Proteasome-mediated Degradation of Rac1-GTP during Epithelial Cell Scattering[□]

Emma A. Lynch,* Jennifer Stall,* Gudila Schmidt,[†] Philippe Chavrier,[‡] and Crislyn D'Souza-Schorey*

*Department of Biological Sciences and the Walther Cancer Institute, University of Notre Dame, Notre Dame, IN 46556; [†]Institut für Experimentelle und Klinische Pharmakologie und Toxikologie der Albert-Ludwigs-Universität Freiburg, D-79104 Freiburg, Germany; and [‡]Membrane and Cytoskeleton Dynamics Group, Institute Curie, 75248 Paris, France

Submitted August 18, 2005; Revised February 6, 2006; Accepted February 8, 2006
Monitoring Editor: Asma Nusrat

Epithelial cells disassemble their adherens junctions and “scatter” during processes such as tumor cell invasion as well as some stages of embryonic development. Control of actin polymerization is a powerful mechanism for regulating the strength of cell–cell adhesion. In this regard, studies have shown that sustained activation of Rac1, a well-known regulator of actin dynamics, results in the accumulation of polymerized actin at cell–cell contacts in epithelia and an increase in E-cadherin-mediated adhesion. Here we show that active Rac1 is ubiquitinated and subject to proteasome-mediated degradation during the early stages of epithelial cell scattering. These findings delineate a mechanism for the down-regulation of Rac1 in the disassembly of epithelial cell–cell contacts and support the emerging theme that UPS-mediated degradation of the Rho family GTPases may serve as an efficient mechanism for GTPase deactivation in the sustained presence of Dbl-exchange factors.

INTRODUCTION

Epithelial cells disassemble their contacts with neighboring cells and “scatter” during processes of embryonic development and wound healing but also when tumor cells become invasive during metastases progression (Perez-Moreno *et al.*, 2003). The intercellular adherens junctions are specialized subapical structures that function as principle mediators of epithelial cell–cell adhesion. The E-cadherin–catenin complex of the adherens junctions effectively links the actin cytoskeleton of adjacent cells to form the subapical adhesion belt of epithelial tissues. Epithelial cell scattering involves the breakdown of adherens junctions, the concomitant loss of the epithelial phenotype and the acquisition of a motile phenotype. The loss of cell–cell contacts during these “epithelial to mesenchymal transitions” has largely been attributed to mutations in components of the adherens junctions or their transcriptional repression (Perez-Moreno *et al.*, 2003). As migrating epithelia do not always exhibit changes in the composition of cadherin–catenin complexes, it is likely that posttranscriptional regulation of cellular processes can impinge on the assembly/disassembly of adherens junctions and contributes to the loss of cell polarity and the acquisition of migratory potential.

Several posttranscriptional mechanisms are implicated in the destabilization of epithelial cell–cell adhesion (D'Souza-Schorey, 2005). Although the phosphorylation of the cadh-

erin–catenin complex has been reported to weaken the interactions between these molecules (Shibamoto *et al.*, 1994; Kinch *et al.*, 1995), the endocytosis of the E-cadherin has also been shown to facilitate the disassembly of cell–cell contacts (Palacios *et al.*, 2001, 2002; Paterson *et al.*, 2003). In addition, control of actin polymerization at sites of cell–cell contact represents a powerful mechanism for regulating the strength of cell–cell adhesion (Vasioukhin *et al.*, 2000; Kovacs *et al.*, 2002). An accumulation of polymerized actin at the adherens junctions stabilizes cell–cell contacts and its disruption leads to the disassembly of the adherens junctions. Epithelial cell scattering has also been shown to require a step mediated by the proteasome (Tsukamoto and Nigam, 1999). The latter study showed that treatment of cells with proteasome inhibitors blocked epithelial cell scattering, although the mechanism by which this occurs is not known. Here we show that Rac1-GTP, a well-known regulator of actin dynamics that localizes to cell junctions, is subject to ubiquitin/proteasome-mediated degradation during the early steps of cell scattering. Deactivation of Rac1 by the ubiquitin/proteasome system (UPS) could represent a decisive mechanism to turn off Rac1-GTP effects and its sustained activation at the adherens junctions.

MATERIALS AND METHODS

Plasmids, Cell Culture, and Transfection

Rac2 was amplified by PCR from Rac2-myc p5Rα (a kind gift from Dr. G. Gacon, Institut Cochin, INSERM, Paris, France) and subcloned into the EcoRI and BamHI sites of pEGFP-C1 (Clontech, Mountain View, CA). Plasmids encoding constitutively active Rac1 (Rac1-G12V pCGT) and dominant negative Rac1 (Rac1-T17N pCGT) were kindly provided by Linda Van Aelst (Cold Spring Harbor, NY) and that encoding POSH-RBD, was provided by Gudila Schmidt (Albert Ludwigs Universität Freiburg, Germany). Madin Darby canine kidney II (MDCK II) cells and BSC-1 African Green Monkey kidney cells were cultured in DMEM supplemented with 10% fetal bovine serum,

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05-08-0779>) on February 15, 2006.

[□] The online version of this article contains supplemental material at *MBC Online* (<http://www.molbiolcell.org>).

Address correspondence to: Crislyn D'Souza-Schorey (D'Souza-Schorey.1@nd.edu).

Figure 1. HGF-induced scattering is blocked by inhibition of proteasome activity. MDCK II cells grown in colonies were first incubated with or without 10 μ M lactacystin (LAC) or 30 μ M ALLN and then treated with 50 ng/ml HGF for 8 h at 37°C to induce scattering. Cells were fixed, labeled for actin using rhodamine phalloidin and for E-cadherin using an anti-E-cadherin mAb, and processed for immunofluorescence microscopy. Images shown are along a single confocal plane. Prior treatment with lactacystin blocks the disassembly of cell-cell contacts and HGF-induced scattering.

penicillin, and streptomycin. Transient transfections were performed using 8 μ l of Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) and 6 μ g of purified plasmid DNA following manufacturer's instructions. Lactacystin, a proteasome inhibitor (Calbiochem, San Diego, CA), ALLN (Calbiochem) and hepatocyte growth factor/scatter factor (HGF; Calbiochem) were used at a final concentration of 10 μ M, 30 μ M, and 50 ng/ml, respectively.

Immunofluorescence

Immunofluorescence staining was conducted as described previously (Palacios *et al.*, 2002). Cells were visualized using a Nikon fluorescence microscope (Melville, NY) and a Bio-Rad confocal scanning imaging system (Richmond, CA). Antibodies against the adherens junction components were obtained from Transduction Laboratories (San Diego, CA). Rhodamine phalloidin was obtained from Molecular Probes (Eugene, OR).

Immunoprecipitations and Western Blotting

MDCK II cells grown in 60-mm culture dishes were incubated with 500 μ l RIPA lysis buffer (150 mM sodium chloride, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris-HCl, pH 7.5, and 1% mammalian protease inhibitor cocktail; Sigma, St. Louis, MO) for 4 min on ice with gentle rocking. Cells were scraped and the lysates were centrifuged at 14,000 rpm for 5 min. Cell lysates were incubated with an anti-ubiquitin mouse monoclonal antibody (mAb; Covance, Richmond, CA) for 1 h on ice. Fifty percent slurry of agarose-linked protein A beads (Amersham Biosciences, Piscataway, NJ) was added to the lysate, which was incubated for 1 h at 4°C with rocking. The beads were collected by centrifugation followed by three washes with RIPA lysis buffer. After the final wash the supernatant was discarded and 50 μ l of 2 $\times$ SDS Laemmli buffer was added to the beads. The samples were then resolved by SDS-PAGE. Proteins were transferred onto nitrocellulose membranes and probed for Rac1 using mouse mAb against Rac1 (Transduction Laboratories). In a separate set of experiments, MDCK II cells were transiently transfected with plasmids encoding Rac1-G12V pCGT and Rac1-T17N pCGT. Exogenous Rac1 mutants were immunoprecipitated from cell lysates as described above using a mouse mAb against the T7 tag (Novagen, Madison, WI). Immunoprecipitates were probed for ubiquitin with anti-ubiquitin antibodies. In separate experiments, total lysates were probed

for ERK and phospho-ERK using anti-ERK and anti-phospho-ERK (Cell Signaling Technology, Beverly, MA) or for the His-tag using with anti-His antibodies (Upstate Biotechnology, Lake Placid, NY).

PAK Pulldown Assay

Cells were grown in 60-mm dishes and treated with lactacystin and/or HGF for indicated times. Cells were quickly rinsed in phosphate-buffered saline and lysed using 500 μ l lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl₂) plus mammalian protease inhibitors. Cells were scraped, lysates were centrifuged for 5 min at 14,000 rpm. To determine the levels of Rac1-GTP, 400 μ l of each cell lysate was incubated with PAK (CRIB)-GST beads for 1 h with rocking at 4°C. Samples were centrifuged for 2 min at 2000 rpm and supernatant was discarded. The beads were washed three times with wash buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 10 mM MgCl₂) plus mammalian protease inhibitors. Finally, 50 μ l 2 $\times$ Laemmli buffer was added to the beads and boiled for 10 min. The levels of active Rac1, Rac1-GTP, were detected by Western blotting using specific Rac1 mAb (Transduction Laboratories). Approximately 25 μ g of total lysate was analyzed to examine the levels of total Rac1 and α -tubulin (Sigma).

RESULTS

As stated above, a previous study by Tsukamoto and Nigam demonstrated that epithelial cell scattering requires a step mediated by the proteasome (1999). The study showed that inhibitors of proteasome activity such as lactacystin and MG132 blocked cell scattering induced either by v(viral)-Src expression or treatment of cells with HGF/scatter factor. Consistent with these observations, we found that treatment of the MDCK cells with proteasome inhibitors such as lactacystin and ALLN (N-acetyl-Leu-Leu-Nle-CHO, also known as MG101 or LLNL) completely blocked HGF-induced cell scattering. In the absence of lactacystin, HGF treatment promoted the breakdown

Figure 2. Proteasome inhibition reverses the decrease in Rac1-GTP levels upon growth factor stimulation. MDCK II (A) or BSC-1 (B) cells were incubated with lactacystin (right) or without lactacystin (left) for 1 h before treatment with 50 ng/ml HGF for the indicated time periods. Rac1-GTP was precipitated from cells using the PAK pulldown assay. Rac1-GTP levels were detected by immunoblotting procedures. Cell lysates were also probed for total Rac1 levels. To normalize for total protein loading, cell lysates were also labeled for α -tubulin. Band intensities were quantified by densitometry. The average data of three separate experiments is shown.

of E-cadherin-based cell-cell contacts and the acquisition of a motile phenotype (Figure 1). On the basis of these observations, we hypothesized that proteasome-mediated degradation of a junction-stabilizing component is required for cell scattering to proceed.

Sustained activation of Rac1 in epithelial monolayers results in the accumulation of polymerized actin at cell-cell contacts and an increase in E-cadherin-mediated adhesion (Braga *et al.*, 1997; Takaishi *et al.*, 1997; Noren *et al.*, 2001). Furthermore, Tiam 1, a nucleotide exchange factor of the Rac1 GTPase, localizes to the adherens junctions and its overexpression has similar effects as activated Rac1 on intercellular adhesion (Hordijk *et al.*, 1997). Analysis of Rac1 activation during the sequential steps of cell scattering in MDCK I cells revealed that the Rac1-GTP levels in polarized epithelia are relatively high but that the disassembly of adherens junctions is accompanied by a transient decrease in the cellular levels of Rac1-

GTP (Palacios and D'Souza-Schorey, 2003). This is followed by the restoration of high Rac1-GTP as cells become migratory and exhibit lamellipodia and membrane ruffles. The dip in Rac1-GTP levels correlated with a loss of polymerized actin at sites of cell-cell contact (Palacios and D'Souza-Schorey, 2003) and therefore represents potentially a key step in the diminution of adherens junction stability. On the basis of these observations, we investigated whether Rac1-GTP might be subjected to proteasome-mediated degradation during cell scattering. We first examined whether blocking proteasome function reverses the decrease in Rac1-GTP. For these investigations, MDCK II cells were treated with HGF in the presence or absence of lactacystin for increasing time periods and active GTP-bound Rac1 was precipitated from cell lysates using the PAK pulldown assay as previously described (Benard and Bokoch, 2002). As seen in Figure 2A, the level of Rac1-GTP was transiently decreased at 2 and 4 h posttreatment with HGF. This dip in Rac1-GTP levels

was also reflected in the cellular levels of total Rac1. However, in the presence of lactacystin, the transient decrease in Rac1-GTP (and total Rac1) was not observed (Figure 2A). This finding indicated that proteasome activity has a role in the down-regulation of Rac1-GTP during cell scattering. In contrast to Rac1, RhoA levels remained unperturbed during cell scattering in the presence or absence of lactacystin (Supplementary Figure 1). The decline in Rac1-GTP levels was not restricted to HGF treatment only, because EGF treatment also induced a transient decrease in Rac1-GTP levels (Supplementary Figure 2). A similar effect on Rac1-GTP levels during cell scattering was also observed in other epithelial cell lines, such as BSC-1 African Green monkey kidney cells (Figure 2B). It should be noted however, that although BSC-1 cells exhibited the decline in Rac1-GTP levels, the "total" Rac1 levels remained constant. This profile for total Rac1 is consistent with that previously described in the MDCK I cell line (Palacios *et al.*, 2003). One explanation for this is that Rac1-GAP activity may be significantly higher in the MDCK I and BSC I cell lines during the early stages of cell scattering. This in turn leads to a substantial pool of Rac1-GDP that is less susceptible to proteasome degradation (see below). Nevertheless, Rac1-GTP, which is responsible for the maintenance of cell-cell contacts, is subject to proteasome-mediated degradation during cell scattering, and this appears to be essential for cell scattering, irrespective of the cell type. It should also be noted that the down-regulation of Rac1-GTP is likely dependent on growth factor-mediated signaling because physical disruption of cell-cell contacts by depletion of extracellular calcium did not produce a down-regulation of Rac1-GTP levels (unpublished data). The effectiveness of lactacystin treatment was tested by analyzing lysates of cells treated with proteasome inhibitors on SDS gels. These studies showed smears of ubiquitinated proteins in lactacystin-treated cells, suggesting an increase in the cellular levels of ubiquitinated proteins upon proteasome inhibition (Supplementary Figure 3).

We also determined whether the effect of proteasome inhibition was specific to growth factor-induced Rac1 degradation or whether other cellular processes activated by HGF during cell scattering were also perturbed. To this end, we analyzed the levels of ERK (extracellular signal regulated kinase) activation in response to HGF. As shown in Figure 3, HGF-induced phosphorylation and activation of ERK was not altered in the presence of proteasome inhibitors. No changes were detected even at more prolonged time periods (unpublished data). In addition, tyrosine phosphorylation of E-cadherin, which also occurs in response to HGF, was not perturbed by proteasome inhibition (unpublished data).

To demonstrate the importance of Rac1 degradation in cell scattering, we tested the hypothesis that overexpression of the Rac GTPase would "saturate the system" and thus may rescue the disassembly of cell-cell contacts during cell scattering. Interestingly, a recent study showed that specific amino acids in Rac1, but not in Rac2 or Rac3, makes Rac1 susceptible to proteasome-mediated degradation, suggesting that Rac1 degradation is isotype-specific (Pop *et al.*, 2004). Thus, we examined whether a nondegradable isoform of Rac will protect against HGF-induced cell scattering by substituting for Rac1. Thus, MDCK cells transiently expressing Rac2-GFP were treated with HGF under conditions where nontransfected cells exhibited a scattered phenotype (Figure 4A). We found that almost all Rac2-GFP-expressing cells remained adhered to adjoining cells and actin staining between cells was stabilized. In contrast, untransfected cells, or cells expressing GFP alone, detached from the cell colony. Representative images are shown (Figure 4A). We also attempted to examine the effects of the Rac1 mutants [Rac1(K147R), Rac1(R174A,L177A)] resistant

Figure 3. Effect of lactacystin on HGF-stimulated ERK activation. MDCK II cells were incubated with or without 10 μ M lactacystin for 1 h before treatment with 50 ng/ml HGF for the indicated time periods. Cell lysates were analyzed for total ERK and phospho-ERK levels. To normalize for total protein loading, cell lysates were also labeled for α -tubulin.

to proteasome degradation. These mutants did not dominantly inhibit degradation of endogenous Rac1 (unpublished data), but we could not examine their effects on cell scattering due to low transfection efficiency.

Previous studies have indicated that effector interaction is necessary for Rac1 degradation (Pop *et al.*, 2004). As such, overexpression of a specific GTPase-binding domain of the Rac-specific effector, POSH (POSH-RBD), prevented Rac1-GTP degradation in HEK293 cells treated with CNF1. Thus we transfected MDCK II cells with a plasmid expressing His-tagged POSH-RBD and then induced them to scatter by treatment with HGF. As shown in Figure 4B, endogenous Rac1-GTP degradation was clearly inhibited in POSH-RBD-expressing cells. Furthermore, the percentage of cells that scattered was significantly decreased (Figure 4C). These data suggest that blocking the interaction of Rac1-GTP with its downstream effector by overexpressing POSH-RBD prevents degradation of the GTPase and attests to the importance of functional Rac1 levels during cell scattering.

Next, we examined whether Rac1-GTP itself might be a substrate of the proteasome during cell scattering. Thus, we tested whether Rac1 was modified by ubiquitin-tagging during the early steps of cell scattering. To this end, ubiquitin-tagged proteins in HGF-treated cell lysates were analyzed specifically for ubiquitinated Rac1 by immunoprecipitation with a mAb directed against ubiquitin, followed by probing of the immunoprecipitates for Rac1 using Western blotting procedures. As seen in Figure 5, a significant amount of ubiquitin-tagged Rac1 was observed at 2 h after HGF treatment, which then subsequently declined likely because of its degradation by the proteasome. In contrast, in the presence of the proteasome inhibitor, ubiquitinated Rac1 was detected in cell lysates even at prolonged periods after HGF treatment. The sizes of ubiquitin-tagged Rac1 bands were approximately 34 and 40 kDa, consistent with short-chain polyubiquitination. These modified higher molecular weight forms of Rac1 were not observed in Rac1-GTP pulldown assays, which may be because ubiquitin-tagged Rac1 is not efficiently recognized by PAK. A very low level of ubiquitinated Rac1 was also detected in polarized cells before scattering, suggesting that a low basal level of Rac1-GTP degradation might occur even under normal conditions. When the anti-ubiquitin antibody was replaced with a nonrelevant control antibody in the experiments described above, and

Figure 4. (A) Exogenous Rac2 blocks HGF-induced cell scattering. MDCK cells transiently expressing wild-type Rac2-GFP, or GFP alone as indicated, were treated with HGF to induce scattering. Cells were fixed, labeled for actin using rhodamine phalloidin, and processed for immunofluorescence microscopy. Images shown are along a single confocal plane. The expression of a nondegradable isoform of Rac, Rac2, stabilized actin at sites of cell-cell contacts and blocked HGF-induced scattering. (B) POSH-RBD blocks the transient decline in Rac1-GTP levels upon HGF stimulation: MDCK II cells with and without POSH-RBD-His expression were treated with 50 ng/ml HGF, for indicated times. Cells were lysed using RBD lysis buffer and subjected to the PAK pulldown assay. Samples were analyzed by SDS-PAGE and probed for Rac1 with a mouse anti-Rac1 and for His with a mouse anti-His antibodies, by Western blotting techniques. Lysates were probed for α -tubulin to normalize for equal protein loading. This figure is representative of three separate experiments. (C) POSH-RBD inhibits cell scattering upon HGF stimulation: MDCK cells were transfected with or without POSH-CRIB-His and treated with and without HGF as indicated. Cells were fixed, labeled for actin using rhodamine phalloidin, and processed for immunofluorescence microscopy. Percentage of unscattered colonies and scattered cells in each experimental condition is plotted. Cells that had at least two neighboring junctions intact were defined as part of a colony (arrowhead) and migrating cells (arrow) were counted as scattered. Western blot confirms POSH-CRIB-His expression.

then probed for Rac1 by Western blotting, ubiquitin-tagged Rac1 was not observed in the immunoprecipitate (Supplementary Figure 4).

We also examined whether Rac1-GTP rather than Rac1-GDP was specifically modified with ubiquitin and targeted to the proteasome for degradation. MDCK II cells were

Figure 5. Rac1 is modified by ubiquitin during the early stages of cell scattering. MDCK II cells were incubated with lactacystin (right) or without lactacystin (left) and then treated with 50 ng/ml HGF for indicated time periods. Cell lysates were incubated with mouse monoclonal ubiquitin and the immunoprecipitates were analyzed for Rac1 by immunoblotting with anti-Rac1 mAb. The level of ubiquitin-tagged Rac1 is maximum at 2 h and these levels subsequently decline in the absence of lactacystin. However in the presence of lactacystin, ubiquitinated Rac1 persists at later time points post-HGF treatment. H.C., IgG heavy chain; L.C., IgG light chain.

transfected with mammalian expression plasmids encoding constitutively active Rac1, Rac1-G12V, or dominant negative Rac1, Rac1-T17N, that were tagged with the viral T7 epitope. Exogenous Rac1-GTP and Rac1-GDP were immunoprecipitated using an anti-T7 mAb. Immunoprecipitates were resolved on SDS gels and labeled for ubiquitin using Western blotting techniques. As seen in Figure 6, the Rac1-GTP mutant, Rac1-G12V is a significantly better substrate for ubiquitination compared with the GDP-bound mutant, Rac1-T17N, and although the expression of Rac1-T17N is higher than Rac1-G12V, minimal labeling of Rac1-T17N with ubiquitin was observed. Thus, Rac1-GTP is selectively targeted to the UPS system for its deactivation during epithelial cell scattering. These results are consistent with previous findings showing nucleotide specific degradation of Rac1 by the proteasome (Doye *et al.*, 2002, Pop *et al.*, 2004).

uitination compared with the GDP-bound mutant, Rac1-T17N, and although the expression of Rac1-T17N is higher than Rac1-G12V, minimal labeling of Rac1-T17N with ubiquitin was observed. Thus, Rac1-GTP is selectively targeted to the UPS system for its deactivation during epithelial cell scattering. These results are consistent with previous findings showing nucleotide specific degradation of Rac1 by the proteasome (Doye *et al.*, 2002, Pop *et al.*, 2004).

DISCUSSION

The study described here supports the emerging theme that UPS-mediated degradation of the Rho family GTPases may serve as an efficient mechanism for Rho-GTPase deactivation in the sustained presence of Dbl-exchange factors (Doye *et al.*, 2002; Lerm *et al.*, 2002; Wang *et al.*, 2003). Ubiquitin tagging of Rac1-GTP during scattering may involve the activation of a ubiquitin ligase or the modification of the Rac1 GTPase itself to render it a better substrate for ubiquitin tagging. It is also possible that additional proteins besides Rac1 may also be subject to proteasome-mediated degradation to facilitate the destabilization of the adherens junctions. In this regard, α -catenin contains a PEST sequence making it a potential target for proteasome-mediated degradation (Takahashi *et al.*, 2000). The cytoplasmic domain of E-cadherin also contains a PEST sequence although the latter is masked by β -catenin and p120 catenin (Huber *et al.*, 2001). However, analyses of protein expression by Western blotting (Supplementary Figure 5) and immunofluorescence microscopy (unpublished data), using antibodies directed against α -catenin, β -catenin, p120 catenin, and E-cadherin, revealed that the adherens junction components were not degraded at early stages of cell scattering, i.e., at 2 h after treatment with HGF. However, we cannot exclude that other proteins in addition to Rac1-GTP might also be degraded during the dissolution of cell-cell contacts. Identification of the ubiquitin ligase that is responsible for Rac1 modification during adherens junction disassembly will shed significant light in this regard.

The deactivation of Rac1 as described above, could serve to promote the destabilization of the actin cytoskeleton at the subapical adhesion belt of epithelial monolayers. This in turn might "loosen up" the adherens junctions to facilitate processes such as the endocytosis of E-cadherin. It would seem more efficient if adherens junction disassembly is accompanied by the dissolution of actin bundles in order to facilitate membrane internalization at sites of cell-cell contact. What might cause the transient deactivation of Rac1 to promote cytoskeletal destabilization at the adherens junctions? Although the ubiquitination and proteasome-mediated degradation of Rac1 might serve as the initial step to

Figure 6. Rac1-GTP is preferentially ubiquitinated during cell scattering. MDCK II cells transiently expressing Rac1-G12V and Rac1-T17N each fused to a T7 tag were treated with HGF and lysed, and exogenous Rac1 in cell lysates was immunoprecipitated with anti-T7 mAb. The immunoprecipitates were probed for ubiquitin with anti-ubiquitin antibody. Rac1-GTP is a significantly better substrate for ubiquitin modification than Rac1-GDP. Cell lysates were also probed for exogenous Rac1 expression using anti-T7 mAb. Note that the T7 Tag contributes a 4-kDa molecular-weight shift, thus ubiquitinated bands of Rac1V12 pCGT construct are ~38 and 44 kDa. HC, IgG heavy chain, LC, IgG light chain, Ub, ubiquitin.

"set off" the disassembly of adherens junctions, it is likely that other downstream mechanisms also contribute toward the down-regulation of Rac1 to bring about the disassembly of epithelial cell-cell contacts. Although, little is known about Rac1-GAP activities during the disassembly of cell-cell contacts it is possible that GAP activities might also serve to decrease the Rac1-GTP pool. Furthermore, previous work in our laboratory has shown that activation of the ARF6 GTPase promotes the decrease in Rac1-GTP levels in addition to facilitating the endocytosis of E-cadherin. These effects of ARF6 are mediated by the recruitment of nm23-H1, a nucleoside diphosphate kinase, shown to function as a GTP-source for dynamin to facilitate endocytosis (Krishnan *et al.*, 2001), as well as to down-regulate cellular levels of Rac1-GTP by binding and sequestering Tiam-1 (Otsuki *et al.*, 2001). Thus several cellular mechanisms exist to bring about the depletion of Rac1-GTP pools and subsequently actin cytoskeleton disassembly, during the breakdown of adherens junctions. The relative activity of each of these processes in different cell types is an area for future investigation.

ACKNOWLEDGMENTS

We thank the Schmidt laboratory for the plasmid encoding POSH-RBD, the Hinchcliffe laboratory at ND for the BSC-1 epithelial cell line, and Linda Van Aelst for critical reading of the manuscript. This work was supported in part by a grant from the American Cancer Society (RSG 03-023-01-CSM) to C.D.-S.

REFERENCES

- Benard, V., and Bokoch, G. M. (2002). Assay of Cdc42, Rac, and Rho GTPase activation by affinity methods. *Methods Enzymol.* 345, 349–359.
- Braga, V. M., Machesky, L. M., Hall, A., and Hotchin, N. A. (1997). The small GTPases Rho and Rac are required for the establishment of cadherin-dependent cell-cell contacts. *J. Cell Biol.* 137, 1421–1431.
- Doye, A., Mettouchi, A., Bossis, G., Clement, R., Buisson-Touati, C., Flatau, G., Gagnoux, L., Piechaczyk, M., Boquet, P., and Lemichez, E. (2002). CNF1 exploits the ubiquitin-proteasome machinery to restrict Rho GTPase activation for bacterial host cell invasion. *Cell* 111, 553–564.
- D'Souza-Schorey, C. (2005). Disassembling adherens junctions: breaking up is hard to do. *Trends Cell Biol.* 15, 19–26.
- Hordijk, P. L., ten Klooster, J. P., van der Kammen, R. A., Michiels, F., Oomen, L. C., and Collard, J. G. (1997). Inhibition of invasion of epithelial cells by Tiam1-Rac signaling. *Science* 278, 1464–1466.
- Huber, A. H., Stewart, D. B., Laurents, D. V., Nelson, W. J., and Weis, W. I. (2001). The cadherin cytoplasmic domain is unstructured in the absence of beta-catenin. A possible mechanism for regulating cadherin turnover. *J. Biol. Chem.* 276, 12301–12309.
- Kinch, M. S., Clark, G. J., Der, C. J., and Burridge, K. (1995). Tyrosine phosphorylation regulates the adhesions of ras-transformed breast epithelia. *J. Cell Biol.* 130, 461–471.
- Kovacs, E. M., Goodwin, M., Ali, R. G., Paterson, A. D., and Yap, A. S. (2002). Cadherin-directed actin assembly: E-cadherin physically associates with the Arp2/3 complex to direct actin assembly in nascent adhesive contacts. *Curr. Biol.* 12, 379–382.
- Krishnan, K. S., Rikhy, R., Rao, S., Shivalkar, M., Mosko, M., Narayanan, R., Etter, P., Estes, P. S., and Ramaswami, M. (2001). Nucleoside diphosphate kinase, a source of GTP, is required for dynamin-dependent synaptic vesicle recycling. *Neuron* 30, 197–210.
- Lerm, M., Pop, M., Fritz, G., Aktories, K., and Schmidt, G. (2002). Proteasomal degradation of cytotoxic necrotizing factor 1-activated rac. *Infect. Immun.* 70, 4053–4058.
- Noren, N. K., Niessen, C. M., Gumbiner, B. M., and Burridge, K. (2001). Cadherin engagement regulates Rho family GTPases. *J. Biol. Chem.* 276, 33305–33308.
- Otsuki, Y., Tanaka, M., Yoshii, S., Kawazoe, N., Nakaya, K., and Sugimura, H. (2001). Tumor metastasis suppressor nm23H1 regulates Rac1 GTPase by interaction with Tiam1. *Proc. Natl. Acad. Sci. USA* 98, 4385–4390.
- Palacios, F., and D'Souza-Schorey, C. (2003). Modulation of Rac1 and ARF6 activation during epithelial cell scattering. *J. Biol. Chem.* 278, 17395–17400.
- Palacios, F., Price, L., Schweitzer, J., Collard, J. G., and D'Souza-Schorey, C. (2001). An essential role for ARF6-regulated membrane traffic in adherens junction turnover and epithelial cell migration. *EMBO J.* 20, 4973–4986.
- Palacios, F., Schweitzer, J. K., Boshans, R. L., and D'Souza-Schorey, C. (2002). ARF6-GTP recruits Nm23-H1 to facilitate dynamin-mediated endocytosis during adherens junctions disassembly. *Nat. Cell Biol.* 4, 929–936.
- Paterson, A. D., Parton, R. G., Ferguson, C., Stow, J. L., and Yap, A. S. (2003). Characterization of E-cadherin endocytosis in isolated MCF-7 and chinese hamster ovary cells: the initial fate of unbound E-cadherin. *J. Biol. Chem.* 278, 21050–21057.
- Perez-Moreno, M., Jamora, C., and Fuchs, E. (2003). Sticky business: orchestrating cellular signals at adherens junctions. *Cell* 112, 535–548.
- Pop, M., Aktories, K., and Schmidt, G. (2004). Isotype-specific degradation of Rac activated by the cytotoxic necrotizing factor 1. *J. Biol. Chem.* 279, 35840–35848.
- Shibamoto, S., Hayakawa, M., Takeuchi, K., Hori, T., Oku, N., Miyazawa, K., Kitamura, N., Takeichi, M., and Ito, F. (1994). Tyrosine phosphorylation of beta-catenin and plakoglobin enhanced by hepatocyte growth factor and epidermal growth factor in human carcinoma cells. *Cell Adhes. Commun.* 1, 295–305.
- Takahashi, N., Ishihara, S., Takada, S., Tsukita, S., and Nagafuchi, A. (2000). Posttranscriptional regulation of alpha-catenin expression is required for Wnt signaling in L cells. *Biochem. Biophys. Res. Commun.* 277, 691–698.
- Takaishi, K., Sasaki, T., Kotani, H., Nishioka, H., and Takai, Y. (1997). Regulation of cell-cell adhesion by rac and rho small G proteins in MDCK cells. *J. Cell Biol.* 139, 1047–1059.
- Tsukamoto, T., and Nigam, S. K. (1999). Cell-cell dissociation upon epithelial cell scattering requires a step mediated by the proteasome. *J. Biol. Chem.* 274, 24579–24584.
- Vasioukhin, V., Bauer, C., Yin, M., and Fuchs, E. (2000). Directed actin polymerization is the driving force for epithelial cell-cell adhesion. *Cell* 100, 209–219.
- Wang, H. R., Zhang, Y., Ozdamar, B., Ogunjimi, A. A., Alexandrova, E., Thomsen, G. H., and Wrana, J. L. (2003). Regulation of cell polarity and protrusion formation by targeting RhoA for degradation. *Science* 302, 1775–1779.