



PII: S0959-8049(99)00293-2

European Journal of Cancer
**Millennium
 Review**
 2000

Rho-like GTPases: Their Role in Epithelial Cell-Cell Adhesion and Invasion[★]

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The family members of small Rho-like GTPases, RhoA, Rac1 and Cdc42Hs, are regulators of diverse cellular signalling pathways, including cytoskeletal organisation, transcription and cell-cycle progression. Recent research has given insight into the complex regulation of cell-cell adhesion and migratory responses of epithelial cells. The Rho-like GTPases RhoA, Rac1 and Cdc42Hs as major determinants of cytoskeletal organisation have been identified as key regulators of epithelial architecture, as well as of cell migration. These findings highlight the complex regulation and cross-talk of GTPase-dependent signalling pathways arising from cell-cell and cell-matrix interactions. The molecular mechanism of how Rho-like GTPases couple to molecules mediating either cell-cell adhesion or cell migration will be of particular interest to understand the invasive phenotype of epithelial tumours. © 1999 Elsevier Science Ltd. All rights reserved.

Key words: Rho-like GTPases, adherens junction, cell-cell adhesion, E-cadherin, migration, Tiam1, PI3-kinase, Rac, Rho, Cdc42

Eur J Cancer, Vol. 35, No. 14, pp. 1905–1911, 1999

[★]Reprinted from *Eur J Cancer* 1999, 35(9), 1302–1308. Please use this reference when citing this article.

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Received 1 Jun. 1999; accepted 6 Jun. 1999.

RHO-LIKE GTPases AS ORGANISERS OF THE CELLULAR CYTOSKELETON

CELL MIGRATION is an essential process during, for instance, development and wound healing. Aberrations in signalling pathways involved in the regulation of cell migration, cell-cell and cell-matrix interactions contribute to tumour invasion and metastasis. The regulation of cytoskeletal changes associated with migratory behaviour of cells has recently become focused on the family of small Rho-like GTPases. Cdc42Hs, Rac1 and RhoA regulate signal transduction pathways that mediate distinct cytoskeletal rearrangements required for cellular motility [1]. In quiescent Swiss 3T3 fibroblasts, activation of RhoA is linked to the assembly of stress fibres and focal adhesions [2], whereas activation of Cdc42Hs and Rac1 results in the formation of filopodia and lamellipodia, respectively [3–5]. Formation of such cytoskeletal structures often correlates with cell migration. The cytoskeletal changes induced by constitutively active V12Cdc42Hs and V12Rac1 are also associated with cell spreading and the formation of focal complexes, specific integrin-based adhesion complexes [5–7]. Besides these direct effects on the cytoskeleton, Rho-like proteins are involved in transcriptional activation, endo- and exocytotic pathways, cell-cycle progression, oncogenic transformation and metastasis formation. Signalling pathways implicated in these processes have recently been extensively reviewed [1, 8, 9].

Similar to Ras proteins, Rho-like GTPases function as molecular switches by cycling between an active GTP-bound state and an inactive GDP-bound state (Figure 1). The function of this switch is the regulated transmission of a signal perceived upon receptor stimulation of the cell to a downstream signalling pathway. This is achieved by the selective binding of the active GTPase to a downstream effector protein. Guanine nucleotide exchange factors (GEFs) stimulate the exchange of bound GDP for GTP leading to activation of the GTPases and binding of downstream effectors, thereby leading to transmission of the signal. GTPase-activating proteins (GAPs) promote inactivation of the GTPases by stimulating the intrinsic GTP-hydrolysis rate of Rho-like proteins. Guanine nucleotide dissociation inhibitors (GDIs) can block the exchange of GDP for GTP as well as the hydrolysis of GTP, as has been shown for Rho GDI [8].

GEFs are characterised by the catalytic Dbl-homology (DH) domain, which is C-terminally flanked by a Pleckstrin-homology (PH) domain. PH domains have been implicated in binding to specific phosphoinositol lipids, as well as to proteins [10]. In addition to the DH-PH combination, many GEFs contain other domains (e.g. SH2-, SH3-, PDZ domains) that are commonly found in signalling molecules, allowing them to assemble in multimolecular signalling complexes.

RHO-LIKE GTPases MEDiate CELL-CELL ADHESION

Epithelial organisation

Epithelial cells are characterised by the formation of a cellular monolayer, consisting of densely packed cells with pronounced intercellular contacts. Epithelial tissues fulfil a barrier function, e.g. by limiting diffusion of soluble, intercellular components as well as regulating the passage of lymphoid cells. The strong cell-cell contacts of epithelial cells are based on two types of apical intercellular junctions, the zonula adherens [11] and the zonula occludens junction [12].

Zonula adherens junctions are based on cadherin-mediated cell-cell adhesion, whereas zonula occludens junctions are formed by tight junctional proteins including the transmembrane protein occludin and the cytoplasmic plaque proteins ZO-1 and ZO-2. Cadherin family members carry a large extracellular domain, a short transmembrane domain and a cytoplasmic tail. The extracellular domain is composed of five cadherin repeats that are involved in mediating the calcium-dependent homophilic cell-cell adhesion, whereas the cytoplasmic tail interacts with members of the catenin family. In adherens junctions, cadherins are complexed with β -catenin (or γ -catenin) and α -catenin, thereby linking the cadherins to the F-actin cytoskeleton. Catenins are structurally important as part of the cadherin complex, but play a similar important role in cellular signalling [13]. The typical cadherin present in epithelial cells is E-cadherin and loss or mutations of E-cadherin or its associated complex members are often associated with metastasis of epithelial tumours [14, 15]. E-cadherin has been recognised as an invasion suppressor molecule, as re-expression of E-cadherin in tumours lacking this molecule reconstituted E-cadherin-mediated cell-cell adhesion and consequently inhibited motility and invasion of these tumour cells [16, 17]. Moreover, loss of E-cadherin was found to be

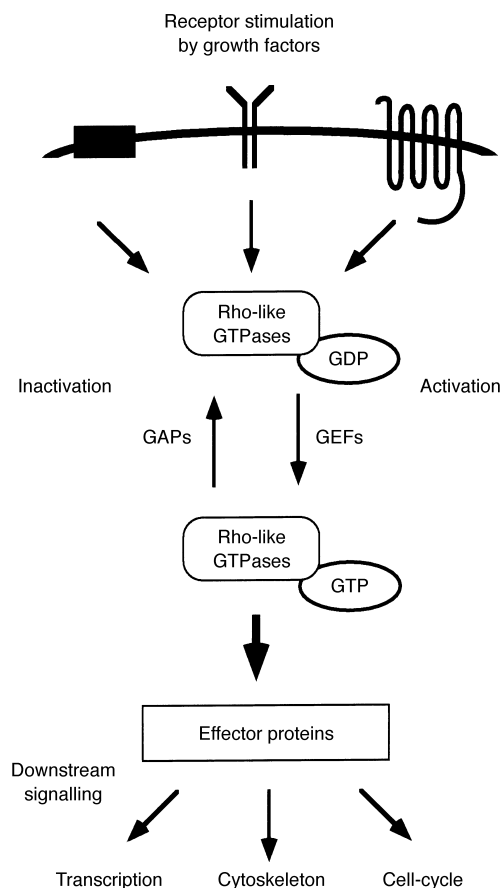


Figure 1. Rho-like GTPases as regulators of cellular signalling pathways. Rho-like GTPases function as molecular switches by cycling between an active GTP-bound and an inactive GDP-bound state. Activation results in the transmission of a signal perceived after receptor stimulation by binding to a downstream effector protein that further communicates with different signalling pathways controlling, for example, cytoskeleton organisation, activation of transcription and cell-cycle progression.

causal for the transition from adenomas to carcinomas [18], substantiating the importance of E-cadherin-mediated cell-cell adhesion for the epithelial architecture.

Formation of intercellular contacts depends on the activity of Rho-like proteins

Recently, family members of the Rho-like GTPases have been recognised to be required for the maintenance of the cytoskeletal organisation of fully differentiated epithelia. In human keratinocytes and Madin-Darby canine kidney cells (MDCK), dominant-negative N17Rac as well as C3 transferase, which inactivates RhoA by ADP-ribosylation on Asn⁴¹ [19], have been found to perturb the organisation of actin filaments at sites of cell-cell adhesion. Inactivation of Rac or Rho results in dislocation of E-cadherin and its complex members from the adherens junctions [20–23]. The efficiency of dominant-negative mutants of Rho-like GTPases in perturbing the architecture of adherens junctions varies with respect to the maturation state of cell-cell contacts and to the cell type [24]. In MDCK cells, expression of constitutively active V12Rac leads to increased accumulation of members of the E-cadherin complex and F-actin at sites of cell-cell adhesion [21, 25]. The GEF Tiam1, a specific activator of Rac [26, 27], is localised to sites of cell-cell contact in MDCK cells, and inhibits the hepatocyte growth factor (HGF)-induced scatter response of MDCK cells, which is due to an increase in E-cadherin-based cell-cell contacts [25]. Moreover, activation of Rac is able to induce E-cadherin-mediated cell-cell adhesion in transformed epithelial cells. Tiam1-mediated activation of Rac restores the epithelial morphology of fibroblastoid, Ras-transformed MDCK-f3 cells, thereby inhibiting motility and invasion [25, 28]. Taken together, these data implicate in particular Rac in the formation and maintenance of epithelial morphology.

Rho-like GTPases play a role in the structural integrity of adherens junctions. As discussed above, inhibition of endogenous Rac and Rho function affects the architecture of adherens junctions and their marker protein E-cadherin, but has no effect on desmosome morphology (desmoplakin) in keratinocytes [20]. In addition, inactivation of endogenous GTPases as well as mutant GTPases interfere with tight junctional morphology and fence function, when measuring transepithelial resistance [21, 29, 30]. Functional activity of the Rac and Rho protein is thus required for the integrity of both tight junctions and adherens junctions.

The role of the third Rho family member, Cdc42Hs, in the formation of cell-cell junctions appears less clear. In MDCK cells, Cdc42 mutants do not affect the localisation of E-cadherin [21]. Similarly, activated V12Cdc42 is unable to restore an epithelial phenotype in fibroblastoid Ras-transformed MDCK-f3 cells (our unpublished results). However, from a similar localisation of activated V12Cdc42 and V12Rac to cell-cell contacts in MDCK cells, a role for Cdc42 in the formation of adherens junctions has been suggested [31]. In cervical carcinoma HtTA-1 HeLa cells, expression of V12Cdc42 partly suppresses the transformed phenotype by inducing cell-cell contacts resembling typical adherens junctions that accumulate N-cadherin and F-actin. V12Rac induces a distinct type of adherens junctions, characterised by intermingled lateral membranes that are similarly enriched in N-cadherin and its associated complex members [32]. At present it is not clear if Cdc42 exerts these effects on cell-cell adhesion directly or indirectly via the activation of Rac [2]. In

the *Drosophila* wing disc epithelium, Dcdc42 is required for processes involving polarised cell shape changes during pupal and larval development, as well as for the apico-basal elongation of epithelial cells in later larval stages [33]. This is in agreement with a role for Cdc42 in establishing cell polarity, as has been found for yeast mating [34], T-cell polarisation [35] and chemotaxis of macrophages [36]. Recently, Cdc42 has been shown to control secretory and endocytotic transport to the basolateral plasma membrane of epithelial MDCK cells [37]. It thus appears that Cdc42 is crucial for the establishment and maintenance of cell polarity.

Downstream targets

The mechanism of how Rho-like GTPases influence cell-cell adhesion remains to be elucidated. Among the target molecules for Cdc42 and Rac that have been identified so far, only IQGAP1, a downstream effector for Rac and Cdc42 [38–40], has been reported to localise to adherens junctions in MDCK cells. IQGAP1 is associated with the E-cadherin complex, by binding to E-cadherin and β -catenin [41], and directly binds actin filaments [42, 43]. GTP-loaded Cdc42 enhances the actin filament crosslinking activity of IQGAP1 by promoting oligomerisation of IQGAP1 [43]. By concomitant crosslinking of actin and binding to the E-cadherin complex, IQGAP1 could represent a GTPase-regulated link between molecules mediating cell-cell adhesion and the cortical cytoskeleton. Other Rac and Cdc42 target molecules such as PAK and WASP have been implicated in the organisation of the actin cytoskeleton [44–47], but so far no data are available with respect to their role in epithelial cell-cell adhesion. Similarly, there is no information available yet about the role of known RhoA downstream effectors [9] in intercellular adhesion.

V12Rac- or Tiam1-induced cell-cell adhesions are characterised by strong accumulation of F-actin at the sites of cell-cell contact [20, 21, 25, 28]. The recruitment of F-actin to artificially clustered cadherin complexes has been shown to require Rac but not Rho proteins [20]. This recruitment of F-actin depends on prior clustering of cadherin receptors at sites of cell-cell contact in keratinocytes [24]. Alternatively, Tiam1-mediated Rac activation induces functional E-cadherin-mediated cell-cell adhesion in transformed MDCK cells, which is accompanied by strong accumulation of F-actin at sites of cell-cell contact [25, 28]. Although the exact order of events in the formation of adhesive contacts appears unclear, this indicates that Rac-mediated polymerisation of cortical F-actin is central to Rac-induced cell-cell adhesion. Rac-dependent actin polymerisation in lamellipodia and membrane ruffles has been shown to require PI3-kinase [2, 48, 49]. Tiam1-mediated activation of Rac and the induction of cell-cell adhesion in transformed MDCK cells is also dependent on PI3-kinase activity [28], suggesting that similar regulatory mechanisms participate in actin polymerisation at cell-cell contacts and in the formation of lamellipodia required for cell migration.

RHO-LIKE GTPases IN EPITHELIAL CELL MIGRATION AND INVASION

Signalling networks of Rho-like proteins

Rho-like GTPases have not only been shown to regulate epithelial cell-cell adhesion, but have also been implicated in migratory responses of epithelial cells. RhoA seems to play a dual role with respect to epithelial cell-cell adhesion and

motility. V12Ras-transformed mammary epithelial cells exhibit high levels of RhoA activity, as demonstrated by increased phosphorylation of the myosin light chain and analysis of stress fibre and focal adhesion formation [22]. Consequently, inhibition of endogenous Rho activity results in inhibition of the motile phenotype of these transformed mammary epithelial cells, by inhibiting formation of focal contacts and Rho-mediated contraction. Inhibition of RhoA activity only partly restores the normal epithelial phenotype [22], reflecting the requirement of Rho activity for the establishment and maintenance of epithelial cell-cell contacts [20–22]. This indicates that Rho activity is required for both the maintenance of epithelial cell-cell contacts and migration of epithelial cells.

The apparent upregulation of RhoA activity by oncogenic V12Ras [22] raises questions about the crosstalk of Ras and Rho-like GTPases in normal and malignant cells. Successful transformation by oncogenic Ras requires Rho activity, as Rho suppresses induction of the cyclin-dependent-kinase inhibitor p21^{WAF1/Cip1} and thereby allows Ras-induced DNA synthesis and entry into S-phase [50]. Similarly important for the determination of the cellular phenotype is the crosstalk between Rac and Rho proteins. Using biochemical activity assays to determine the activation state of Rho-like GTPases, we found that constitutive activation of Rac results in down-regulation of Rho activity in fibroblasts and MDCK cells. Rac-induced inactivation of Rho is associated with a non-migratory phenotype in fibroblasts. The balance of Rac and Rho activities seems thus to determine the epithelioid or migratory phenotype (E.E. Sander, The Netherlands Cancer Institute, Amsterdam, The Netherlands).

Epithelial MDCK cells break up their cell-cell junctions and scatter in response to HGF, which is the ligand of the *c-met* proto-oncogene transmembrane tyrosine kinase receptor [51, 52]. HGF activates the small GTPase Ras by increasing the levels of bound GTP [53]. Both HGF and V12Ras-induced membrane ruffling and lamellipodia formation require Rac activity [54]. However, activated V12Rac is not sufficient to induce cell scattering, in agreement with the established role of Rac in promoting cell-cell adhesion (see above). The failure of activated Rac to induce cell motility in MDCK cells indicates that HGF in addition to Rac stimulates other signalling pathways to induce cell scattering. This pathway has been identified as the MAPK pathway [55], consistent with a role of MAPK in carcinoma cell migration [56]. HGF-induced motility of MDCK cells furthermore requires PI3-kinase activity [57], which is most likely acting downstream of Ras and upstream of Rac [28], similar to PDGF-mediated Rac activation in fibroblasts [48, 49]. In T47D mammary carcinoma cells, activated V12Rac and V12Cdc42 stimulates motility on collagen, requiring PI3-kinase and the integrin $\alpha_2\beta_1$ [58]. The integrin $\alpha_6\beta_4$ has been shown to activate PI3-kinase and to stimulate invasion of colon carcinoma cells. Rac is required downstream of PI3-kinase for cell motility [59]. Similarly, Tiam1-mediated activation of Rac and migration of Ras-transformed MDCK cells on collagen substrates requires PI3-kinase activity acting upstream of the GEF Tiam1 and Rac [28]. Furthermore, Rac has been found downstream of integrin signalling in fibroblasts [60] and lymphoid Jurkat cells [7]. In integrin β_1 knockout neuroepithelial cells, re-expression of β_1 results in cell scattering and activation of both Rac1 and RhoA (C. Gimond, The Netherlands Cancer Institute, Amsterdam). Similarly, HGF-induced cell migration of MDCK cells is

preceded by transient activation of both Rac and Rho GTPases, whereby HGF-induced signalling towards Rac1, but not Rho, is PI3-kinase sensitive (E.E. Sander, The Netherlands Cancer Institute, Amsterdam, The Netherlands). It thus appears that the activation of integrin extracellular matrix receptors as well as growth factor stimulation can lead to activation of both Rac and Rho GTPases, resulting in (epithelial) cell migration. This activation pattern of Rac and Rho proteins furthermore suggests that the balance of Rac and Rho activities determines the epithelial or mesenchymal phenotype of epithelial cells.

Matrix-dependent signalling of Rho-like proteins

The composition of the extracellular matrix can contribute to the regulation of these different cellular responses with respect to GTPase signalling, cell-cell adhesion or migration in epithelial cells. Epithelial bladder carcinoma cells show migratory behaviour, mediated by the integrin $\alpha_2\beta_1$, on collagens but not on fibronectin or laminin substrates [61, 62]. On a fibronectin or laminin 1 matrix, Tiam1-mediated activation of Rac reverts the transformed phenotype and inhibits migration of MDCK-f3 cells, due to restoration of E-cadherin adhesions. In contrast, on collagen substrates, Tiam1-mediated activation of Rac stimulates motility of these cells. Thus, integrin signalling by the extracellular matrix determines the cellular response towards Rac activation in epithelial cells, which is either inhibition or promotion of migration [28]. These matrix-dependent effects of Rac signalling may explain the reported controversial results with respect to Rac-induced suppression or promotion of migration (Figure 2).

In non-migratory MDCK-f3 cells, the GEF Tiam1 is localised to sites of cell-cell contact, whereas the protein is associated with membrane ruffles and lamellae in migratory cells on collagen. In spite of the substrate-dependent localisation of the Rac activator Tiam1, Tiam1-mediated activation of Rac is substrate-independent [28], suggesting that localisation and/or composition of the Rac signalling complex is differentially regulated by the cell substrate. Migration of pancreatic carcinoma cells on collagen is mediated by assembly of a signalling complex containing the adaptor proteins p130^{CAS} (Crk-associated substrate) and Crk. Formation of this complex is similarly observed in a subpopulation of these cells that have been selected for migration on vitronectin. Expression of p130^{CAS} is sufficient to promote Rac-dependent migration and localisation of this complex to membrane ruffles in Cos cells [63]. Kiyokawa and colleagues [64] have shown that the assembly of a complex containing p180^{DOCK}, p130^{CAS} and Crk results in activation of Rac, as shown by GTP-loading of the GTPase. This suggests that proteins with adaptor function assemble (different) Rac signalling complexes and are involved in their targeting to specific intracellular locations. In addition, integrin signalling might positively or negatively cooperate with Rac-dependent pathways required either for migration or adhesion. The cell-type specific expression pattern of integrins most likely accounts for different cellular responses with respect to a migratory phenotype, due to different or overlapping substrate specificities and signalling properties of integrins [65].

Crucial for integrin-mediated migratory responses is the proteolytic breakdown of the cell substrate, and tumour cells often harbour increased or altered metalloprotease production and secretion [66]. In fibroblasts, constitutively active V12Rac induces the production of collagenase-1 (MMP1) via

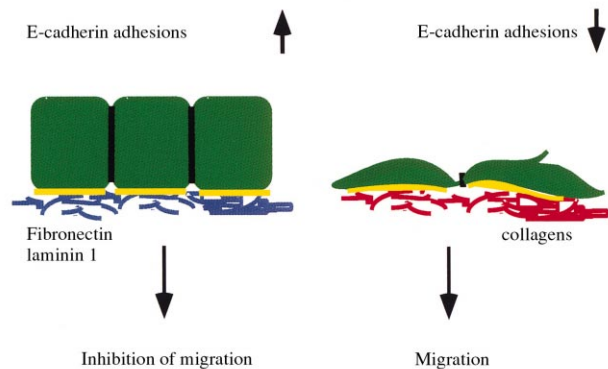


Figure 2. Cell-matrix interactions determine Rac-mediated effects on E-cadherin-based cell-cell adhesion and cell migration in epithelial tumour cells. Activation of Rac on fibronectin or laminin 1 promotes the formation of E-cadherin-mediated adhesions and results in inhibition of migration in transformed MDCK cells. In contrast, collagen substrates contribute to the disassembly of E-cadherin-based cell-cell contacts and synergise with Rac signalling to stimulate cell motility.

a NF κ B/interleukin 1 α -dependent pathway [67]. This finding strengthens the importance of Rho-like proteins in the induction and control of cellular migration. The intimate control of molecules involved in cell-cell adhesion and migration is demonstrated by the cleavage of E-cadherin by the matrix metalloprotease stromelysin-1, as has been found in mammary epithelial cells [68]. Synthetic peptides of E-cadherin, containing the HAV (histidine-alanine-valine) sequence that is crucial for homophilic adhesions, and E-cadherin fragments present in conditioned medium can induce invasion of epithelial cells, suggesting that E-cadherin fragments generated by proteolysis may harbour signalling function and thereby stimulate invasion of E-cadherin-expressing cells [69].

FUTURE RESEARCH DIRECTIONS

Recent research has accomplished important insight into the complex relationship between cell-cell and cell-substrate interactions and their signalling properties. Family members of the Rho-like GTPases have been identified as regulators of both cell-cell adhesion and cell migration in epithelial cells. The mechanistic link of how Rho-like proteins couple to cadherin-mediated adhesion is still unknown. The identification of δ -catenin in adherens junctions points to the complexity of the control of cell-cell adhesion. δ -catenin has been found to associate with cadherin thereby negatively modulating cell-cell adhesion and promote cell scatterings. The regulatory mechanism underlying the structural and signalling function of Rho-like proteins will become a challenging line of research. It becomes clear now that the integration of many incoming signals, received from cell-cell, cell-substrate and classical growth factor receptors, is required to elicit migratory responses. This will include identification of often cell-type specific signalling pathways from integrins and cadherins which are linked by Rho-like GTPases. Research will focus especially on the crosstalk between these adhesion and signalling molecules, as the cellular phenotype appears to be determined by a balance composed of integrated, different signalling pathways. Signalling by the Ras GTPase requires cytoskeletal and transcriptional changes that are mediated by Rho-like GTPases [22, 50, 54, 71, 72]. Unravelling mechanisms of cellular transformation by oncogenic Ras will require

the dissection of potentially altered signalling pathways from oncogenic Ras to Rho-like proteins. The recently developed biochemical activity assays for GTP-bound Rho-like GTPases [28, 73] will provide new avenues to dissect different activation patterns of these proteins and to gain insight into their crosstalk. Also the effects of Rho proteins on gene expression, e.g. production and secretion of matrix metalloproteases, proliferation and loss of contact inhibition, need to be addressed. Normal morphogenic and developmental processes require co-ordinated movement of cells, which is deregulated in tumour cells. Therefore, elucidating the complex network of different receptor signalling in normal and tumorigenic cells is central to the development of novel cancer therapies for metastatic carcinomas.

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Acknowledgements—Research by J.G. Collard is supported by grants from the Dutch Cancer Society and The Netherlands Organisation for Scientific Research. E.E. Sander is supported by a fellowship from the Deutsche Forschungsgemeinschaft.