Ras induces NBT-II epithelial cell scattering through the coordinate activities of Rac and MAPK pathways

Natacha Edme[1], Julian Downward[2], Jean-Paul Thiery[3] and Brigitte Boyer[1,*]

[1]Laboratoire de Régulations Cellulaires et Oncogénése UMR146, Institut Curie Section de Recherche, Centre Universitaire Paris-Sud, 91405 Orsay, France
[2]Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK
[3]Laboratoire de Compartimentation et Dynamique Cellulaires UMR144, Institut Curie Section de Recherche, 26 rue d'Ulm, 75248 Paris, France
*Author for correspondence (e-mail: Brigitte.Boyer@curie.u-psud.fr)

Accepted 12 April 2002
Journal of Cell Science 115, 2591-2601 (2002) © The Company of Biologists Ltd

Summary

Cell dissociation and cell migration are the two main components of epithelium-mesenchyme transitions (EMT). We previously demonstrated that Ras is required for the accomplishment of both of these processes during the EGF-induced EMT of the NBT-II rat carcinoma cell line in vitro. In this study, we examined the downstream targets of Ras that are responsible for the dissociation and motility of NBT-II cells. Overexpression of activated forms of c-Raf and MEK1 (a component of the mitogen-activated protein kinase pathway, MAPK) led to cell dissociation, as inferred by the loss of desmosomes from the cell periphery. By contrast, active PI3K, RalA and RalB did not induce desmosome breakdown. The MEK1 inhibitor PD098059 inhibited EGF- and Ras-induced cell dispersion, whereas the PI3K inhibitor LY294002 had no effect. Accordingly, among the partial loss-of-function mutants of Ras (RasV12) that were used to distinguish between downstream targets of Ras, we found that the Raf-specific Ras mutants RasV12S35 and RasV12E38 induced cell dissociation. The PI3K- and RalGDS-activating Ras mutants had, in contrast, no effect on cell dispersion. However, MEK1 was unable to promote cell motility, whereas RasV12S35 and RasV12E38 induced cell migration, suggesting that another Ras effector was responsible for cell motility. We found that the small GTPase Rac is necessary for EGF-mediated cell dispersion since overexpression of a dominant-negative mutant of Rac1 (Rac1N17) inhibited EGF-induced NBT-II cell migration. All stimuli that promoted cell migration also induced Rac activation. Finally, coexpression of active Rac1 and active MEK1 induced the motility of NBT-II cells, suggesting that Ras mediates NBT-II cell scattering through the coordinate activation of Rac and the Raf/MAPK pathway.

Key words: EMT, Signal transduction, Cell dissociation, Cell motility

Introduction

Epithelium to mesenchyme transition (EMT) is a process during which cells loose their epithelial characteristics and acquire a fibroblast-like phenotype. EMT participates in several morphogenetic processes during embryonic development and is also likely to play a key role during the metastasis of carcinoma cells. EMT is a complex process that comprises the disruption of intercellular junctions, including adherens junctions, desmosomes and tight junctions, and cell migration. Cell dissociation and motility are induced by stimuli provided by either growth factors or extracellular matrix components. Activation of transmembrane receptors to these extracellular ligands initiates cascades of events that lead ultimately to cytoskeleton reorganization and to gene transcription. Understanding how these cellular changes are orchestrated during EMT requires characterization of the signal targets engendered by the activated receptors.

The rat bladder carcinoma line NBT-II undergoes EMT in response to growth factor stimulation (EGF, FGF-1 and TGFα) (Vallés et al., 1990; Gavrilovic et al., 1990). Cell-cell dissociation is preceded by the internalization of desmosomal components and their progressive loss from cell-cell contact areas. It has been previously demonstrated that the Ras proto-oncogene is required during the EGF-induced NBT-II desmosome breakdown (Boyer et al., 1997). Ras is known to participate in several signalling pathways leading to activation of the phosphoinositide-3-kinase (PI3K) (Rodriguez-Viciana et al., 1994; Rodriguez-Viciana et al., 1997; Kotani et al., 1994), RalGDS and Raf/MAPK phosphorylation cascades (Treisman, 1996). In addition, Ras has been shown to activate the small GTP-binding protein Rac (Van Aelst and D'Souza-Schorey, 1997).

PI3K is required during the hepatocyte growth factor/scatter factor (HGF/SF)-induced scattering of epithelial MDCK cells (Royal and Park, 1995). More recent studies have shown the involvement of the MAPK pathway during the HGF/SF- and PI3K-induced scattering of MDCK cells (Potempa and Ridley, 1998; Khwaja et al., 1998). For example, keratinocyte cell dispersion depends upon, at least, the sustained activation of MAPK (McCawley et al., 1999). Members of the Rho family of small GTPases also exhibit specific roles during epithelial cell scattering. The small GTPase Rac is required downstream of HGF/SF and Ras, as a dominant-negative Rac1 inhibits SF- or Ras-induced MDCK cell scattering (Potempa and Ridley,

1998; Ridley et al., 1995), although activated Rac1 alone does not induce this response (Ridley et al., 1995; Hordijk et al., 1997). By contrast, activation of RhoA inhibits MDCK cell spreading and scattering (Ridley et al., 1995). Ras and Rac seem to be involved in cell dissociation by reducing the stability of adherens junctions in primary BRK cells (Quinlan, 1999). In apparent contradiction, other studies have demonstrated a role for Rac in enhancing the formation of adherens junctions in MDCK cells (Ridley et al., 1995; Takaishi et al., 1997) and in keratinocytes (Braga et al., 1997). Although the role of signalling molecules downstream of Ras in destabilizing adherens junctions has been extensively investigated, the Ras effectors responsible for desmosome breakdown have still to be characterized.

The Ras pathway has also been implicated in the cytoskeletal rearrangements and cellular motility that accompany cell dispersion. Among the Ras effectors, PI3K mediates the Ras control of the cytoskeleton (Rodriguez-Viciana et al., 1997) and is responsible for actin rearrangement induced by activated FAK and by Rac1 and Cdc42 (Keely et al., 1997). Furthermore, the increased motility observed in response to HGF treatment of the human breast epithelial cell line 184BJ is blocked by the PI3K inhibitor wortmannin (Day et al., 1999), and carcinoma invasion promoted by $\alpha 6\beta 4$ integrin involves PI3K activity (Shaw et al., 1997). The Raf/MAPK pathway promotes the migration of fibroblastic cells and is necessary for Ras- and TC21-induced transformation and metastasis of NIH3T3 cells (Webb et al., 1998; Rosario et al., 1999). Moreover, the MAPK pathway is known to affect the actin-myosin system and leads to an increased motility of COS-7 cells (Klemke et al., 1997). Finally, the Rho family members of small GTPases are implicated in the cytoskeletal reorganization that occurs during cell migration (Ridley et al., 1995; Ridley et al., 1992; Ridley and Hall, 1992; Nobes and Hall, 1995; Nobes and Hall, 1999). Rac1 activation induces the formation of lamellipodia, and active Cdc42 promotes the protrusion of filopodia, leading to increased motility of Swiss3T3 cells (Nobes and Hall, 1995). Accordingly, activation of Cdc42 and Rac1 promotes motility and invasion of mammary epithelial cells on collagen matrices (Keely et al., 1997). Rac1 may regulate cell migration not only by influencing actin organization but also by potentiating MAPK activation (Leng et al., 1999).

In this report, we show that the Raf/MAPK pathway and Rac play crucial roles during EGF- and Ras-induced NBT-II cell dispersion, whereas PI3K and RalGDS activations are dispensable for cell scattering. Activation of MAPK is sufficient to promote cell dissociation by destabilising desmosomal junctions but is unable to induce cell motility. On the other hand, Rac is necessary for EGF- and Ras-induced NBT-II cell scattering but alone cannot promote cell dissociation. The full programme of EMT (i.e., cell dissociation and motility) is achieved by the simultaneous activation of MAPK and Rac, suggesting that the two pathways downstream of Ras are coordinately regulated during EMT events in NBT-II cells.

Materials and Methods

Constructs, antibodies and biochemical products

Expression vectors coding for the small GTPases Rac1V12, Rac1N17, Cdc42V12 and Cdc42N17 (all tagged with the myc epitope) were kindly provided by A. Hall (University College, London, UK). Plasmids encoding the active forms of RalA and RalB were provided by J.Camonis (Institut Curie, Paris, France). Vectors coding for the myc-tagged active and kinase dead rCD2-p110 chimeras were provided by K.Reif (ICRF, London, United Kingdom). Plasmids coding for the active c-Raf-CAAX was provided by A. Eychene (Institut Curie, Orsay, France). Plasmids encoding wild-type MEK1 and MEK1S218DS222D (MEK1SSDD) were a kind gift of J. Pouyssegur (Centre de Biochimie du CNRS, Nice, France). pEGFP-C1 plasmid was purchased from Clontech Laboratories (GmbH, Heidelberg). Monoclonal rat anti-Ras antibody was kindly provided by S. Roche (Centre de Biochimie du CNRS, Montpellier, France) (Roche et al., 1996). Polyclonal rabbit anti-ERK1 and rabbit anti-MEK1 antibodies were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Anti-RalA and RalB antibodies were the generous gift of J. Camonis (Institut Curie, Paris, France). Monoclonal mouse anti-desmoplakin antibody was a gift from W. W. Franke (Heidelberg, Germany). Anti-GFP was from Roche Molecular Biochemical (Roche Diagnostics Corporation, Meylan). Texas-Red-conjugated anti-mouse fluorescein isothiocyanate (FITC)-coupled anti-mouse, anti-rabbit and anti-rat antibodies were purchased from Jackson Laboratories (West Grove, PA). PD98059 and LY80052 were from Calbiochem, and the FTase2 inhibitor was supplied by P. Elvin (Zeneca Pharmaceuticals, Alderley Park, UK). EGF was purchased from Sigma (St Louis, MO).

Cell culture, microinjection and immunofluorescence

NBT-II cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). A stable RasV12-expressing clone was obtained by transfection of the NBT-II cell line by the calcium phosphate precipitation method. This clone, designated Ras32, was routinely cultured in DMEM containing 10% FCS and geneticin at 400 μg/ml (G418 sulfate; Gibco BRL, Gaithersburg, MD). Microinjection experiments were performed as described previously (Boyer et al., 1997). Briefly, cells were microinjected into the nucleus with 100 ng/μl of plasmids 2 days after seeding. Cells were incubated at 37°C for 4 hours prior to stimulation with EGF at 30 ng/ml. At least 200 cells were injected on each coverslip. After overnight incubation, cells on coverslips were rinsed in phosphate-buffer saline (PBS) before a 5 minute fixation at –20°C in methanol/acetone. Coverslips were dried and processed for immunofluorescence studies as described previously (Boyer et al., 1997). Digitized pictures were recorded and processed by Adobe Photoshop software. The percentage of cells having lost their desmosomes from the cell periphery was estimated by counting the number of cells with desmoplakin (DP) immunoreactivity within the cytoplasm as compared to cells expressing DP in a punctate pattern along the cell periphery (Boyer et al., 1989).

When needed, cells were treated with FTase2 at 25 μM, UO126 and PD98059 at 100 μM and LY80052 at 20 μM for 48 or 24 hours before fixation in methanol/acetone or addition of EGF for an additional 15 hours before fixation. Cells were processed for immunofluorescence studies as described above. Cell viability in the presence of each agent was assessed by cell morphology under an inverted microscope. At the concentrations used for each drug, no toxic effect was observed on cells.

Ras-injected cells were immunostained with monoclonal rat anti-Ras antibody followed by FITC-conjugated anti-rat antibody. Wild-type MEK1 and MEK1SSDD-injected cells were visualized with rabbit anti-MEK1 antibody and FITC-conjugated anti-rabbit antibody. Myc-tagged CD2-p110-, Rac1V12-, Rac1N17-, Cdc42V12- and Cdc42N17-injected cells were immunolabelled with the 9E10 mouse monoclonal anti-myc antibodies and FITC-conjugated anti-mouse antibodies. The RalAV23- and RalBV23-injected cells were visualized with mouse anti-RalA and rabbit anti-RalB antibodies respectively, followed by FITC-conjugated anti-mouse or anti-rabbit antibodies. RasV12-, RasV12A38-, RasV12C40-, RasV12G37-,

RasV12S35- and RasV12E38-injected cells were immunostained with rat monoclonal anti-Ras antibodies followed by FITC-conjugated anti-rat antibodies. The presence of desmosomes at the cell periphery was monitored by immunofluorescent staining with anti-desmoplakin antibodies and Texas-Red-coupled anti-mouse antibodies, as described previously (Boyer et al., 1989).

For biochemical assays and migration experiments, NBT-II cells were transiently cotransfected with the indicated expression vectors and EGFP reporter construct by the PEI (polyethylenimine) method (Boussif et al., 1995). Briefly, NBT-II cells were plated on a 35 mm dish, transfected with 5 μl of PEI and 2.5 μg of the desired contructs, along with 0.5 μg of EGFP reporter construct. Cells were allowed to incorporate the DNAs for 5 hours, washed and analyzed 24 hours later.

Cell migration assay

To analyse cell migration, two conditions were used: cells in clusters and isolated cells. Cells in clusters were microinjected with 100 ng/μl of the constructs of interest along with 50 ng/μl of the construct coding for EGFP reporter gene. For motility of isolated cells, the constructs of interest and the EGFP construct were transiently overexpressed by the PEI method (see above), and 24 hours later, cells were trypsinized and plated under low confluence conditions. Four hours after microinjection or 10 hours after plating, cells were placed on the motorized stage of a Leica inverted microscope equipped with a chamber providing a controlled temperature and CO_2 concentration and connected to a Princeton MicroMax CCD camera (Princeton Instruments Inc., Trenton, NJ). Phase contrast and epifluorescence images were collected and analysed with the Metamorph software (Metamorph Imaging System, Universal Imaging Corporation, West Chester, PA) running on a PC workstation. The motility of cells was evaluated by tracking their movement over 15 hours with images recorded every 4 minutes. For each overexpressed construct, 40 to 60 cells were analysed in at least three independent experiments.

MAPK and Rac activity assays

The assay of MAPK activity was performed as described previously (Boyer et al., 1997), using myelin basic protein (MBP) as an exogenous substrate for MAPK.

For the Rac activity assay, bacteria transformed with the GST-PAK2 construct were activated with 100 μM IPTG for 3 hours at 37°C. After centrifugation, the bacterial pellets were resuspended in 10 ml prelysis buffer [50 mM Tris, pH 7.5, 1% Triton X-100, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 10 μg/ml aprotinin and leupeptin and 0.1 mM phenylmethylsulfonidefluoride (PMSF)] and lysed by adding lysosyme (0.1 volume of a 10 mg/ml solution in 25 mM Tris-HCl, pH 8.0) for 30 minutes at 4°C. DNA was degraded by sonication at 4°C. Lysates were centrifuged at 15,000 ***g*** for 15 minutes, and the cleared lysates were frozen at –80°C. Twenty five μg of lysate was incubated with 30 μl of glutathione-sepharose beads (Pharmacia Biotech, Uppsala) at 4°C for 30 minutes before processing for pull-down experiments.

When needed, NBT-II cells were activated with 30 ng/ml of EGF for 90 minutes or transiently transfected with the constructs of interest 24 hours before pull-down experiments. Cells were washed three times with cold TBS and scraped in 100 μl of lysis buffer (50 mM Tris, pH=7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 100 μM PMSF, 100 μM sodium orthovanadate and 500 μM sodium fluoride). Lysates were centrifuged at 15,000 ***g*** for 3 minutes at 4°C, and the supernatants were incubated with GST-PAK2 beads for 60 minutes at 4°C. The beads and proteins bound to the fusion protein were washed with a Tris buffer (50 mM pH 7.2) containing 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 0.1 mM PMSF, eluted in Laemmli sample buffer, separated by gel electrophoresis and analysed for Rac1 expression by western blot using Rac1 monoclonal antibodies (Transduction Laboratories).

Results

The MAPK pathway is necessary and sufficient to induce desmosome internalization but does not promote cell motility

Ras is known to activate several effectors, such as PI3K, which produces phosphoinositides (Rodriguez-Viciana et al., 1994; Kodaki et al., 1994), and RalGDS, which enhances the exchange of GDP for GTP of the Ras-related RalA and RalB factors (Kikuchi et al., 1994). It also induces the activation of the serine/threonine kinase Raf, which activates the MEK/ERK phosphorylation cascade (Schaeffer and Weber, 1999). To investigate which of the signalling pathways controlled by Ras may be responsible for the EMT of NBT-II cells, the first approach was to examine cell dissociation monitored by the internalization of desmoplakin (DP) immunostaining (Boyer et al., 1989) in NBT-II cells overexpressing downstream effectors of Ras. C-Raf can be constitutively activated by the addition of a C-terminal farnesylation motif (CAAX), which targets the protein to the cell membrane. MEK1 is phosphorylated at Ser218 and Ser222 upon activation by C-Raf (Zheng and Guan, 1994). The substitution of Ser218 and Ser222 with Asp mimics the negatively charged phosphate group and constitutively activates MEK (Pages et al., 1994). To test whether active MEK1 (MEK1SSDD) mediates NBT-II cell dissociation, the corresponding construct was transiently expressed by microinjection into NBT-II cell nuclei. Subsequently, DP immunolocalization was analysed in the microinjected cells. Transient overexpression of MEK1SSDD promoted desmosome breakdown, as inferred from the loss of DP immunoreactivity at intercellular junctions (Fig. 1Aa,b). By contrast, overexpression of wild-type MEK1 did not lead to DP internalization, suggesting that activation of the Raf/MAPK pathway is necessary to induce cell dissociation. The CD2-p110 chimera is a constitutively active form of PI3K that targets the catalytic subunit p110 to the plasma membrane (Reif et al., 1996). When overexpressed in NBT-II cells, the chimera did not induce desmosome disruption (Fig. 1Ac,d). Microinjection of RalAV23 and RalBV23 mutants, which are constitutive GTP-bound forms of RalA and RalB, had no effect on DP internalization (Fig. 1Ae,f, Fig. 1B). In addition, coexpression of MEK1SSDD, CD2-p110 and RalAV23 did not increase the percentage of DP-negative cells above that of cells expressing MEK1SSDD alone, indicating that the three effectors do not cooperate to induce cell dissociation. Statistical analysis of data obtained from several independent experiments are shown in Fig. 1B. These data indicate that the activation of the Raf/MAPK pathway is sufficient to promote cell dissociation.

To confirm that the Raf/MAPK pathway, but not the PI3K pathway, is required during NBT-II cell dissociation, we next tested the effect of their pharmacological inhibition. When cells were pretreated for 24 hours with the MEK inhibitors PD089059 or UO126, which specifically prevent MEK1 from promoting both threonine and tyrosine phosphorylation of MAPK (Pang et al., 1995; Dudley et al., 1995), EGF stimulation of NBT-II cells was no longer able to promote desmosome disruption (Fig. 2Ae,f). By contrast, incubation of

cells with LY294002, a specific PI3K inhibitor, had no effect on EGF-induced desmosome breakdown (Fig. 2Ac). However, when cells were incubated with 3 μM UO126, a concentration that prevented EGF-induced cell dissociation in only 10% of cells, addition of 20 μM LY294002 protected more than 90% of cells from EGF-induced desmosome breakdown (data not shown). Thus, when MEK activity was reduced below the level required for NBT-II cell dissociation, PI3K was able to play a role in desmosome internalization. As a control, a farnesyl transferase inhibitor that prevents the activation of Ras by inhibiting its membrane localisation was tested under the same experimental conditions (Fig. 2Ad). The effects of PD089059 and LY294002 were also examined in Ras32, a stable RasV12-overexpressing clone of NBT-II cells. Ras32 cells exhibit a fibroblast-like phenotype that is clearly different from the epithelial morphology of the parental NBT-II cells (Fig. 2B). Immunofluorescence analysis of Ras32 cells revealed a cytoplasmic punctate pattern of DP immunostaining, with few immunofluorescent dots at the cell surface, which is indicative of desmosome disruption (Fig. 2Ag). Twenty-four hours after addition of PD089059 or UO126 to the culture medium, desmosomes were reformed at intercellular junctions (Fig. 2Aj,k) whereas, under the same conditions, LY025052 had no effect on DP localization (Fig. 2Ah). However, the intensity of intracellular DP immunostaining was markedly higher in LY025052-treated Ras 32 cells than in their untreated counterparts, suggesting that PI3K could be involved in preventing desmosomal proteins from degradation. The same effect was observed in parental NBT-II cells (Fig. 2, compare b with c). As a control, addition of FTase2 relocalized DP to cell-cell junctions (Fig. 2Ai).

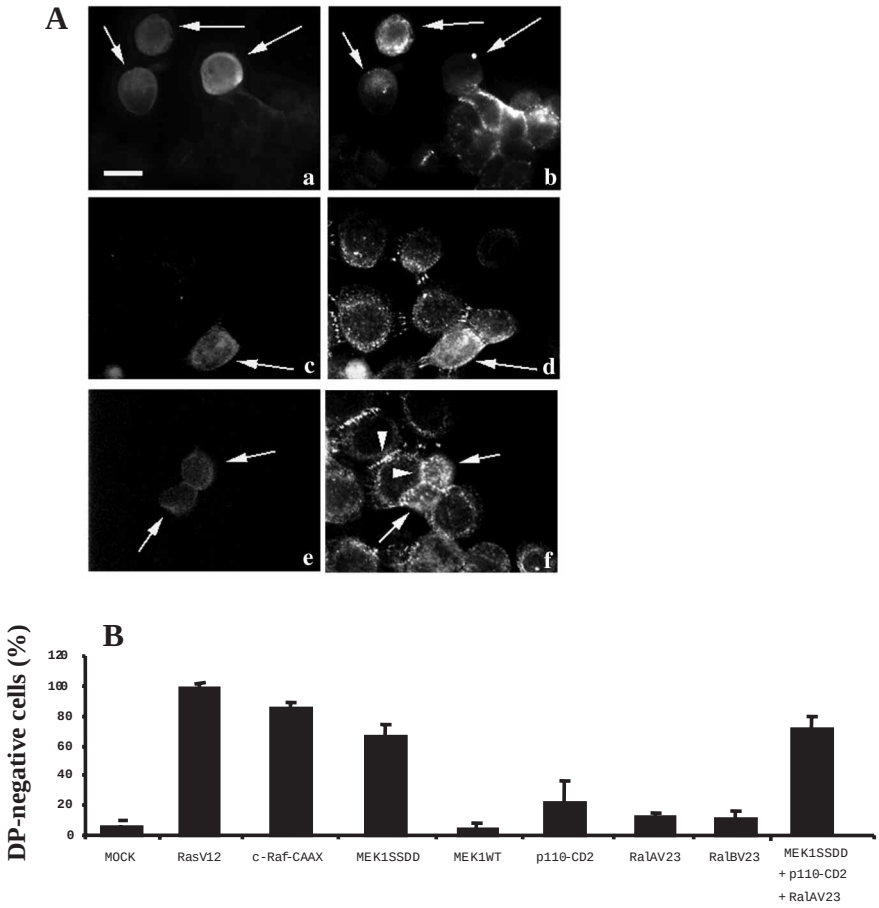

Fig. 1. Active Mek1 promotes NBT-II cell dissociation. (A) Cells were microinjected into the nucleus with 100 ng/μl of constructs coding for MEK1SSDD (a,b), CD2-p110 (c,d) and RalBV23 (e,f), incubated for 18 hours, fixed and double labelled for the expression of the injected construct (a,c,e) and desmoplakin (DP) (b,d,f). Arrows point to microinjected cells, and arrowheads indicate the presence of desmoplakin immunostaining at the cell periphery. (B) For each experiment, several coverslips were analysed and the percentage of desmosome-negative cells was calculated as the ratio between the number of cells without DP at the cell surface and the total number of cells. The results have been averaged from several experiments (n>3) in which at least 100 cells were microinjected. The means and s.e.m. are shown. Bar, 10 μm.

Another way to confirm that Ras induces NBT-II cell dissociation through the Raf/MAPK pathway was to test the effects of partial loss-of-function Ras mutants that allow characterization of the various effector pathways downstream of Ras (White et al., 1995). RasV12C40 selectively activates the PI3K pathway; RasV12G37 is specific for RalGDS effector, whereas RasV12S35 and RasV12E38 selectively activate the Raf/MAPK pathway (Rodriguez-Viciana et al., 1997; White et al., 1995). Transient overexpression of RasV12 led to DP internalization in 100% of cells. A similar result was obtained after microinjection of RasV12S35 and RasV12E38, whereas transient expression of RasV12C40 and RasV12G37 did not induce DP internalization (Fig. 3). These data confirmed the specific contribution of the Raf pathway to cell dissociation. As a control, microinjection of RasV12A38, an inactive Ras mutant that does not interact with an effector (Rodriguez-Viciana et al., 1997), had no effect on DP internalization (Fig. 3).

Altogether, these results demonstrate that the Ras-Raf-MAPK pathway is necessary and sufficient to promote desmosome breakdown in NBT-II cells.

MEK1-induced cell dissociation is a prerequisite for Rac1-mediated cell motility

To determine whether active MAPK also participates in the migration response of cells to EGF, we used two assays. In the first one, we microinjected the desired constructs into clustered cells along with a EGFP plasmid, and four hours later, their motility was recorded on a motorized Leica microscope. In the second one, cells transfected with the different constructs were trypsinized and plated at low density to obtain individual cells, thus bypassing the cell dissociation step. Cell motility was then measured for the isolated cells. In both assays, overexpression

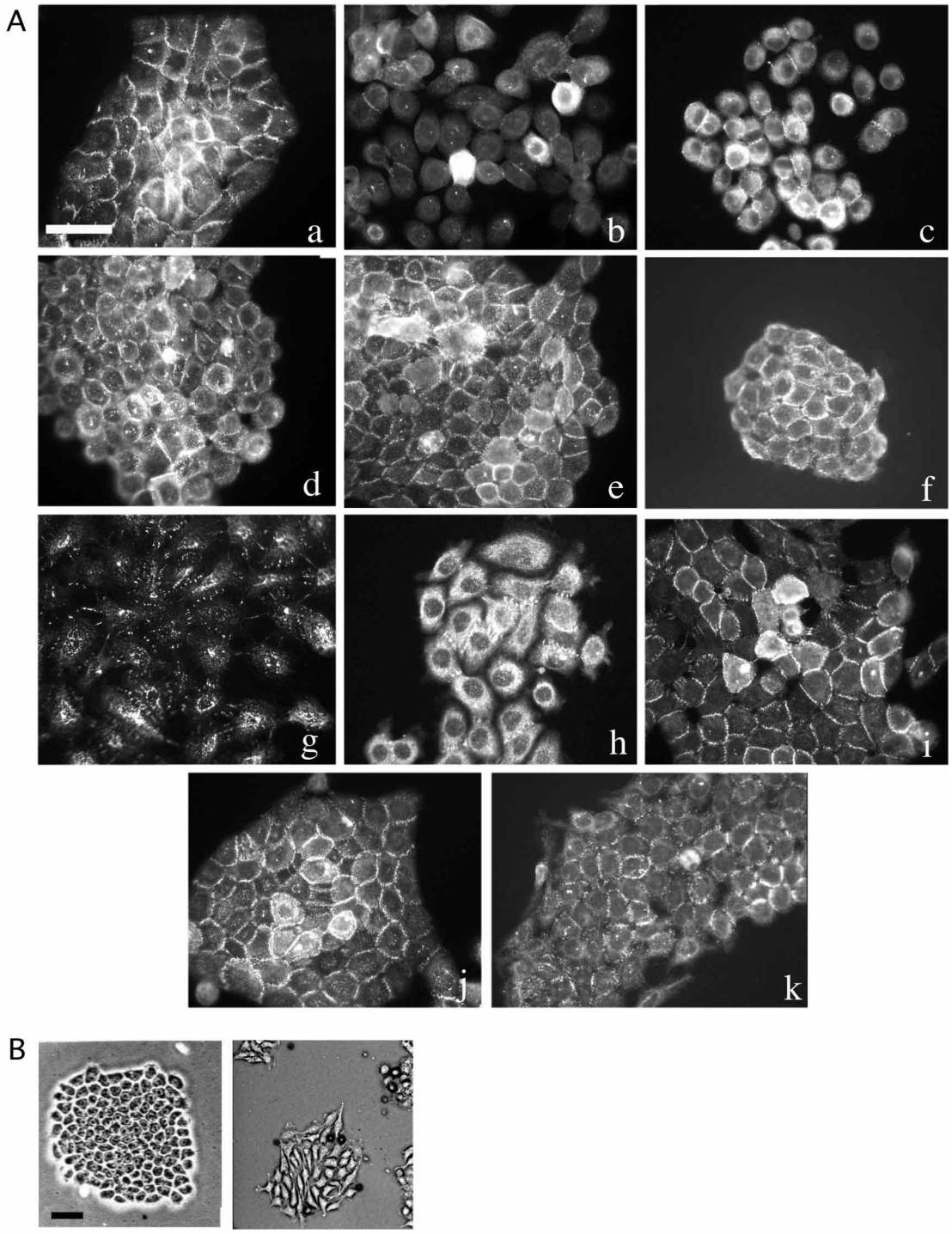

Fig. 2. PD98059 and UO126 inhibit EGF-induced desmosome disruption and promotes desmosome reformation in stable RasV12 transfectants. (A) Control NBT-II cells were cultured without (a) or with 30 ng/ml EGF (b) for 15 hours. Alternatively, 2 days after seeding, cells were incubated with 20 μM of LY80052 (c), 25 μM of FTase2 (d), 100 μM of PD098059 (e) or 100 μM of UO126 (f) for 24 hours before an additional 15 hours incubation with 30 ng/ml of EGF. Cells were fixed and stained for DP expression. Stable RasV12-overexpressing cells (Ras32) (g) were incubated with 20 μM of LY80058 (h), 100 μM of PD098059 (i) or 100 μM of UO126 (j) for 24 hours and 25 μM of FTase2 (k) for 48 hours. (B) Phase contrast micrographs of NBT-II cells (left) and Ras32 cells (right). Note that RasV12 cells exhibit a fibroblastoid phenotype in sharp contrast to the epithelial morphology of the parental NBT-II cells. Bar, 30 μm.

of RasV12 alone was sufficient to induce cell motility, suggesting that Ras was endowed with both cell dissociation- and motility-promoting activities. Whereas MEK1SSDD induced desmosome breakdown in NBT-II cells, its overexpression did not promote cell migration (Fig. 4B,C). This result demonstrates that cell dissociation alone is not sufficient to promote cell motility and suggests that a component of the EGF-induced Ras-dependent transduction machinery distinct from the MEK/MAPK pathway is responsible for inducing cell motility.

In a search for signalling molecules that cooperate with the active MEK/MAPK signal to induce EMT, we examined Rac involvement, as small GTPase proteins of the Rho family have been shown to induce actin cytoskeleton remodeling (Nobes and Hall, 1995; Nobes and Hall, 1999) and have been implicated in processes involving cell dissociation and motility (Potempa and Ridley, 1998). When microinjected into clustered cells, Rac1V12 did not induce cell dissociation in more than 15% of the cells (data not shown), unlike the high rate of cell dissociation induced by MEK1SSDD (60%).

Accordingly, coexpression of MEK1SSDD and Rac1V12 induced 60% of cells to dissociate, a figure similar to that obtained with MEK1SSDD alone. This result confirmed that Rac1 does not contribute to cell dissociation. Cdc42 was also tested and did not promote DP internalization in more than 13% of cells (data not shown). However, cells overexpressing Rac1V12 together with MEK1SSDD exhibited an elongated morphology that was clearly different from that of cells injected with MEK1SSDD alone (Fig. 4A). This result suggested that expression of Rac1 resulted in dramatic changes towards a fibroblast-like phenotype often associated with cell motility. Therefore, we asked whether Rac1 activity could be sufficient to promote cell migration. Coexpression of Rac1V12 with MEK1SSDD in clustered cells or overexpression of Rac1V12 in isolated cells was sufficient to induce cells to migrate at a speed similar to that of RasV12-expressing cells, suggesting that the Ras-mediated scattering activity was fully reproduced by coexpression of active MEK and Rac. Cdc42V12 expression did not promote cell motility of MEK1SSDD-expressing cells or of isolated cells (Fig. 4B,C).

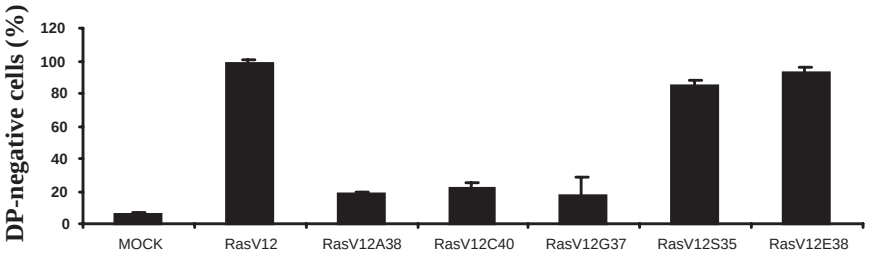

Fig. 3. Raf-activating Ras mutants promote desmosome breakdown in NBT-II cells. Cells seeded on coverslips were microinjected into the nucleus with 100 ng/μl of the indicated expression vectors. 18 hours later, they were fixed and immunostained for both Ras and DP expression as described in the Materials and Methods. For each experiment, several coverslips where analysed and the percentages of desmosome-negative cells were calculated as in Fig. 1B. The results have been averaged for several experiments (n>3) in which at least 100 cells were microinjected. The means and s.e.m. are shown.

We also tested whether activated p110 with or without active RalA could substitute for Rac1V12 in the induction of motility. Coexpression of active p110 with MEK1SSDD did not elicit cell motility (speed of transfected cells: 8.4±5.2 μm/h; speed

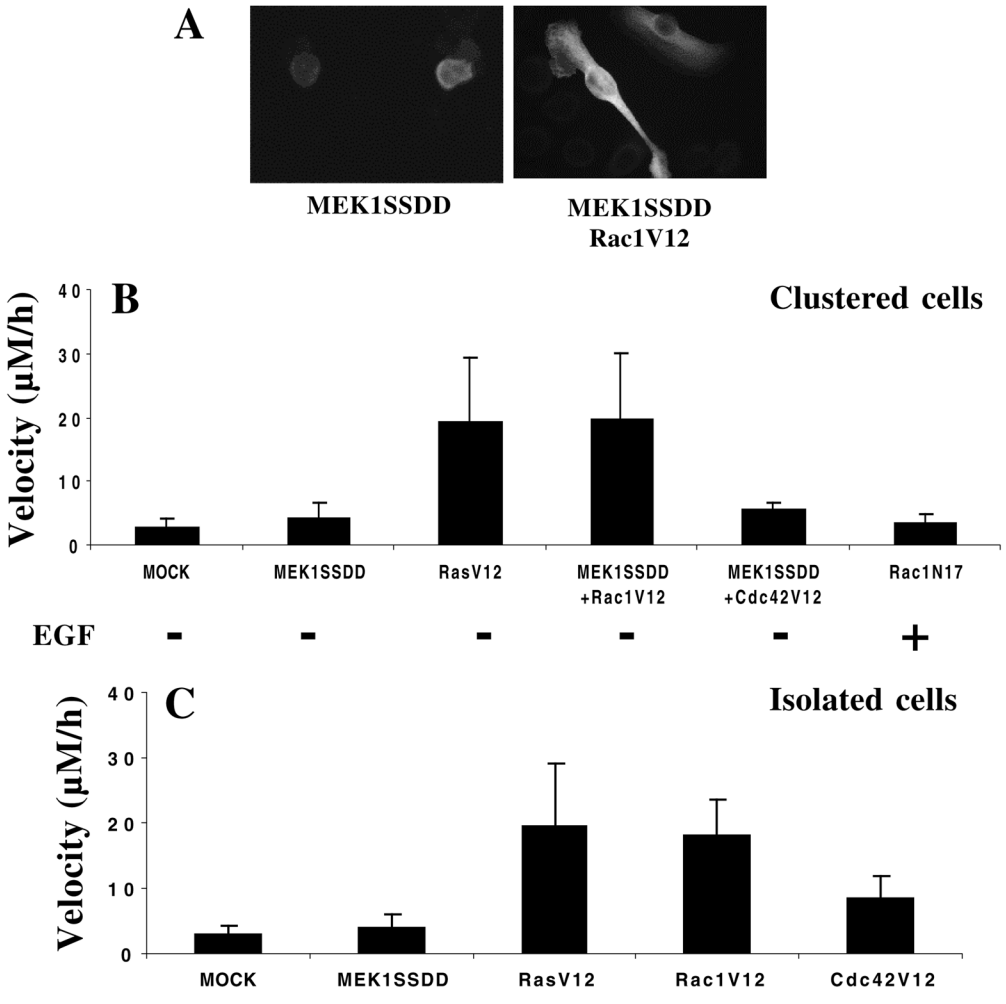

Fig. 4. Rac promotes cell locomotion of MEK1-dissociated cells. (A) Cells were microinjected with 100 ng/μl of the indicated constructs, incubated for 18 hours and then labelled for MEK1 expression. Note that MEK1SSDD-expressing cells retain the round morphology of epithelial NBT-II cells, whereas coexpression of MEK1SSDD and Rac1V12 causes cell spreading and elongation. (B) Cells were microinjected with both 100 ng/μl of the indicated constructs and 25 ng/μl of EGFP encoding construct. After 4 hours of incubation, cells were placed on a Leica motorized microscope connected to a computer using the Metamorph software, and cell motility was recorded by time-lapse videomicroscopy. Velocities were calculated by tracking EGFP-positive cells. Results are expressed as the mean of at least three independent experiments, where at least 40 EGFP-positive cells were recorded±s.e.m. (C) Cells were transfected by the PEI method with the indicated constructs together with the EGPF construct at a 1:4 ratio. 24 hours later, cells were trypsined and re-plated under sparse conditions. Cell migration was recorded 10 hours later as described above. At least three independent experiments were done, and the velocities were averaged for at least 40 cells.

of control cells: 7.2±3.4 μm/h). Moreover, coexpression of active p110 with MEKSSDD and active RalA had no effect on the speed of locomotion of clustered cells (speed of transfected cells: 12.4±3.6 μm/h; speed of control cells: 9.6±3.2 μm/h). Consistently, coexpression of active p110 and active RalA in isolated cells did not promote cell movement (speed of transfected cells: 12.2±5.6 μm/h; speed of control cells: 7.9±4.4 μm/h). Conversely, inhibition of PI3K activity by LY80052 did not block EGF- or Ras-induced migration (data not shown). Altogether, these results suggested that the motility-activating role of Rac was not dependent upon PI3K activity. They also demonstrated that PI3K, alone or in association with RalA, was not endowed with a migration-promoting activity.

Rac is activated under conditions leading to EMT

In order to investigate whether EGF and Ras were able to activate Rac in NBT-II cells, we used a Rac pull-down assay that relies on the capacity of activated Rac to bind to its cellular target PAK2 (p21 activated protein kinase). EGF promoted a robust and sustained activation of Rac, starting 15 minutes after addition of EGF and progressively increasing thereafter (Fig. 5A). For comparison, MAPK activity was induced earlier, starting 5 minutes after addition of EGF, and persisted over time. Moreover, transient overexpression of RasV12 induced Rac activation (Fig. 5B). The potential activation of Rac by the Ras-PI3K pathway was investigated by pull-down assay. Whereas overexpression of Rac1V12 and RasV12 induced a strong Rac activation, the amount of active Rac bound to PAK-GST beads was almost undetectable in cells expressing active p110, suggesting that PI3K is not a good inducer of Rac activation in NBT-II cells (Fig. 5B). This result correlated with the absence of migration of NBT-II cells overexpressing active p110 (Fig. 4B) and suggested furthermore that EGF- or Ras-dependent activation of Rac was not mediated by PI3K activity. Furthermore, the Ras-Raf-MAPK signalling pathway was not likely to be responsible for Rac activation, as active c-Raf and active MEK1 had no effect on Rac binding to PAK-GST (Fig. 5C). Accordingly, MEK and PI3K inhibitors did not inhibit Rac activation induced by EGF or RasV12 (Fig. 5D), thus confirming that Rac activation by EGF or Ras is independent of the MAPK and PI3K pathways in NBT-II cells.

Partial loss-of-function mutants of Ras activate Rac

We also examined whether the partial loss-of-function mutants of Ras trigger cell motility. When overexpressed in NBT-II cell clusters, RasV12S35 and RasV12E38 promoted cell movement, with a speed of locomotion that was not significantly different from that of RasV12-expressing cells. In contrast, neither RasV12C40 nor RasV12G37 mutants were able to elicit cell migration (Fig. 6A). As a control, the RasV12A38 mutant had no effect on cell motility. On the other hand, when coexpressed with MEK1SSDD, which promotes cell dissociation, all Ras mutants were able to induce cell motility (Fig. 6B). Altogether, these data suggested that the partial loss-of-function mutants of Ras were endowed with a motility-promoting function. To test whether this function could be caused by Rac activation, a Rac pull-down assay was done on extracts of NBT-II cells transfected with the various partial loss-of-function mutants of Ras. All of them elicited robust Rac activity, similar to that produced by RasV12 expression (Fig. 6C). This result demonstrated that all partial loss-of-function mutants of Ras were able to activate Rac.

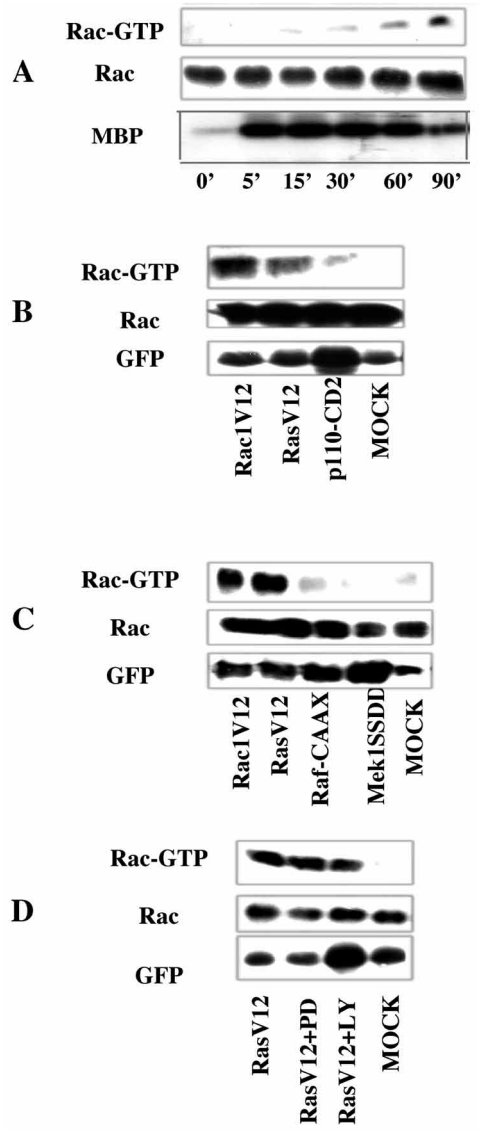

Fig. 5. Rac1 activation by downstream pathways of Ras. (A) NBT-II cells were activated with 30 ng/ml of EGF for the indicated times, then lysed with lysis buffer as indicated in the Materials and Methods. Cell extracts were incubated with GST-PAK2 Sepharose beads, washed and immunoblotted with anti-Rac1 antibodies. Levels of endogenous Rac were estimated by immunoblotting total cell extracts with Rac1 antibodies. Alternatively, equivalent amounts of cell extracts were immunoprecipitated with anti-MAPK antibodies and subjected to a kinase assay using MBP as an exogenous substrate. (B-D) Rac pull-down was done on NBT-II cells transiently transfected with the indicated constructs. The upper panel shows the amounts of Rac bound to PAK-GST beads as revealed by Rac1 antibodies. The middle panel shows the levels of Rac as estimated by Rac immunoblotting on total cell extracts. The lower panel shows the levels of EGFP expression measured by EGFP immunoblotting on total cell extracts with anti-GFP antibodies.

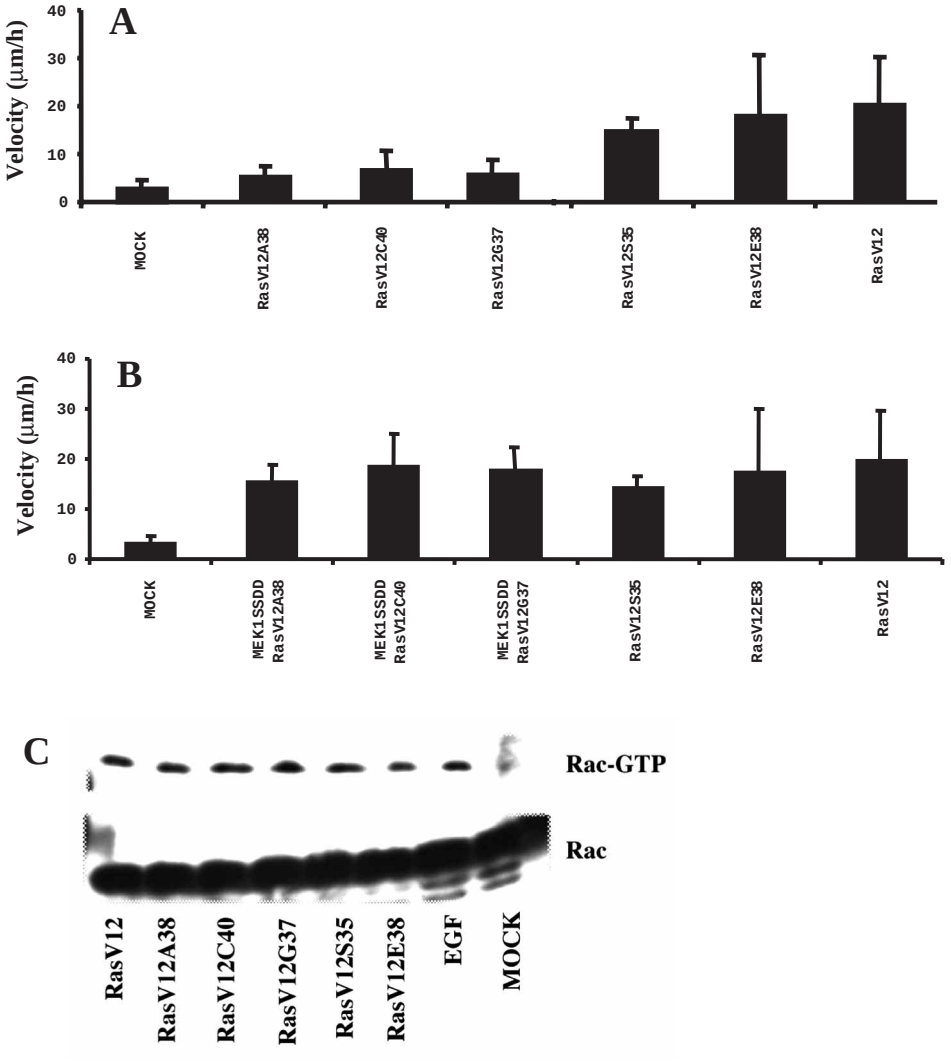

Fig. 6. Raf-specific mutant forms of Ras promote NBT-II cell migration. Two days after seeding, cells were microinjected with 100 ng/μl of the Ras expression vectors and 25 ng/μl of the EGFP construct. Four hours after microinjection, cells were placed on the motorised stage of a Leica inverted microscope and recorded by videomicroscopy. Velocities were obtained by tracking individual EGFP-positive cells. Each column represents the mean of at least three independent experiments in which at least 40 cells were recorded. (A,B) Clusters of NBT-II cells were transiently transfected with the indicated constructs along with the EGFP reporter construct prior to videocinematography recording. Velocities were measured by tracking at least 40 EGFP-positive cells. For each construct, at least three independent experiments were performed. The mean velocity and s.e.m. are shown. (C) The Rac pull-down assay was done on NBT-II cells transiently transfected with the indicated constructs. The upper panel shows a Rac immunoblot performed on cell extracts purified on PAK-GST Sepharose beads. The lower panel shows a Rac immunoblot of total cell extracts.

Discussion

EMT is a complex event that requires coordinate changes in cell physiology. One of the first signs of EMT is the loss of cell-cell adhesive contacts (Boyer et al., 1989). When cells are completely dissociated they start scattering owing to active cell movements. Understanding the cellular mechanisms underlying EMT events therefore requires us to distinguish between the signal implicated in the breakdown of intercellular junctions and those involved in cell motility. We had previously demonstrated that EGF-induced NBT-II cell scattering depends on a Ras signal (Boyer et al., 1997). In this work, we confirmed that expression of an active form of Ras (RasV12) is sufficient to promote the full range of changes that are characteristic of EMT processes. In order to dissect the mechanisms involved in cell dissociation and in acquisition of cell motility, we have examined which effector molecule of the Ras signal is responsible for the cellular changes occurring during EMT. Implication of the Raf/MEK/MAPK pathway in cell dissociation was demonstrated by a full range of concordant data: expression of the partial loss of function mutants RasV12E38 and RasV12S35 and of the constitutively active MEK1 mutant induced respectively 96%, 85% and 60% of cells to dissociate, whereas treatment of EGF-stimulated cells with the MEK1 inhibitors PD098059 and UO126 blocked desmosome breakdown in the vast majority of cells. These data suggested that the signalling pathway initiated by Ras or EGF stimulation induces a Raf/MEK/MAPK activation responsible for desmosome breakdown. Accordingly, HGF-induced scattering of HT29 cells (Herrera, 1998) and of MDCK cells (Potempa and Ridley, 1998) has been shown to be inhibited by PD098059. Also, downregulation of the MAPK pathway with PD098059 and UO126 in Ras-transformed MDCK cells causes reformation of adherens and tight junctions and restoration of the epithelial phenotype (Chen et al., 2000). Epithelial gap junctions are also sensitive to MAPK signals as PDGF-induced disruption of gap junctions can be inhibited by PD098059 (Hossain et al., 1998). These data combined with ours suggest that the MAPK pathway may be directly involved in the regulation of several types of epithelial junctions.

The precise mechanisms whereby the MAPK pathway controls the integrity of intercellular junctions remain elusive. However, the Ras signal has been demonstrated to be upstream of the transcription factor Slug (Boyer et al., 1997), the expression of which has been shown to induce desmosome breakdown in NBT-II cells (Savagner et al., 1997). Nevertheless, a direct link between MAPK activity and

induction of Slug expression remains to be investigated. Disruption of the other types of epithelial junctions is also expected to depend on the ability of the Ras/MAPK pathway to activate the transcription of target genes.

In the NBT-II system of epithelial cell scattering, there is no evidence that other known effectors of Ras contribute to desmosome breakdown and cell dissociation. Several lines of evidence ruled out the involvement of PI3K in the dissolution of cell-cell junctions: microinjection of RasV12C40 and p110 contructs did not lead to desmosome breakdown and, conversely, addition of the PI3K inhibitor LY80052 to EGF-stimulated NBT-II cells or to RasV12-expressing transfectants did not restore desmosomes at the cell surface. Our results are in agreement with recent data showing that MAPK but not PI3K activity is responsible for the disruption of desmosomes and adherens junctions in EGF-treated ovarian cancer cells (Ellerbroek et al., 2001). Apart from PI3K, neither Rac nor RalGDS displayed a desmosome-dissociating activity, as inferred from the fact that microinjection of Rac1V12 or RasV12G37, RalAV23 or RalBV23 did not lead to DP internalization. Therefore, the Raf/MAPK signal appeared as the only downstream effector of Ras capable of dissociating NBT-II cells. However, RasV12 expression induced 100% of cells to dissociate, whereas MEK1SSDD expression led to DP internalization in only 60% of cells. This difference may reveal the hypothetical existence of a cell-dissociating Ras effector distinct from Raf. Alternatively, it may simply result from the greater efficiency of the RasV12 construct, compared with MEK1SSDD.

Even though they were dissociated, MEK1SSDD-expressing NBT-II cells were not induced to migrate. In other models, the role of MEK/MAPK in mediating cell migration is controversial. It is necessary for EGF-induced chemotaxis (Slack et al., 1999), for PDGF-induced migration of RPE epithelial cells (Hinton et al., 1998) and for collagen and fibronectin-induced haptotactic migration of Rat1 fibroblasts (Klemke et al., 1997; Anand-Apte et al., 1997). By contrast, PDGF induction of chemotaxis of Rat1 fibroblasts (Anand-Apte et al., 1997) and EGF stimulation of migration of mouse NR6 cells cannot be mimicked by active MAPK (Xie et al., 1998). The apparent discrepancy between these data may be explained by cell migration not occurring below a certain threshold of MAPK activation (Krueger et al., 2001). In contrast to active MEK1, RasV12 promoted vigorous cell movements. These results suggested that cell dissociation and motility are distinct events that require specific signalling pathways to occur and that RasV12 is likely to activate an effector molecule specifically involved in cell motility.

In a search for a Ras effector that triggers cell motility, we found that overexpression of Rac1V12 was sufficient to activate cell movement, provided that cell-cell contacts were not present at the cell surface. The loss of desmosomes was achieved by either culturing cells under sparse conditions or transiently expressing MEK1SSDD. The involvement of the small GTPase Rac in triggering cell motility has already been reported (Nobes and Hall, 1999; Anand-Apte et al., 1997; Kjoller and Hall, 2001; Bourguignon et al., 2000; Sander et al., 1998). However, the role of Rac in cell motility is still controversial since downregulation of Rac by active Ras leads to EMT (Zondag et al., 2000), and, conversely, Rac activation in NIH3T3 fibroblasts induces an epithelium-like morphology and inhibits cell migration (Sander et al., 1999).

In the NBT-II cell system, it is not possible to test whether Rac acts alone or cooperates with the MEK/MAPK pathway to induce cell motility. In COS-7 cells, Rac potentiates the C-Raf-induced cell motility (Leng et al., 1999) by promoting the formation of membrane ruffles, whereas MEK is known to affect the actin-myosin system (Klemke et al., 1997) and Rho-dependent actin stress fiber formation (Sahai et al., 2001). Coordinated activation of MEK1 and Rac1 has also been reported to reproduce the proliferative effect of oncogenic Ras (Cobellis et al., 1998). In NBT-II cells, Rac was strongly activated by EGF or Ras signalling. Interestingly, all Ras partial loss-of-function mutants activated Rac and reproduced the effect of active Rac on NBT-cell motility. These results suggested that the effector molecule that triggers Rac activation does not contact the GTP-bound Ras at conserved residues in the 'effector loop'. Accordingly, a systematic study of contact sites between small GTP-binding proteins and partner proteins has revealed the presence of binding interactions outside the effector loop (Corbett and Alber, 2001). The existence of Ras effectors that use this new type of binding strategy may be linked to specific functions.

In contrast to what has been described in previous reports, the molecule mediating Rac activation is not likely to be PI3K (Keely et al., 1997; Buhl et al., 1995; Nobes et al., 1995), as active p110 did not induce Rac activity and did not mimick Rac activation of NBT-II cell motility. Moreover, LY80052 did not decrease Rac activation elicited by RasV12 or EGF and did not interfere with RasV12-induced cell motility. Rac may be activated in a Ras-dependent manner by Rac exchange factors such as Tiam (Hordijk et al., 1997; Bourguignon et al., 2000) or Vav2 (Liu and Burridge, 2000). Tiam/Rac signalling is dependent on extracellular matrix components (Sander et al., 1998), whereas Vav2-mediated activation of Rac1 is dependent on growth factor stimulation (Liu and Burridge, 2000). For that reason, it is reasonable to speculate that EGF- and Ras-induced activation of Rac1 in NBT-II cells is mediated by Vav2. Since Tiam1 but not Vav2 has been demonstrated to be downstream from PI3K (Sander et al., 1998), this could explain why PI3K is not involved in EGF-stimulated cell motility in NBT-II cells. However, this hypothesis awaits further evidence.

We are grateful to A. M. Vallés and R. Moore for critical reading of the manuscript. We also thank J. B. Sibarita and J. De Mey for help with the Metamorph system. This work was supported by the Centre National de la Recherche Scientifique (CNRS), the Association pour la Recherche contre le Cancer (ARC), Astra Zeneca Pharma S. A. and the Ligue Française contre le Cancer. N.E. was supported by doctoral fellowship from the Ministère de l'Enseignement Supérieur et de la Recherche.

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