Brief Report

Oncogenic Ras Downregulates Rac Activity, which Leads to Increased Rho Activity and Epithelial-Mesenchymal Transition

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Abstract. Proteins of the Rho family regulate cytoskeletal rearrangements in response to receptor stimulation and are involved in the establishment and maintenance of epithelial cell morphology. We recently showed that Rac is able to downregulate Rho activity and that the reciprocal balance between Rac and Rho activity is a major determinant of cellular morphology and motility in NIH3T3 fibroblasts. Using biochemical pull-down assays, we analyzed the effect of transient and sustained oncogenic Ras as signaling on the activation state of Rac and Rho in epithelial MDCK cells. In contrast to the activation of Rac by growth factor-induced Ras signaling, we found that sustained signaling by oncogenic RasV12 permanently downregulates Rac activity, which leads to upregulation of Rho activity and epithelial-mesenchymal transition. Oncogenic Ras decreases Rac activity through sustained Raf/MAP kinase signaling, which causes transcriptional downregulation of Tiam1, an activator of Rac in epithelial cells. Reconstitution of Rac activity by expression of Tiam1 or RacV12 leads to downregulation of Rho activity and restores an epithelial phenotype in mesenchymal RasV12- or RafCAAX-transformed cells. The present data reveal a novel mechanism by which oncogenic Ras is able to interfere with the balance between Rac and Rho activity to achieve morphological transformation of epithelial cells.

Key words: Ras signaling • Rho-like GTPases • Madin-Darby canine kidney cells • Raf/MAP kinase • Tiam1

Introduction

Transitions between epithelial and mesenchymal phenotypes of cells are required for morphogenetic processes and tissue remodeling during embryogenesis. These phenotypic conversions are regulated by cadherin-mediated cell-cell adhesion, growth factors, and extracellular matrix components (for recent reviews, see Adams and Nelson, 1998; Vlemmixx and Kemler, 1999). In addition, sustained signaling by oncogenic Ras may result in morphological transformation to a mesenchymal phenotype, which is associated with changes in gene expression, loss of E-cadherin-mediated cell-cell adhesions, and increased invasiveness of tumor cells (Behrens et al., 1989; Vlemmixx et al., 1991). Rho-family proteins, in particular Rac and Rho, mediate distinct cytoskeletal rearrangements in response to receptor stimulation (Hall, 1998), and have been implicated in the establishment and maintenance of cadherin-based cell-cell adhesions (Braga et al., 1997; Hordijk et al., 1997; Takaishi et al., 1997; Kuroda et al., 1998), as well as in the migration of epithelial cells (Keely et al., 1997; Sander et al., 1998). We recently found that Rac is able to downregulate Rho activity and that the balance between Rac and Rho activity determines the cellular morphology and motile behavior of NIH3T3 fibroblasts (Sander et al., 1999). The mechanism by which oncogenic Ras induces morphological transformation of epithelial cells is still poorly understood. Here, we used biochemical pull-down assays to analyze the activation state of Rac and Rho in response to Ras signaling in epithelial MDCK cells. Our data indicate that, in contrast to growth factor-induced Ras signaling, oncogenic Ras downregulates Rac and upregulates Rho activity, leading to epithelial-mesenchymal transition. Oncogenic Ras-mediated downregulation of Rac activity is caused by sustained activation of the
Raf/MAP kinase pathway, which results in transcriptional downregulation of the Rac-specific exchange factor, Tiam1 (Habets et al., 1994; Michiels et al., 1995). Raf reconstitution of Rac activity in mesenchymal MDCK cells transformed by activated mutants of Ras or Raf, downregulates Rho activity and restores the epithelial phenotype. Our data suggest that oncogenic Ras is able to induce a mesenchymal phenotype in epithelial MDCK cells by influencing the balance between the activation state of Rac and Rho proteins.

Materials and Methods

Cells and Retroviral Transductions

MDCK cells, MDCK/F3 cells expressing RasV12, and derivatives thereof were grown in DMEM supplemented with 10% FCS and antibiotics. The retroviral vector encoding C119Tiam1 and transduced MDCK cells, and RasV12-transformed MDCK/F3 cells have been described (Hordijk et al., 1997). Constitutively active, myc-tagged RafCAAX and p110CAAX cDNAs were cloned into a modified retroviral LZRS vector (Kinsella and Nolan, 1996; Michiels et al., 2000) and stable cell lines were generated by retroviral transduction and selection for neomycin resistance as described (Sander et al., 1999). MDCK-RafCAAX cells expressing Tiam1 were generated by superinfection with retrovirus encoding C119Tiam1 and a neo-cDNA-resistance marker and selected in medium containing G418 and zeocin (Cayla). For Rac and Rho activity assays, cells were stimulated with 10 ng/ml recombinant hepatocyte growth factor (HGF) for the indicated times. Where indicated, cells were pretreated for 4 h with 20 μM of the phosphatidylinositol 3 (P13) kinase inhibitor, LY 294002 (Calbiochem).

Rac and Rho Activity Assays

Rac and Rho activity assays were performed as previously described (Sander et al., 1998, 1999; Reid et al., 1999). In brief, 10^6 cells were grown in 10-cm dishes, washed in cold phosphate buffered saline, and lysed on ice in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 100 mM NaCl, 10% glycerol, 5 mM MgCl2) in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 100 mM NaCl, 10% glycerol, 5 mM MgCl2, and protease inhibitors). Cleared lysates were incubated for 30 min at 4°C with glutathione S transferase (GST)-PAK or GST-rhotekin (Sander et al., 1998, 1999; Reid et al., 1999; Ren et al., 1999) precoupled to glutathione-Sepharose beads (Amersham Pharmacia Biotech) to precipitate GTP-bound Rac and Rho, respectively. Precipitated complexes were washed three times in lysis buffer and boiled in sample buffer. Total lysates and precipitates were analyzed by Western blot using mAbs against Rac1 (Transduction Laboratories) and RhoA (Santa Cruz Biotechnology, Inc.).

Protein and RNA Analysis

A cnotated, phosphorylated forms of MAPK and PKB/Akt were detected using antibodies from New England Biolabs, Inc. A anti-MAPK antibody was provided by P. Hordijk (CLB, Amsterdam, The Netherlands), and anti-Akt1 antibody was obtained from Santa Cruz Biotechnology, Inc. Tiam1 protein levels were analyzed by immunoprecipitation using anti-Tiam1 (C16) polyclonal antibody from Santa Cruz Biotechnology, Inc., followed by Western blotting with anti-Tiam1 antibody (Habets et al., 1994). RafCAAX was detected with the myc-tag-specific antibody, 9E10. RNA analysis was performed using standard procedures (Habets et al., 1994).

Immunofluorescence

For immunofluorescence, wild-type MDCK and transduced cell populations were stained with primary antibody recognizing β-catenin (Transduction Laboratories) and rhodamine-conjugated phalloidin (Molecular Probes, Inc.) to stain for F-actin. Images were collected by confocal microscopy (Leica).

Abbreviations used in this paper: GST, glutathione S transferase; HGF, hepatocyte growth factor; P13, phosphatidylinositol 3.

Results

HGF-induced Ras Activation Increases Rac and Rho Activity through Independent Signaling Pathways

Growth factors like HGF and PDGF have been shown to activate Rac through Ras-dependent PI3 kinase signaling. Dominant negative RasN17 was demonstrated to inhibit HGF- or PDGF-induced membrane ruffling, whereas active RasV12 induces membrane ruffling in a PI3 kinase-dependent manner (Ridley et al., 1995; Rodriguez-Viciana et al., 1997; Scita et al., 1999). Consistent with this signaling model, HGF/cMet receptor stimulation of MDCK cells resulted in a rapid and transient increase in Rac activity that returned to basal levels within ten minutes and was inhibited by pretreatment of cells with the PI3 kinase inhibitor, LY 294002 (Fig. 1 a). These findings do not exclude that a possible direct activation of PI3 kinase by HGF/cMet receptor signaling may also contribute to Rac activation. The activation of Rac coincided with the induction of membrane ruffles, but preceded HGF-induced cell scattering by a few hours (data not shown). HGF also stimulated activation of Rho, which was much more prolonged than the activation of Rac (Fig. 1 a). Increased Rho activity was still observed four hours after stimulation, and may be required for the HGF-induced scatter response of MDCK cells. Rho activation was not dependent on PI3 kinase activity (Fig. 1 a), indicating that HGF/cMet receptor stimulation activates Rac and Rho through independent signaling pathways.

Oncogenic Ras Signaling Downregulates Rac Activity and Upregulates Rho Activity

In contrast to the activation of Rac by HGF, we found that sustained Ras signaling in subclones and populations of RasV12-transformed MDCK cells resulted in a permanent downregulation of Rac activity (Fig. 1 b). Others have shown that microinjection of RasV12 in different cell
Oncogenic Ras Downregulates Rac Activity

To investigate the signaling pathways activated by sustained Ras signaling that lead to phenotypic changes and downregulation of Rac activity, we introduced activated forms of the Ras effectors Raf kinase (RafCAAX) and PI3 kinase (p110CAAX) into epithelial MDCK cells by retroviral transduction. Activation of both effector pathways in transduced cell populations was examined using antibodies directed against phosphorylated MAPK and phospho-PKB/Akt. The different lines was determined by Western blotting of total cell lysates using antibodies against phospho-MAPK and phospho-PKB/Akt.

Figure 2. Ras-induced transformation of epithelial MDCK cells is mediated by the Raf/MAP kinase pathway. a, Phase-contrast images of wild-type MDCK cells and MDCK cell populations expressing activated mutants of Ras, Raf kinase, or PI3 kinase. b, A activation of the Raf/MAP kinase and PI3 kinase pathways in the different lines was determined by Western blotting of total cell lysates using antibodies against phospho-MAPK and phospho-PKB/Akt.

Oncogenic Ras Induces Morphological Transformation of MDCK Cells through Sustained Raf/MAP Kinase Signaling

To investigate the signaling pathways activated by sustained Ras signaling that lead to phenotypic changes and downregulation of Rac activity, we introduced activated forms of the Ras effectors Raf kinase (RafCAAX) and PI3 kinase (p110CAAX) into epithelial MDCK cells by retroviral transduction. Activation of both effector pathways in transduced cell populations was examined using antibodies directed against phosphorylated MAP kinase and PKB/Akt, two downstream components of Raf and PI3 kinase signaling, respectively (Marshall, 1996). As expected, both the MAP kinase and the PKB/AKT pathways were activated in RasV12-transformed cells, whereas RafCAAX or p110CAAX expression resulted in specific activation of their downstream targets (Fig. 2b). In contrast to sustained PI3 kinase signaling, constitutive activation of the Raf/MAP kinase pathway by RafCAAX was sufficient to induce transition to a mesenchymal phenotype (Fig. 2a). Similar to oncogenic RasV12 (Fig. 1b), sustained Raf signaling (Fig. 1c), but not PI3 kinase signaling (not shown) resulted in decreased Rac and increased Rho activity. We thus conclude that oncogenic Ras regulates Rac and Rho activities through sustained activation of the Raf/MAP kinase pathway.

Sustained Raf/MAP Kinase Signaling Results in Transcriptional Downregulation of the Rac-specific Exchange Factor, Tiam1

MAP kinase controls cellular behavior by regulating the transcription of a large number of genes. Therefore, we examined whether downregulation of Rac activity was caused by altered expression of Rac-specific exchange factors, like Tiam1, that is endogenously expressed in MDCK cells (Michiels et al., 1995; Hordijk et al., 1997). In both RasV12- and RafCAAX-transformed cells, the levels of Tiam1 protein were strongly reduced or completely absent (Fig. 3, a and c). Consistent with the effects of MAP kinase on gene transcription, downregulation of Tiam1 occurred at the transcriptional level, since Tiam1 mRNA transcripts were barely detectable in RasV12-transformed cells (Fig. 3b). We did not find decreased expression of other putative Rac exchange factors, such as Sos1, PIx, and Vav-2, in RasV12- or RafCAAX-transformed cells, whereas the Tiam1 homologue Stef (Hoshino et al., 1999) was not expressed in MDCK cells (data not shown). However, this does not exclude that other as yet unidentified exchange factors for Rac may be downregulated in RasV12- or RafCAAX-transformed cells.
To substantiate the role of Raf/MAP kinase signaling in the transcriptional downregulation of Tiam1 and decreased Rac activity, we isolated six individual clones from the RafCAAX-transformed cell population. The mesenchymal cell clones 1 and 2, which express relatively high levels of RafCAAX, displayed high levels of MAP kinase phosphorylation and loss of Tiam1 expression (Fig. 3 c). In contrast, the epithelial cell clones 4, 5, and 6, expressed very low levels of RafCAAX, exhibited control levels of MAP kinase activation, and expressed normal levels of Tiam1 (Fig. 3 c). Clone 3 showed an intermediate phenotype and moderate levels of MAP kinase activation that were associated with intermediate levels of Tiam1. The degree of MAP kinase activation in the cell clones thus correlated with decreased Tiam1 protein levels and transition to a mesenchymal phenotype. Similar results were obtained using subclones derived from RasV12-transformed cells (not shown).

It could be argued that Tiam1 downregulation is due to the phenotypic changes induced by RasV12 or RafCAAX expression in cells (see Fig. 2), rather than by sustained Raf/MAP kinase signaling. To exclude this possibility, we analyzed Tiam1 protein levels in RasV12-transformed cells that reverted towards an epithelial phenotype upon introduction of constitutively active RacV12 (Hordijk et al., 1997). Despite their epithelioid morphology, these cells still showed a RasV12-mediated increase in MAP kinase activity and failed to restore normal Tiam1 levels (Fig. 3 a). This strongly suggests that constitutive activation of the Raf/MAP kinase pathway by oncogenic Ras causes transcriptional downregulation of Tiam1 expression, leading to decreased Rac activity and transition to a mesenchymal phenotype. Treatment of fibroblastoid RasV12-transformed MDCK cells with the MEK inhibitor PD98059 (18 h, 20 μM) showed an epithelial-like appearance due to inhibition of polarization and migration of the cells. However, PD-treated cells hardly formed E-cadherin-based adhesions, and no changes in Rac or Rho activity or Tiam1 were found in response to PD treatment (not shown). These data do not exclude that, in addition to the Raf–MAP kinase pathway, other events play a role in epithelial–mesenchymal transition, such as changes in phosphorylation of the myosin II light chain or heavy chain (Klemke et al., 1997; van Leeuwen et al., 1999), which affect cell spreading and cell contraction, or changes in phosphorylation of proteins involved in the formation of E-cadherin adhesions (Kinch et al., 1995).

Reconstitution of Rac Activity Is Sufficient to Revert Ras or Raf-transformed Cells to an Epithelial Morphology

We next examined whether downregulation of Rac activity by sustained Raf/MAP kinase signaling could explain the mesenchymal transition. Reconstitution of Rac activity to approximately the level of wild-type MDCK cells by exogenous expression of Tiam1 reverted the fibroblastoid RafCAAX-expressing cells towards an epithelioid phenotype (Fig. 4 a). In these cells, adherens junctions were restored, as illustrated by the relocalization of the marker proteins E-cadherin (not shown) and β-catenin (Fig. 4, b–d). In addition, the tight junction marker ZO-1 was re-distributed to the sites of cell–cell contact (not shown). Restoration of Rac activity by Tiam1 in RasV12- and RafCAAX-transformed MDCK cells resulted in strong downregulation of Rho activity (Fig. 4 a), a phenomenon that we also observed in Tiam1- and RacV12-expressing NIH3T3 fibroblasts (Sander et al., 1999). From these data we conclude that Rac negatively regulates Rho activity, and that the lack of this negative regulation in Ras- and Raf-transformed cells is the major cause of increased Rho activity. Therefore, our data suggest that oncogenic Ras is able to induce a mesenchymal phenotype in epithelial MDCK cells by influencing the balance between Rac and Rho activity.

Discussion

As illustrated in Fig. 5, our data indicate complex cross-talk between Ras, Rac, and Rho in epithelial MDCK cells.
Short-term activation of Ras by HGF/cMet receptor signaling induces a transient, PI3 kinase-dependent Rac activation, presumably by PI3 kinase-mediated activation or membrane translocation of exchange factors for Rac, as shown for Sos, Vav1, and Tiam1 (Han et al., 1998; Nimnual et al., 1998; Sander et al., 1998). Of note, Eps8 and E3b1, two downstream targets of receptor tyrosine kinases, also have been implicated in growth factor-induced activation of Rac by Ras through regulating the activity of Sos (Scita et al., 1999). HGF induces a more prolonged activation of Rho that occurs independently of PI3 kinase, indicating that distinct signaling pathways are involved in HGF-induced activation of Rac and Rho. Stimulation of NIH 3T3 cells with PDGF also results in a transient activation of Rac. However, in contrast to HGF stimulation of MDCK cells, Rho is transiently inactivated by PDGF receptor signaling (Sander et al., 1999). This indicates that regulation of Rac and Rho activity is dependent on the growth factor receptor involved, and possibly also on the cell type studied.

In RasV12-transformed cells, persistent Ras signaling results in a Raf/MAP kinase-mediated downregulation of the Rac-specific exchange factor Tiam1, leading to decreased Rac activity (Fig. 5). Although the expression of other putative Rac exchange factors like Sos1, PIX, Vav2, and Stef was not affected, we cannot exclude that the expression of as yet unidentified Rac exchange factors is influenced by oncogenic Ras signaling. Decreased Rac activity in RasV12- or RafCAAX-transformed cells is accompanied by increased Rho activity, whereas restoration of Rac activity results in decreased Rho activity in these cells. This indicates that Rac negatively influences Rho activity under conditions where Rho is not activated by separate pathways, such as HGF-receptor stimulation. Antagonistic roles for Rac and Rho have been demonstrated in the control of neuronal morphology (Kozma et al., 1997; van Leeuwen et al., 1997) by regulating myosin light chain or heavy chain phosphorylation (Sanders et al., 1999; van Leeuwen et al., 1999). The present data indicate that Rac can also antagonize Rho in a more direct manner by inhibiting Rho at the GTPase level. Previously, we found that Tiam1-expressing RasV12-transformed MDCK cells showed a fibroblast-like migratory phenotype when seeded on collagen, but an epithelial phenotype when seeded on fibronectin or laminin (Sander et al., 1998). These phenotypes were associated with similar levels of Rac activity on
Discussion for further details.

Regulates Rho activity, and restores an epithelial phenotype. See mesenchymal phenotype. Reconstitution of Rac activity down-accompanied by upregulation of Rho activity and transition to a genic Ras-mediated downregulation of Rac activity is increased Rac activity through Raf/Map kinase mediated transcriptional regulation of cells. Rather, it appears that the balance between Rac and Rho activity is not involved in downregulation of Rho activity (Sander et al., 1999).

A cтированные мутации Rαc и Rho have been shown to cooperate with Ras in the transformation of fibroblasts (Qi et al., 1995a,b; Khozravi-Far et al., 1995). Here, we show that in epithelial MDCK cells, RasV12-induced morphological transformation involves downregulation of Rαc and upregulation of Rho activity. In addition to a role in cytoskeletal reorganization, the observed increase in Rho activity may also contribute to uncontrolled growth by suppressing Ras-mediated induction of the cyclin-dependent kinase inhibitor p21Waf1/Cip1, thereby allowing cell cycle progression (Olson et al., 1998).

We thank T. Reid and S. van Delft for generation and testing of GST-PAK and GST-rotekin fusion proteins; M. areel and W. Birchmeier for providing Ras as-transformed MDCK cells for initial studies; G. Nolan for the retroviral vector and packaging cells; M. van Dijk and J. Downward for providing RafCAAX and p110CAAX constructs; and F. van Leeuwen and other members of the department for stimulating discussions and critical reading of the manuscript.

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Figure 5. Schematic model depicting the differential effects of transient and sustained Ras signaling towards Rac and Rho. Short-term activation of Ras by HGF/Met receptor signaling leads to a transient PI3 kinase-dependent activation of Rac and a more prolonged, PI3 kinase-independent activation of Rho, which is accompanied by membrane ruffling and cell scattering. In contrast, sustained signaling by oncogenic Ras results in decreased Rac activity through Raf/MAP kinase mediated transcriptional downregulation of the Rac exchange factor Tiam1. Oncogenic Ras-mediated downregulation of Rac activity is accompanied by upregulation of Rho activity and transition to a mesenchymal phenotype. Reconstitution of Rac activity downregulates Rho activity, and restores an epithelial phenotype. See Discussion for further details.

The observed Rac–Rho antagonism as found in epithelial cells is consistent with our earlier findings in NIH 3T3 fibroblasts, where Rac was also shown to downregulate Rho activity (Sander et al., 1999). High Rac and low Rho activity is associated with an epithelial-like phenotype in NIH 3T3 fibroblasts, characterized by the formation of cadherin-based cell–cell adhesions and inhibition of cell migration. In contrast, elevated levels of Rac and Rho activities are associated with a fibroblastoid and migratory phenotype of these fibroblasts. The present data show that in epithelial MDCK cells, the balance between Rac and Rho activity determines the cellular phenotype. Oncogenic Ras is able to shift this balance by decreasing Rac and increasing Rho activity, leading to mesenchymal MDCK cells that have lost the capacity to establish E-cadherin-based cell–cell adhesions. It should be noted that the observed downregulation of Rac activity by RasV12 does not exclude a requirement for Rac in invasion and migration of cells. Rather, it appears that the balance between Rac and Rho activity is a major determinant of epithelial-mesenchymal transition. Blocking Ras or Rac pathways using dominant negative mutants inhibits motility (Ridley et al., 1995; Keely et al., 1997; Shaw et al., 1997), and Rac activity has been shown to be required for the formation and maintenance of E-cadherin–based cell–cell adhesions (Braga et al., 1995; Takaishi et al., 1997; Zhong et al., 1997; Sander et al., 1998). Both processes appear to be regulated by Rac-mediated signaling pathways.

How Rac is able to downregulate Rho activity remains a challenge for future research. The signaling pathway involved lies downstream of Rac and upstream of Rho, since expression of RacV12 downregulates Rho activity at the GTPase level. Studies with Rac effector mutants in NIH 3T3 fibroblasts suggest that Rac-mediated signaling pathways leading to reorganization of the cytoskeleton or to stimulation of Jun kinase are not involved in downregulation of Rho activity (Sander et al., 1999).

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