Modulation of Rac1 and ARF6 Activation during Epithelial Cell Scattering*

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Felipe Palacios‡ and Crislyn D’Souza-Schorey§

From the Department of Biological Sciences and the Walther Cancer Institute, University of Notre Dame, Notre Dame, Indiana 46556-0369

Epithelial cell scattering encompasses the dissolution of intercellular junctions, cell-cell dissociation, cell spreading, and motility. The Rac1 and ARF6 GTPases have been shown to regulate one or more of these aforementioned processes. In fact, activated Rac1 has been shown to promote cell-cell adhesion as well as to enhance cell motility, leading to conflicting reports on the effect of Rac1 activation on epithelial cell motility. In this study, we have examined the activation profiles of endogenous Rac1 and ARF6 during the sequential stages of epithelial cell scattering. Using Madin-Darby canine kidney cells treated with hepatocyte growth factor/scatter factor or cell lines stably expressing activated v-Src, we show that Rac1 and ARF6 exhibit distinct activation profiles during cell scattering. We have found that an initial ARF6-dependent decrease in the levels of Rac1-GTP is necessary to induce cell-cell dissociation. This is followed by a steady increase in Rac1 and ARF6 activation and cell migration. In sum, this study documents the progression of ARF6 and Rac1 activities during epithelial cell scattering.

Epithelial tissues are composed of highly organized sheets of polarized cells that are poorly motile. Cell-cell adhesion is pivotal for the maintenance of structural integrity and function of epithelial tissues. Within the epithelial sheet, polarized cells are held together by intercellular junctions such as the tight junctions and the adherens junctions (1, 2). The adherens type junctions are principally responsible for homotypic cell-cell adhesion and are composed of a transmembrane protein, E-cadherin, that is linked to the actin cytoskeleton via a family of cytoplasmic proteins, the catenins. The disassembly of the adherens junctions has been shown to promote a loss of cell polarity and the acquisition of a more fibroblast-like or mesenchymal phenotype (3, 4). Such a transition, referred to as an epithelial to mesenchymal transition, is a hallmark feature of processes such as tumor cell invasion and wound healing (4–6). A similar change in morphology also occurs during some normal developmental processes, for example, the formation of the neural crest (7). Epithelial to mesenchymal transitions are characterized by cell scattering, a process involving the dissolution of cell junctions followed by cell-cell dissociation and acquisition of a migratory phenotype.

There are several mechanisms for regulation of adherens junction stability. v-Src-mediated tyrosine phosphorylation of the cadherin-catenin complex correlates with decreased cell-cell adhesion, although it has yet to be proven that phosphorylation is directly responsible for these observed effects (4, 8, 9). In addition to stabilizing existing adherens junctions, the actin cytoskeleton can drive the formation of new cadherin-based cell-cell contacts by promoting the appositioning of adjacent cell membranes (10). Thus, actin polymerization represents another means by which adherens junction stability may be regulated. The Rho-family GTPases Rac, Rh, and Cdc42 have also been implicated in regulation of cadherin-based cell-cell adhesion (11). Like all GTPases of the Ras superfamily, these proteins cycle between their active GTP- and inactive GDP-bound forms. Activated Rac1 promotes actin accumulation and cadherin-based adhesion at cell contact sites (12–15). Rh and Cdc42 have been shown to have similar roles as Rac, although their effects have been less consistent (12, 15).

Earlier work from our laboratory has shown that the ARF6 GTPase, via its effect on membrane traffic and the actin cytoskeleton, promotes the disassembly of adherens junctions and membrane ruffling, respectively, and thereby facilitates the acquisition of a motile phenotype (16). We have shown that ARF6 is activated in response to treatment of cells with HGF/ scatter factor, whereas dominant-negative ARF6 expression abolishes Src-induced cell scattering. These findings implicate an important role for ARF6 during cell scattering. More recently, we have also shown that activation of ARF6 is coupled to the down-regulation of Rac1 activity during adherens junction disassembly (17). Thus, ARF6-GTP serves to facilitate adherens junction disassembly and epithelial scattering in part by decreasing the cellular pool of Rac1-GTP. On the other hand, the activation of Rac1 has also been linked to promoting cell migration. For example, gain of a motile phenotype in response to treatment of cells with hepatocyte growth factor or by wounding results in the activation of Rac1 (6, 18, 19). In addition, the expression of ARNO, an ARF6-GEF, has been shown to facilitate cell motility in part by activating Rac1 (20). Taken together, activation of Rac1 in epithelia is associated with opposite outcomes: one, the promotion of cell-cell junctions and enhancement of the epithelial phenotype, and two, the formation of lamellipodia during epithelial cell migration. It is apparent, therefore, that an intricate and spatial coordination of

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‡ A predoctoral fellow of the Walther Cancer Institute.
§ To whom correspondence should be addressed: Dept. of Biological Sciences, University of Notre Dame, Box 369, Galvin Life Sciences Bldg., Notre Dame, IN 46556-0369. Tel.: 574-631-3735; Fax: 574-631-7413; E-mail: D’Souza-Schorey.1@nd.edu.

1 The abbreviations used are: ARF, ADP-ribosylation factor; ARNO, ARF nucleotide-binding site opener; MDCK cells, Madin-Darby canine kidney cells; GEF, guanine nucleotide exchange factor; GST, glutathione S-transferase; PAK, p21-activated protein kinase; CRIB, Cdc42/Rac-interactive binding; HGF, hepatocyte growth factor; HA, hemagglutinin.
Rac1 and ARF6 Activation during Cell Scattering

In this study, we have examined the activation profiles of endogenous Rac1 and ARF6 during the sequential stages of HGF- and Src-induced epithelial cell scattering. We have found that during cell scattering, an initial decrease in the levels of active Rac1 is necessary to induce cell-cell disassociation. This transient down-regulation of Rac1 is ARF6-dependent. Subsequently, a steady increase in Rac1 and ARF6 activation and cell migration is observed. We present a model that brings together disparate observations and delineates the progression of ARF6 and Rac1 activities during epithelial cell scattering.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—The MDCKpp60°-Src cell line was kindly provided by W. Birchmeier. Cells were grown at 41 °C on glass coverslips or tissue culture plastic as described previously (21) and kindly provided by W. Birchmeier. Cells were grown at 41 °C on glass coverslips or tissue culture plastic as described previously (21) and allowed to form small colonies of ~40–60 cells/colony. To induce cell scattering, MDCKpp60°-Src cells were incubated at the permissive temperature of 35 °C for time periods as indicated. MDCK type I cells (Clontech, a gift of A. Zahraoui) were grown as described previously (16). Transfection of cells with cDNA plasmids was carried out using the LipofectAMINE transfection reagent according to the manufacturer's instructions. HA (hemagglutinin)-tagged Rac1 and FLAG-tagged nm23-H1/ΔKpn expression plasmids have been described previously (17, 22).

**Microscopy**—MDCKpp60°-Src cells were allowed to scatter, and stages of cell scattering were monitored by time-lapse microscopy. All phase images were obtained using an inverted Zeiss Axiovert TV-135 microscope equipped with a cool CCD camera (Princeton Instruments, Trenton, NJ). Time-lapse imaging was carried out for a maximum of 4 h in a temperature-controlled environment (~35 °C) with 5% CO2 maintained in an insulated plexiglass chamber encasing the microscope. Cells were returned to the cell culture incubator, and after an additional 4 and 12 h, cells were visualized by phase-contrast microscopy. Image acquisition and processing were carried out with MetaMorph imaging software (Universal Imaging Corp.). For immunofluorescence microscopy, MDCK cells were fixed, permeabilized, and processed for immunofluorescence as described (16). Anti-HA rabbit polyclonal antibodies were from Covance, anti-FLAG mouse monoclonal was from Stratagene, and rhodamine phalloidin was from Molecular Probes. Anti-E-cadherin mouse monoclonal antibody was a generous gift from William Gallin. Stained cells were analyzed using a Bio-Rad confocal scanning laser microscopy system.

**GTPase Activation Assays**—Cells were grown in 6-well dishes in islands (~40% confluency). After inducing cell scattering, cells were quickly rinsed in ice-cold phosphate-buffered saline and incubated with 0.5 ml of lysis buffer (25 mM Tris-HCl, pH 7.4, 300 mM sucrose, 25 mM NaF, 10 mM Na3P2O7, 2 mM Na3VO4, 0.5% Triton X-100) plus protease inhibitors for 30 min on ice with gentle rocking. Cells were then scrapped and centrifuged for 5 min at 14,000 rpm at 4 °C. To determine the levels of Rac1 GTP, 400 µl of the cell lysates were incubated with PAK(CRIB)-GST beads as described (23), and the levels of active Rac1, Rac1-GTP, were detected by Western blotting using specific Rac1 monoclonal antibodies (Transduction Laboratories). In parallel, the level of ARF6-GTP in 400 µl of cell lysates was measured using the ARF6-GTP pull-down assay recently described (24). Additionally, 20 µl of cell lysates were examined for the distribution of total Rac1 and ARF6 by Western blot analysis.

**RESULTS AND DISCUSSION**

v-Src activation is a potent inducer of epithelial cell scattering. Thus, to monitor the different phases of epithelial cell scattering, we made use of an MDCK cell line stably transfected with a temperature-sensitive v-Src mutant (MDCKpp60°-Src) (21). At the non-permissive temperature of 41 °C, the cells assemble into compact and polarized colonies, as shown in Fig. 1. When switched to the permissive temperature of 35 °C, the cell colonies undergo scattering within hours. For our investigations, cells were seeded at low confluency and allowed to develop into small colonies (~40–60 cells/colony) prior to incubation at 35 °C for increasing time periods. Cell scattering was monitored by phase-contrast microscopy. After 30 min of Src activation, colony compaction was reduced, and cells within the colony were observed to “loosen” cell-cell contacts. By 1 h, the cells were clearly less adherent, and the colonies exhibited intercellular spaces. Such changes have been reported in past studies and are thought to be caused by an initial collapse in cell-cell adhesion (26). After 4 h of Src activation, cells appeared more flat and spread, and a centrifugal detachment of cells was observed. Most of the cells at the edges of the disrupted colony formed extensive lamellipodia. After 8 h of Src activation, cells within a colony were completely dispersed, and finally, at 16 h of Src activation, cells were migratory and showed a more fibroblast-like phenotype.

We proceeded to monitor the activation profiles of ARF6 and Rac1 during cell scattering. The levels of active, GTP-bound Rac1 were measured using the PAK-GST pull-down assay. We observed that in polarized cell colonies, the levels of Rac1-GTP were high. These results are in agreement with previous studies showing that confluent monolayers of epithelial cells have high levels of active Rac1 (13, 14). Upon initial activation of Src, the levels of active Rac1 decreased as the cells started to
scatter. After 1 h of Src activation, the levels of Rac1-GTP were decreased by ~50%. However, during subsequent stages of cell scattering, Rac1-GTP levels steadily increased. The total levels of Rac1 were not significantly altered during cell scattering.

From the above observations, we conclude that there is a transient decrease followed by a subsequent rise in Rac1 activation during Src-induced epithelial cell scattering.

We next examined the levels of GTP-bound ARF6 during Src-induced cell scattering. To measure the levels of ARF6-GTP, we utilized an ARF6-GTP pull-down assay recently developed in our laboratory (24). As shown in Fig. 2B, in confluent epithelia, ARF6-GTP levels were minimal. However, upon Src activation, there was an exponential increase in ARF6-GTP. In studies describing a role for the ARF6-GEF, ARNO, in MDCK cells, it was shown that overproduction of ARNO did not perturb cell-cell adhesion within the colony but rather promoted lamellipodia extensions in migrating epithelial cells via the activation of Rac1 (19). Thus, it is likely that ARNO functions to activate ARF6 during the latter stages of cell scattering. In fact, the apical distribution of ARNO in polarized epithelia may serve to sequester the protein, which later becomes accessible for ARF6 activation as the cells become non-polarized. Activation of ARF6 in non-polarized cells could serve to translocate at least a subpopulation of cytoplasmic Rac1 to the cell surface as has been described previously (22, 27).

To complement the above investigations, we used a second experimental system in which the parental clone of MDCK cells described earlier was treated with HGF. Observation of cells under a phase-contrast microscope revealed that cells exhibited similar morphological alterations during cell scattering except that the kinetics appeared to be delayed. For instance, the initial stages of cell-cell dissociation and formation of intercellular spaces were more evident at 4 h after HGF treatment (data not shown). The GTPase activation profiles during HGF-induced cell scattering were examined. As shown in Fig. 2, C and D, the unique profiles for ARF6 and Rac1 activation detected earlier for Src-induced scattering were also observed upon treatment of cells with HGF. Moreover, the transient decrease in Rac1 activation coincided with an increase in cellular levels of ARF6-GTP and dissolution of cell-cell contacts. The decrease in Rac1-GTP levels was significantly inhibited when MDCKpp60--src cells were transfected with the dominant-negative ARF6(T27N) mutant (Fig. 3A), suggesting that the decrease in Rac1-GTP levels is dependent on ARF6.

Our previous work has shown that expression of a constitutively activated ARF6 mutant in epithelial cells induces a
breakdown of cell-cell contacts. These effects of ARF6 are mediated by recruitment of nm23-H1, a nucleoside diphosphate kinase, that facilitates junction disassembly in part by sequestering the Rac1 nucleotide exchange factor, Tiam1, leading to decreased levels of Rac1-GTP (17). Disassembly of cell-cell contacts and down-regulation of Rac1-GTP is blocked by expression of an nm23-H1 mutant, nm23-H1(ΔKpn), which prevents oligomerization and hence the function of wild type nm23-H1 (28). To determine whether the decrease in Rac1-GTP might be due to the ARF6-regulated recruitment of nm23-H1, we examined the effect of expressing nm23-H1(ΔKpn) on cell scattering and Rac1 activation. Transfection of cells with plasmid encoding nm23-H1(ΔKpn) inhibited scattering of MDCKpp60v-Src cells at permissive temperatures (Fig. 3). As observed in Fig. 3B, the expression of nm23-H1(ΔKpn) elicited only a partial “rescue” of Rac1-GTP levels during the scattering response. This could be because not all the cells express nm23-H1. Alternatively, it is possible that there are additional mechanisms that lead to a decrease in activated Rac1 levels during the onset of cell scattering.

Taken together, the above studies demonstrate that Rac1 activity is modulated during cell scattering and in part is regulated by ARF6 activation. The unique activation profile of Rac1 appears to be reflected in the morphological changes and polymerized actin redistribution that occurs during cell scattering. At sites of cell-cell contacts, increased accumulation of actin and Rac1-GTP facilitates cell-cell junction formation (29). Rac1-GTP also promotes the formation of actin-rich lamellipodia at the leading edge of migrating cells (6, 18, 19). Indeed, staining of MDCKpp60v-Src cells with rhodamine-phalloidin during scattering shows increased labeling for polymerized actin at the cell junctions at very early time points (Fig. 4). However, actin staining between cells decreases by 1 h. Other studies have also shown that during the initial stages of the

Fig. 3. Effect of ARF6(T27N) and nm23-H1(ΔKpn) expression in MDCKpp60v-Src cells at permissive temperatures. Cellular levels of endogenous Rac1-GTP were determined in MDCKpp60v-Src cells expressing ARF6 (T27N) (A) and nm23-H1(ΔKpn)-FLAG (B) at permissive temperatures for varying time periods as indicated. Cells lysates were also probed for total Rac1 by immunoblotting procedures. Cells expressing nm23-H1(ΔKpn)-FLAG at 4 h were labeled with anti-FLAG antibodies and stained for actin using rhodamine phalloidin (C).

Fig. 4. Polymerized actin distribution in MDCKpp60v-Src cells during cell scattering. Cells grown at a permissive temperature of 35 °C were fixed at the indicated time periods, and actin filament distribution was visualized by staining with rhodamine phalloidin followed by immunofluorescence microscopy. Arrows indicate actin accumulation at intercellular junctions at early time points, and arrowheads point to lamellipodia, which form as cells begin to scatter.
The breakdown of cell-cell contacts, the actin cytoskeleton is disrupted at the adherens junctions (10). Within 4 h after Src activation, actin filaments have been markedly remodeled, and cells exhibit actin-rich lamellipodia at leading edge (Fig. 4). This is likely mediated by the increase in Rac1-GTP and ARF6-GTP levels during the later stages of cell scattering.

To compliment our observations of the biphasic activation profile of Rac1, we have examined the distribution of wild type Rac1 in MDCKpp60v-Src cells during cell scattering. We find that a significant pool of Rac1-GTP shifts from cell junctions to the perinuclear cytoplasm in MDCKpp60v-Src cells at ~1 h after Src induction at permissive temperatures. At later time points, when cell-cell contacts are practically non-existent, the majority of the Rac1 is found at lamellipodia of migrating cells (Fig. 5). Consistent with these observations, studies by Nakagawa et al. (30) showed that upon Ca2+ chelation, the dissolution of cell junctions is accompanied by the redistribution of Rac1 to the cytoplasm. Thus, the spatial distribution of Rac1 in the cell may be important for temporal control of Rac1 activation during epithelial to mesenchymal transitions.

What is the significance of the changes in the activities of ARF6 and Rac1 during cell scattering? Significant progress has been made toward elucidating the role of the actin cytoskeleton either to facilitate cell-cell adhesion or to promote surface protrusions and ruffles required for cell migration. The dynamic process of cell scattering necessitates dynamic remodeling, from intercellular adhesion to the migratory phenotype. The activation of the ARF6 and Rac1 GTPases are likely critical determinants that facilitate this transition. In organized polarized epithelial sheets, ARF6-GTP levels are minimal, whereas Rac-GTP is high and E-cadherin cycling and turnover is minimal. This is consistent with our earlier observations that a dominant-negative ARF6-GDP mutant is localized almost exclusively to cell-cell junctions in polarized epithelia, and its expression enhances the epithelial phenotype (16). Addition of migratory stimulus induces the activation of ARF6. Further acquisition of the migratory phenotype is facilitated by subsequent increases in ARF6 and Rac1 activities (Fig. 5).

From the above investigations, we also infer that the cellular phenotype can markedly affect the activation profile of individual GTPases. In individual or small clusters of loosely adherent epithelial cells that do not exhibit colony compaction, basal levels of Rac-GTP are lower than those observed in larger and polarized epithelial colonies that exhibit signifi-
cantly higher cell-cell contacts. Moreover, loosely adherent cells will likely exhibit a steady increase in Rac1 activation as opposed to a transient down-regulation of Rac1 that is observed in larger colonies. These findings also support previous reports that cadherin engagement or cell-cell adhesion can modulate the activation profiles of the Rho family GTPases (14).

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