be associated with colorectal cancer. While both deleted in liver cancer-1 and -2 (DLC-1 or ARHGAP7 and DLC-2) are downregulated [Ullmannova and Popescu, 2006], the Rho GTPase activating protein 8 (ARHGAP8) is upregulated in colorectal cancer [Johnstone et al., 2004]. This differential expression of proteins that should play the same function suggests that specific Rho GTPase members are regulated differently by GAPs. For example, DLC-1 targets RhoA, indicating that its downregulation is related to the hyperactivity of this member of Rho family. Additionally, it seems that a specific subcellular localization of GAP is required as DLC-1 is a tumor suppressor when located in the focal adhesion (FA) [Kim et al., 2009]. However, despite the overexpression/activation of RhoA that correlates with DLC-1 downregulation, it remains to be determined whether a negative feedback loop exists to explain the overexpression of ARHGAP8. Among the GDIs, a family with three members (α, -β, and -γ RhoGDI), in colorectal cancer, RhoGDIα is upregulated [Zhao et al., 2008] while RhoGDIβ is downregulated [Fujita et al., 2011]. Again, this differential expression indicates a fine-tuning regulation of specific Rho members important for colorectal cancer progression. Recently, it was proposed that RhoGDIs protect Rho GTPases from degradation [Boulter et al., 2010]; therefore, the overexpression of RhoGDIα might represent a mechanism to compensate for the increase in Rho member expression levels in cells, as it is ubiquitously expressed and interacts with several Rho GTPases, including RhoA, RhoC, Rac1, Rac2, and Cdc42 [Garcia-Mata et al., 2011]. Additionally, we cannot rule out the hypothesis that RhoGDIα compensates for the loss of RhoGDIβ. In fact, RhoGDIs were first described as Rho inhibitors because they sequestrate small GTPases at the cytosol. According to this finding, the observed downregulation of RhoGDIβ correlates with the theory that Rho GTPases are activated in colon cancer samples. However, it seems to be more complex as RhoGDIs display similar affinities for both the active and inactive forms of Cdc42 and Rac [Nomanbhoy and Cerione, 1996]. Furthermore, the depletion of RhoGDIα reduces the levels of plasma membrane-associated GTPases, where they are predominantly active, thereby suggesting that RhoGDIs are involved in the transport of Rho GTPases to the plasma membrane [Garcia-Mata et al., 2011]. The implications of this regulatory mechanism in colon tumor cells are still unknown.

Active Rho GTPases acquire a conformational change that allows them to interact with approximately 100 downstream effector proteins and subsequently execute specific cellular functions [Vega and Ridley, 2008]. Most of these effector proteins are serine–threonine kinases that phosphorylate downstream target proteins and thereby activate various signaling pathways. Table I summarizes the observed altered expression levels of Rho GTPases as well as the regulatory/effector proteins that trigger numerous signaling pathways important for colorectal carcinoma development and progression. In addition, it is thought that Rho GTPases are transcription factor regulators as they are capable of modulating SRF, NFκB, STATs, CREB, and other transcription factors, which are involved in various stages of tumorigenesis [as reviewed by Benitah et al., 2004]. Moreover, a recent study has demonstrated RhoA localization in the nucleus in SW480 colon cancer cells and cancer tissues [Li et al., 2010]; however, the role that the protein plays in this cellular compartment is still unknown.

Transcriptional upregulation of Rho proteins have been extensively described in the literature, although no mutations in Rho GTPase genes in colorectal cancer have been identified. Recent miRNA (miR) studies may clarify this issue as aberrant expression of miRs has been shown in human tumors. For instance, high expression levels of miR-185 and low expression levels of miR-133b were significantly associated with poor survival and metastasis in colorectal cancer patients [Akçakaya et al., 2011]. The high expression levels of miR-185 could be of great interest because it directly regulates both RhoA and Cdc42 expression and is associated with both proliferation and invasion in colon cancer cells [Liu et al., 2011].

Rho GTPases and accessory proteins are also regulated by post-translational modification including phosphorylation, ubiquitylation, and lipid modification. For example, PKA inhibits RhoA via phosphorylation of serine residue 188 [Qiao et al., 2008], and Src phosphorylates RhoGDI at tyrosine 156, which impairs the interaction of RhoGDI mainly with RhoA and Rac [DerMardirossian et al., 2006]. Smurf1 induces ubiquitin-mediated proteasomal degradation of activated RhoA in a site-specific manner. Cancer cell lines displaying lack of RhoA, Rac 1, and Cdc42 ubiquitylation display sustained activation of these GTPases [Boyer et al., 2006]. Ubiquitin/proteasome-mediated degradation of active Rac1 has also been observed during early steps of epithelial cell scattering [Lynch et al., 2006]. Finally, the activity and subcellular localization of classical Rho GTPases are regulated by prenylation of farnesyl or geranylgeranyl isoprenoid lipids in a reaction mediated by farnesyltransferase (Ftase) and geranylgeranyltransferase type 1 (GGTase-I), respectively, at the cysteine residues of their carboxyl-terminal CAAX motif (C, cysteine; A, aliphatic amino acids; and X, a

TABLE I. Altered Expression of RhoGTPases and Regulatory/Effector Proteins and Cellular Function Response in Colorectal Carcinoma

Gene/protein	Expression	Cellular function	References
GTPase			
RhoA	Up[a]	Cell–cell adhesion disruption Migration/metastasis	Tatsuta et al. [2005], Carothers et al. [2006], Takami et al. [2008], Wang et al. [2009]
RhoC	Up[a,c]	Cell–cell adhesion disruption Invasion	Bellovin et al. [2006], Wang et al. [2009]
Rac1	Up[a]	Migration/invasion	Takami et al. [2008], Espina et al. [2008]
Rac1b	Up[c]	Cell cycle progression Survival/anti apoptotic	Jordan et al. [1999], Esufali et al. [2007], Matos et al. [2008]
Rac1b	Up[c]	Cell cycle progression Survival/anti apoptotic	Jordan et al. [1999], Esufali et al. [2007], Matos et al. [2008]
Cdc42	Up[b]	Migration/invasion	Gomes Del Pulgar et al. [2008]
Effector			
IQGAP1	Up[a]	Cell–cell adhesion disruption Migration/invasion	Nabeshima et al. [2002], Watanabe et al. [2004], Noritake et al. [2005]
N-WASP	Up[d]	Migration/metastasis	Yanagawa et al. [2001]
WAVE2	Up[a]	Metastasis	Iwaya et al. [2007]
PAK1	Up[a]	Cell–cell adhesion disruption Migration/metastasis	Carter et al. [2004], Lozano et al. [2008]
PAK4	Up[b]	Tumor growth Transformation	Liu et al. [2008]
PAK5	Up[a]	Differentiation and adhesion lost/migration	Gong et al. [2009]
ROCKI-II	Up	Cell–cell adhesion disruption Tumor growth/migration/metastasis Angiogenesis/survival	Croft et al. [2004],* Carothers et al. [2006], Vishnubhotla et al. [2007]
Regulators			
GDI			
RhoGDIα	Up[b,e]	Cell proliferation Migration/metastasis	Zhao et al. [2008]
RhoGDIβ	Down[c]	Metastasis	Fujita et al. [2011]
GAP			
DLC-1 and -2	Down[c]	Cell proliferation Migration	Ullmannova and Popescu [2006], Jin et al. [2008]
ARHGAP8	Up[c]	Unknown	Johnstone et al. [2004]
RhoGAP6	Up[b,c]	Unknown	Guo et al. [2010]
GEF			
Tiam1	Up[a]	Cell–cell adhesion disruption Tumor growth Anoikis resistance Migration/invasion	Minard et al. [2005], Minard et al. [2006], Malliri et al. [2006], Jin et al. [2011]
P190RhoGEF	Up[a]	Migration	Yu et al. [2011]
Asef 1 and -2	Up[a,c]	Cell–cell adhesion disruption Migration	Kawasaki et al. [2009]

*This study was performed using activated form of ROCK-expressing colon carcinoma cells in nude mice. Rock was not used with human colorectal samples.
[a]Immunohistochemistry.
[b]Western blotting.
[c]Real time PCR.
[d]cDNA microarray.
[e]Proteomic.

terminal amino acid). After prenylation, the other three C-terminal amino acids (-AAX) are cleaved by a specific protease termed Ras-converting enzyme 1 (Rce1) and subsequently methylated by isoprenylcysteine carboxyl methyltransferase (Icmt). Together, these modifications increase protein hydrophobicity and facilitate the membrane anchorage of Rho GTPases, which is associated with their activity [Roberts et al., 2008]; it is for this reason that prenylation inhibitors have been used in therapeutics. Although these regulator mechanisms have been reported for various cell types, the mechanism remains unclear in colorectal cancer.

These findings demonstrate the involvement of Rho GTPases in a wide variety of regulatory mechanisms and indicate that these proteins play an important role in complex signaling pathways mediating events during the development of colon cancer, thereby suggesting that Rho GTPases may constitute important therapeutic targets.

Rho GTPases ASSOCIATED WITH COLORECTAL CANCER DEVELOPMENT

The intestinal homeostasis is a dynamic process in which stem cells at the base of crypts divide and migrate to the apical region, where they differentiate. This allows for the self-renewal of the colon epithelium with equilibrium between cell proliferation and death, which is loosely controlled by the Wnt pathway [van der Flier and Clevers, 2009]. Both the abnormal maturation and inflammation of the mucosa can give rise to benign polyp formation, and eventually, malignancy can take place through an ordered set of mutations referred to as the adenoma–carcinoma sequence [Fearon and Vogelstein, 1990]. Throughout this process, there are successive mutations of tumor suppressor genes and oncogenes, which are controlled by either transcriptional or posttranscriptional regulators. Among them, the most important genes for colon cancer development are the tumor suppressor *p53*, which is mutated in

approximately half of all colorectal cancers [Lacopetta, 2003], the oncogene *K-Ras*, which is mutated in more than 60% of colorectal cancer patients [Takayama et al., 2001], and the adenomatous polyposis coli (*Apc*), which is mutated in 80% of patients with this cancer type [Shitashige et al., 2008]. Interestingly, Rho GTPases are associated with the alteration of these three genes that regulate various events involved in the development of colorectal cancer. These aspects are discussed below and illustrated in Figure 1.

Some studies have discussed the role that Rho GTPases play on gene expression regulated by the Wnt pathway, which negatively regulates Apc. A common model used to study the biology of colorectal cancer in vivo is the Min mouse strain, which presents a hereditary adenoma formation and a heterozygous point mutation in the *Apc* gene leading to the development of multiple polyps in both the small and large intestines. Using this model, the intestinal mucosa of adult Min/+ mice have displayed elevated expression levels of Rho-GTP [Carothers et al., 2006]. Additionally, Rho GTPases have been shown to modulate the expression of Wnt target genes in colon cancer cells. A comparative microarray analysis has shown that Rac1 inhibition suppresses the expression of the Wnt-induced genes *NKD1*, *BAMBI*, and *MMP-7*, which are all associated with cell invasion and metastasis [Gomes del Pulgar et al., 2007]. Rac1b overexpression has been shown to stimulate the induction of the Wnt target gene, *cyclin D*, a known cell cycle regulator [Esufali et al., 2007]. Rho GTPases also associate with Apc at a post-transcriptional level in colon cancer cells, as truncated Apc interacts and activates both Asef and Asef2, GEFs specific for Cdc42 [Kawasaki et al., 2003; Mitin et al., 2007]. Both genes are aberrantly enhanced in intestinal adenomas and tumors [Kawasaki et al., 2009], and Asef overexpression has been shown to reduce E-cadherin-mediated cell–cell adhesion and promote cell migration [Kawasaki et al., 2003]. Apc also interacts with IQGAP, an effector protein of both Rac and Cdc42, to modulate cell migration [Watanabe et al., 2004]. These results correlate with Cdc42 overexpression/activation observed in colorectal cancer samples. However, it has been shown that the active mutant of Cdc42 inhibits the anchorage-independent growth of colon cancer cells, while Asef has been described as a tumor suppressor [Mitin et al., 2007]. Cdc42 also interacts directly with both the full-length and truncated Apc in colon cancer cells in two processes. On the one hand, Cdc42-Apc serves to localize full-length Apc to the leading edge with F-actin, which correlates with recent studies suggesting that Apc regulates cytoskeletal dynamics to influence cell migration, division, polarization, and adhesion. On the other hand, Cdc42 interaction with a common type of truncated Apc leads Apc to the golgi/lysosome compartments. In this context, it seems that active Cdc42 induces the degradation of truncated Apc, thereby reducing its function as a tumor suppressor [Sudhaharan et al., 2011]. Thus, these studies suggest the possibility of a crosstalk between Rho GTPases and the Apc/Wnt pathway to enhance gene transcription and cellular events associated with the development of colorectal cancer.

Many features of cancer cells emerge as a result of interplay between multiple oncogenic mutations. *Ras* and *p53* mutations occur with high frequency in human colorectal cancer and the presence of both mutations in the same tumor strongly correlates with disease progression to malignancy. A study using colon cells of murine and human origin has shown that cells expressing a constitutively active form of Ras (RasV12) and low p53 activity display increased RhoA activity and cell motility [Xia and Land, 2007]. During colorectal cancer development, the *K-Ras* allele mutation that leads to inhibition of its function, impaired Rho activation and abrogated stress fiber formation, which suggests that

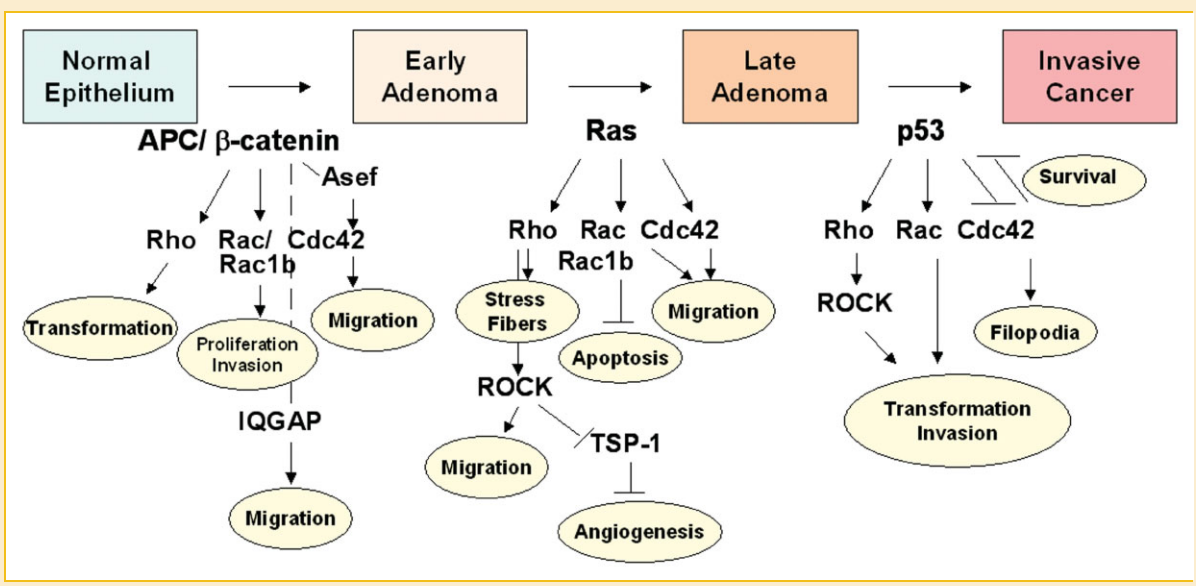

Fig. 1. Rho GTPases in the colorectal adenoma–carcinoma sequence. Activation of the Wnt signaling pathway can occur at the first stage of colorectal cancer progression as a result of mutations in the *APC* gene. Progression to late carcinomas requires activating mutations of the proto-oncogene *KRAS*, and to invasive cancer mutations in *TP53*. Rho, Rac and Cdc42 participate of signaling pathways of these three genes to mediate important cellular events of colon cancer progression (yellow circles). See the text for details. Activation →, inhibition ⊣, interaction —. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

oncogenic *K-Ras* induces increased Rho activity [Pollock et al., 2005]. *K-Ras* also activates Rac to prevent apoptosis. For example, mutations in *BRAF*, a gene that encodes a downstream protein of Ras signaling, has been shown to activate a tumor-specific splice variant of Rac1 GTPase, Rac1b, thereby promoting the survival of colon cancer cells [Matos et al., 2008]. In fact, colon adenocarcinoma cells expressing an oncogenic *Ras* mutation displayed enhanced RhoA, Rac1, and Cdc42 expression, thereby mediating cell migration [Makrodouli et al., 2011]. Moreover, the Rho/ROCK/c-Myc pathway is downstream of Ras and thereby represses the anti-angiogenic factor thromspondin-1 (TSP-1) in epithelial cancers [Watnick et al., 2003]. Interestingly, TSP-1 is strongly expressed in normal colonic epithelial cells, but it is lost in early colonic adenomas and becomes undetectable in invasive colon cancers [Jo et al., 2005]. Therefore, it is possible that Rho overexpression downregulates TSP-1 expression in this cancer type.

Crosstalk between p53 and Rho GTPases has been initially observed in fibroblasts in which both the overexpression of wild-type p53 and the activation of endogenous p53 counteracted Cdc42-induced filopodia formation [Gadéa et al., 2002]. It has been then shown that Cdc42 negatively regulates p53-induced apoptosis in HCT-116 colon cancer cells in response to miR-29 [Park et al., 2008]. As elevated RhoA and Rac activity was identified in $p53^{-/-}$ mouse fibroblasts [Guo et al., 2003; Guo and Zheng, 2004], loss of function of p53, a common feature in colorectal cancer, may contribute to increased Rho GTPase activity. Furthermore, it has been shown that p53-deficient mouse fibroblasts cultured in three-dimensional matrices displayed amoeboid-like movement with increased invasive properties in a RhoA–ROCK-dependent manner [Gadéa et al., 2007]. Thus, these data suggest a new function for p53, as it modulates cell morphology and migration in addition to cell cycle progression control. However, whether these functions also occur in mutated p53 colon cancer cells remains to be evaluated. Therefore, events leading to Rho GTPase hyperactivation may cooperate with p53-deficiency to promote cell transformation and invasion.

Rho GTPases AND METASTASIS

During the early stages of the metastatic process, epithelial cells display a loss in cell–cell adhesion and polarity, enhanced cell proliferation, reduced cell death, and acquire a migratory and invasiveness potential. Subsequently, the epithelial cells detach from the primary tumor, migrate toward blood or lymphatic vessels (intravasation), exit the vessels (extravasation), colonize secondary sites, and undergo angiogenesis. The current data derived from in vivo and in vitro studies showing that deregulated Rho GTPase signaling represents an important mechanism for dissemination/metastasis in colorectal cancer is discussed below and illustrated in Figure 2.

Rho GTPases IN CELL–CELL ADHESION LOSS AND PROLIFERATION

The epithelium lining the gastrointestinal tract is composed of a monolayer of polarized cells attached to their neighbors via highly

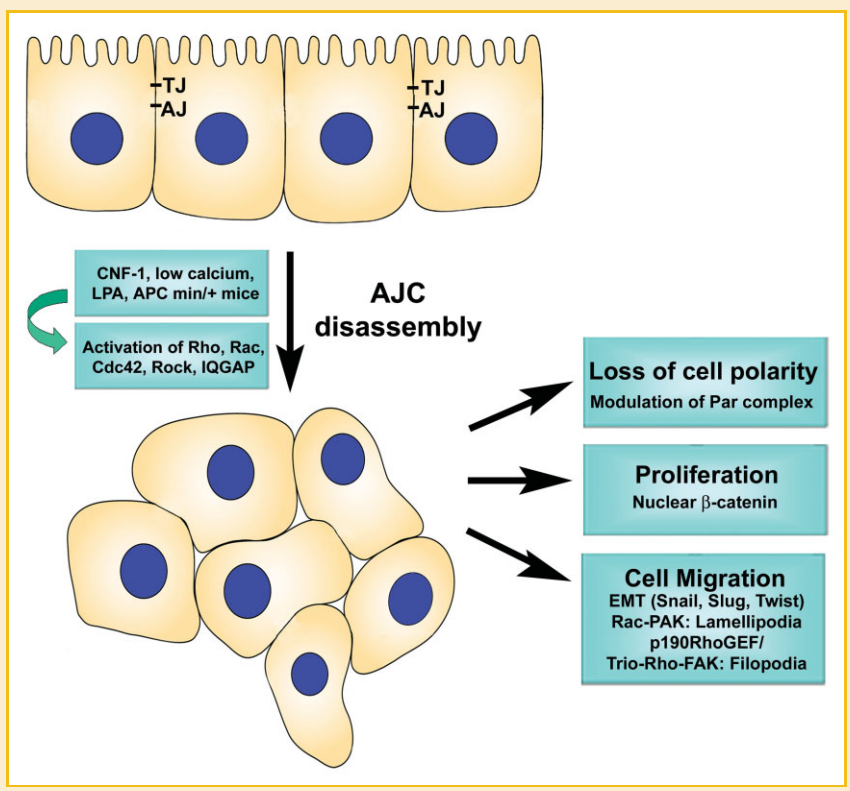

Fig. 2. Rho GTPases inducing cell–cell adhesion lost modulate cell polarity, proliferation and migration in colon cancer cells. Rho GTPases activation by various agents mediates AJC disassembly. The cell–cell junctions disruption constitute an initial event for loss of cell polarity, increased proliferation and migration, as indicated. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

organized intercellular junctions. These adhesive structures are formed by tight junctions (TJs), adherens junctions (AJs), desmosomes, and gap junctions, of which the two former junction types are most apically located and collectively referred to as the apical junctional complex (AJC). Both TJs and AJs are composed of transmembrane and peripheral membrane protein complexes, which interact with the underlying actin cytoskeleton. In this context, Rho GTPases are known to regulate both the assembly/disassembly and function of AJC. It has been well established that AJC disassembly and subsequent cell–cell adhesion loss can trigger cell-signaling pathways leading to epithelial cancer progression.

In colon cancer cells, various stimuli that trigger AJC disruption, such as the cytotoxic necrotizing factor-1 [Hopkins et al., 2003], extracellular calcium depletion [Leve et al., 2008], and LPA [Leve et al., 2011] have been shown to lead to activation of the Rho GTPases. Furthermore, in vivo studies using either mice treated with LPA or ex vivo intestines of mice treated with a calcium chelator mimicked the *Apc* Min/+ mice phenotype, which exhibit increased Rho GTPase activity and decreased AJ-dependent adhesion [Carothers et al., 2006]. Moreover, as apico-basal polarity in epithelial cells is sustained by the organization of the actin cytoskeleton and AJC, it is not surprising that Rho also plays a role in this process. A detailed review regarding Rho GTPase interaction with proteins of the polarity complex in various cell types can be found in Iden and Collard [2008]. In addition, dominant-negative Rac cells have been shown to display inversion of cell polarity in a dependent form of RhoA–Rho-kinase activation [Yu et al., 2008]. However, there are few studies linking cell polarity components and Rho GTPases during colorectal cancer development.

It is well known that Rho GTPases regulate important cellular events such as cell-extracellular matrix (ECM) adhesion as well as cortical actin ring and microtubule dynamics, which are involved with cell proliferation control. In colorectal cancer, only a few studies have demonstrated the direct participation of Rho GTPases in the regulation of both proliferation and apoptosis, although it is possible to speculate that it could be a consequence of cell–cell adhesion disruption because functional AJC inhibits cell proliferation. For example, it has been shown that the functional E-cadherin-β-catenin complex impairs the nuclear location of β-catenin, thereby inhibiting transcriptional factors associated with colon cancer cell division [Vidal et al., 2011]. In agreement with this hypothesis, Rho activation with LPA enhances cell proliferation by activating the β-catenin signaling pathway in HCT-116 colon cancer cells [Yang et al., 2005], and this biolipid also prevented apoptosis in colonic epithelial cells [Rusovici et al., 2007]. Other AJC proteins, such as α-catenin [El-Bahrawy et al., 2002], ZO-1 [Balda et al., 2003] and symplekin [Buchert et al., 2010], are associated with enhanced proliferation of colon cancer cells, although the role that Rho GTPases play in this context is still unknown. These results suggest that Rho GTPases play a role in the processes of proliferation and apoptosis via AJC disruption in colon cancer cells.

The study of Rho effector proteins has contributed to the understanding of the mechanism by which Rho GTPases may modulate AJC organization. The Rho-kinases (ROCK I and II) were the first Rho effector proteins identified and were reported to be modulators of stress fibers and FA formation. As the actin cytoskeleton is AJC-linked, some studies using in vitro and in vivo models of colon cancer have demonstrated the participation of ROCKs in AJC disassembly [Carothers et al., 2006; Leve et al., 2008; Ivanov et al., 2009]. Other GTPase effector proteins that modulate cell–cell disruption include IQGAP and P21-activated kinases (PAK). IQGAP is an effector of Rac and Cdc42 that localizes at cell–cell contacts. Under conditions of decreased Rac activity, IQGAP binds to β-catenin to dissociate α-catenin from the E-cadherin-catenin complex. However, when Rac activity is increased, this protein interacts with IQGAP to prevent the disruption of this complex [Noritake et al., 2005]. Rac activity promotes E-cadherin-dependent adhesion disassembly via a mechanism involving PAK1. As PAK1 is crucial for lamellipodia formation, this protein is considered to be an interface between AJC destabilization and increased motility [Lozano et al., 2008]. As indicated in Table I, IQGAP1, PAK1, PAK4, and PAK5 are upregulated in colorectal cancer; however, the role of this protein upregulation remains to be elucidated.

Finally, downregulation or redistribution of E-cadherin leads to cell–cell adhesion disruption with concomitant increase of cell migration, invasiveness, and metastasis in colorectal cancer. E-cadherin expression is negatively regulated by various transcriptional factors such as Snail, Slug, Twist, and ZEB, which also act as inducers of the epithelial-to-mesenchymal transition (EMT) [Medici et al., 2006]. EMT is a morphogenetic process that also occurs in cancer and triggers the loss of cell polarity, cell–cell adhesion disruption, actin cytoskeleton reorganization, and cell migration. In this context, a study using colon cancer cells has shown that the expression and subsequent activation of the RhoC protein, concomitantly with downregulation of E-cadherin and a marked decrease of RhoA activation, is associated with EMT development [Bellovin et al., 2006]. Additionally, Tiam, an activator of both Rac and Cdc42, has been shown to induce E-cadherin downregulation and increase the expression of vimentin, an EMT marker [Minard et al., 2006]. Moreover, in colorectal cancer tissue samples, E-cadherin downregulation and translocalization of the AJ protein p120 catenin from cell–cell junctions to the cytoplasm has been shown to be associated with a reduced patient survival time and an increase in tumor stage and metastasis [Bellovin et al., 2005]. Although EMT is a difficult process to analyze in vivo, studies have described the expression of EMT markers in cells localized to the invasive tumor front. Such EMT markers include nuclear β-catenin [Hlubek et al., 2007] high vimentin [Chen et al., 2008], and N-cadherin [Van Aken et al., 2001] expression levels.

These studies indicate that Rho GTPases are involved in AJC disassembly, which may modulate cell polarity, proliferation, EMT, and migration in colon cancer cells, although additional investigation is required to determine the role that Rho GTPases play in vivo in this cancer type.

Rho GTPases IN TUMOR CELL MIGRATION AND INVASION

Early studies have shown that Rho activation increases the cell migration activity of colon cancer cells. It has also been shown that Tiam1, a GEF of both Rac and Cdc42, enhances cell migration by activating Rac [Minard et al., 2006]. Thus, these data suggest that the activation of Rho, Rac, and Cdc42 mediate colon cancer cell

migration, which is a controversial hypothesis because Rho and Rac antagonize each other in a variety of cell types. However, it is known that coherent cell movement requires a dynamic coordination of adhesion and motility, thereby indicating that a tight spatiotemporal regulation of these three members of the Rho GTPase family occurs [Pertz et al., 2006]. The concept that Rho, Rac, and Cdc42 regulate the actin cytoskeleton and mediate the formation of stress fibers, lamellipodia, and filopodia, respectively, has been well established. Here, we do not intend to describe the complicated signaling pathways and protein complexes that constitute the machinery of protrusions important for cell migration; instead, we will highlight scientific advances regarding the understanding of the involvement of Rho GTPases in the mediation of cell migration and the invasion of colon cancer cells.

Various types of cell migration exist depending on both the cell type and cellular context. In general, during tumor progression, epithelial cells that lose cell–cell contacts use two types of migration: (a) mesenchymal migration, a consequence of the EMT process in which a partial loss of cell polarity and acquisition of a fibroblastic-like morphology occurs; and (b) amoeboid migration, in which cells completely lose cell polarity and acquire a round morphology with protrusions in the direction of the cell movement. It has also been shown that the ability of tumor cells to switch between different modes of motility depends on the response to Rho GTPases [Sanz-Moreno et al., 2008]. In colon cancer cells, Smurf1 activation, which induces Rho degradation, favors mesenchymal migration, while Smurf1 inhibition causes mesenchymal–amoeboid transition movement [Sahai et al., 2007]. These findings suggest that Rho mediates amoeboid motility, although Rho/ROCK is still important for the promotion of mesenchymal migration by mediating the retraction of the cell lagging tail [Pertz et al., 2006]. Therefore, in this type of migration, Rho expression must be reduced only at the cell front, in contrast to Rac and Cdc42, which induce actin polymerization at the leading edge of migrating mesenchymal cells that extend lamellipodia.

The regulation of cell migration is a complex process involving the formation of membrane extensions or protrusions in the direction of migration. Cells with migratory capacity lose cell–ECM adhesion stability partly as a result of stress fiber contractions and develop weak and dynamic adhesion sites known as focal complexes. These complexes are mainly formed at the cell periphery at the end of stress fibers to provide the contractile forces that allow for cell locomotion. In addition, they might mature into FAs, for which the central molecule is the FA kinase (FAK). FAK is a tyrosine kinase that binds to and phosphorylates the small GTPases exchange factors p190RhoGEF and Trio therefore activating Rho [Medley et al., 2003; Zhai et al., 2003]. High expression levels of FAK have been found in a variety of tumor types, including colon carcinomas [Ayaki et al., 2001]. Additionally, increased autophosphorylation of FAK at tyrosine 397 has been observed in a mouse model of intestine adenoma formation [Weyant et al., 2001]. Recently, it has been suggested that the p190 RhoGEF-FAK signaling complex may coordinate or localize RhoA activation and FA formation in quiescent DLD-1 colon carcinoma cells in response to gastrin, thereby regulating cell migration and invadopodia formation [Yu et al., 2011]. We have also found that Rho activation promoted by LPA induces FA autophosphorylation and increased cell migration in Caco-2 colon cancer cells [Leve et al., 2011].

The association between Rho GTPase expression and the invasiveness of colorectal cancer cells was demonstrated by the following findings. ROCK-II knockdown cells have been shown to display a reduced rate of invasiveness [Vishnubhotla et al., 2007]. Additionally, the inoculation of colon cancer cells that overexpress Rac1 in nude mice led to an increase in the development of colorectal cancer tumors, a reduced survival time and an increase in the number of metastasis, while the inhibition of this GTPase completely suppressed tumor formation [Espina et al., 2008]. Finally, using an in vivo study in which intestinal adenocarcinomas were induced in rats using azoxymethane, treatment with LPA caused RhoA activation and led to an increase in the incidence of metastasis [Tatsuta et al., 2005].

In order to invade the stroma and blood vessels during the metastatic process, cells develop ventral membrane protrusions called invadopodia. These structures present an actin-rich core and are composed of a variety of proteins, such as cytoskeleton adaptor proteins, FA molecules, GTPase regulators, and enzymes that degrade ECM. Invadopodias have also been described in colon cancer cells lines [Vishnubhotla et al., 2007] in which the Rho effector protein, ROCK-II, was localized to modulate cell invasion by inducing both MMP-2 and MMP-13 activity [Vishnubhotla et al., 2007].

To date, investigations of the signaling pathways that govern these events in in vivo models of colorectal cancer have only just begun, and new findings may contribute to the identification of new anti-metastatic therapeutic targets for colorectal cancer treatment.

Rho GTPase PROTEINS AS THERAPEUTIC TARGETS

The treatment of colorectal cancer requires a multidisciplinary approach with standardized surgical and chemoradiation therapy; the only standard systemic treatment is 5-fluorouracil (5-FU) alone or combined with oxaliplatin and irinotecan. To date, new therapies, such as those targeting VEGF and EGFR, have contributed to the colorectal cancer treatment protocol, but only subgroups of patients benefit as some tumors have developed drug resistance. Therefore, novel chemotherapeutic combinations for the treatment of this cancer type must be examined.

Current studies using colon cancer cell lines have proposed the use of FTase and GGTase enzyme inhibitors, which block the addition of isoprenoid lipids to GTPases and impair GTPase membrane anchorage and subsequent activation. However, FTase inhibitors have failed to show significant efficacy in refractory colorectal cancer when used as a single agent [Sebti and Adjei, 2004], and GGTase inhibitors have not reached clinical development stages due to dose-limiting toxicity [Lobell et al., 2001]. Compounds that simultaneously inhibit GGTase and FTase have been developed to overcome inhibitor toxicity when co-administrated [Reid et al., 2004], albeit without sufficient potency. A similar compound with potential anticancer drug is geranylgeranylacetone (GGA), which inhibits Rho activity in various cancer cells and induces cell cycle arrest and apoptosis in colon cancer cells [Yoshikawa et al., 2010]. A study using a highly metastatic breast cancer cell line showed that

inhibition of Icmt, the final enzyme of the prenylation pathway, significantly decreased migration by inhibiting both RhoA and Rac1 [Cushman and Casey, 2011]. In colon cancer cells, the inhibition of this enzyme blocked tumorigenicity [Winter-Vann et al., 2005]; however, this study did not focus on Rho signaling. An additional current strategy in cancer treatment includes the use of inhibitors of the enzyme HMG-CoA reductase, also known as statin, which decrease the cholesterol levels and prevent the synthesis of their precursors, farnesyl pyrophosphate, and geranylgeranyl pyrophosphate. Inhibitors of this enzyme reduce the isoprenylation of Ras and Rho proteins, which is necessary for cell membrane anchorage inhibiting GTPase activity. Inhibitors of HMG-CoA have been reported to inhibit colon cancer development in mice [Cho et al., 2008] as well as to overcome cetuximab resistance, a monoclonal antibody that targets EGFR, in clinical trials [Lee et al., 2011]. Although some studies have shown that statins can reduce the risk of colon cancer, it has not been shown to be associated with improved survival in patients with stage III colon cancer [Ng et al., 2011].

As discussed here, Rho proteins and their regulators appear to be promising therapeutic targets for the development of novel anticancer drugs. ROCK inhibition with fasudil has been used to the treatment of cerebral vasospasm in Japanese patients without any serious adverse reactions, and there are a wide variety of ROCK inhibition clinical trials in development, which are mainly focused on cardiovascular diseases [Olson, 2008]. Nevertheless, there are no current clinical trials to determine whether fasudil would be useful in human colorectal cancer treatment. As in vitro studies have shown that fasudil inhibits cell migration and anchorage-independent growth and inhibits tumor progression in tumor models in vivo [Ying et al., 2006], we hypothesize that this drug may be useful for the treatment of colorectal cancer. Indeed, as ROCK inhibition also initiates apoptotic cell death in epithelial cells [Moore et al., 2004], Rho/ROCK may be a potential target for colon cancer treatment. RhoA inactivation also presents other potential clinical applications. As previously reported, downregulation of the RhoA protein in human colon cancer cells is associated with increased sensitivity to doxorubicin, a cytotoxic drug that intercalates DNA [Doublier et al., 2008]. Therefore, Rho inhibition associated with doxorubicin treatment may improve the efficacy of this drug during cancer treatment. Furthermore, as LPA2, a LPA receptor, is involved in Rho activation signaling [Lin et al., 2009], specific LPA2 inhibitors may constitute a therapeutic strategy to control colorectal cancer progression.

Finally, it is important to highlight that biotherapy approaches using a combination of bevacizumab and cetuximab to target VEGF and EGFR, respectively, have been described as a chemotherapeutic option. Interestingly, VEGF targets Rac1 in gastric cancer cells [Xue et al., 2004], and EGFR stimulates Rac to promote colonic cell migration [Dise et al., 2008]. Hence, these drugs also represent new possibilities for colorectal cancer treatment, mainly to inhibit cancer progression. Although further studies are clearly required to understand the cellular response mediated by GTPases, it is important to define specific inhibitors of Rho GTPase-associated functions, which represent potential therapeutic targets to benefit colorectal cancer patients.

CONCLUDING REMARKS

It has now been well established that increased expression of Rho GTPases correlates with tumor progression in colorectal cancer. Nevertheless, as the Rho GTPase family comprises various isoforms and regulators implicated in multiple cellular functions, it is difficult to identify their exact role in tumor promotion and progression. In vivo experimental approaches using gene deletion and the loss of function of the tumor suppressor gene *Apc* in murine models has allowed for the understanding of the direct consequences of inactivation of this gene in colorectal cancer. Furthermore, we emphasize that studies using other models, such as the inducible Cre-Lox-P system, may be useful to both define the earliest events associated with colorectal carcinogenesis and study cancer-associated genes of interest in a tissue-specific manner. Together, these models may also allow for a detailed analysis of the temporal and spatial control of gene expression in vivo and the determination of the physiological and pathological relevance of Rho GTPases, particularly in this cancer type. Finally, these techniques and intravital imaging in combination with inducible ectopic gene expression and orthotopic (xenograft)-transplantation constitutes strategies that may be useful for the identification of Rho GTPase members that are active and overexpressed as well as the Rho GTPase-triggered signaling pathways that represent relevant therapeutic targets for this pathology. Additionally, these techniques may be very useful to determine the efficacy of new chemotherapy agents, such as statins and Rock inhibitors, in colorectal cancer.

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