

Reversal of the Ras-induced Transformed Phenotype by HR12, a Novel Ras Farnesylation Inhibitor, Is Mediated by the Mek/Erk Pathway

Hadas Reuveni,* Tamar Geiger,* Benjamin Geiger,‡ and Alexander Levitzki*

*Department of Biological Chemistry, The Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel; and ‡Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel

Abstract. We have used the selective farnesylation inhibitor HR12 [cysteine-*N*(methyl)valine-*N*(cyclohexyl)glycine-methionine-*O*-methyl-ester] to study the role of oncogenic Ras in cytoskeletal reorganization in Ha-ras^{V12}-transformed Rat1 cells (Rat1/ras). Application of HR12 resulted in complete restoration of the cytoskeleton and associated cell adhesions disrupted by oncogenic Ras. This included an increase in the number and size of focal adhesions, accompanied by massive stress fiber formation and enhanced tyrosine phosphorylation. Furthermore, HR12 induced assembly of adherens junctions and dramatically elevated the level of the junctional components, cadherin and β -catenin. HR12 was unable to restore the nontransformed phenotype in cells expressing farnesylation-independent, myristylated Ras. Examination of the main Ras-regulated signaling pathways revealed that HR12 induced a dose- and time-dependent

decline in Erk1&2 activation ($t_{1/2} \sim 6$ h), which correlated with the accumulation of nonfarnesylated oncogenic-Ras. Inhibition of the Mek/Erk pathway in Rat1/ras cells, using the Mek inhibitor, PD98059, resulted in complete cytoskeletal recovery, indistinguishable from that induced by HR12. Moreover, a constitutively active Mek mimicked the effect of *ras* transformation in Rat1 cells, and prevented HR12-induced cytoskeletal effects in Rat1/ras cells. No such effects were observed after treatment of Rat1/ras cells with the phosphatidylinositol 3-kinase inhibitor LY294002. These findings establish the Mek/Erk pathway as the dominant pathway involved in conferring the cytoskeletal and junctional manifestations of the Ras-induced transformed phenotype.

Key words: adherens junctions • farnesyltransferase • Ras • Erk • cytoskeleton

Introduction

Activating mutations in *ras* genes are found in $\sim 30\%$ of all human cancers, especially in colon and pancreatic carcinomas (50 and 90%, respectively) (Bos, 1989; Der, 1989). Oncogenic Ras is important in tumor initiation as well as in tumor maintenance (Chin et al., 1999). The pivotal role of Ras in human cancer has received attention from both molecular oncologists and drug designers. As the transforming activity of oncogenic Ras is dependent on its anchorage to the plasma membrane (Hancock et al., 1989; Jackson et al., 1990), prevention of Ras translocation to the membrane was established as a strategy for inhibition of its biochemical and pathological activities (Gibbs et al., 1994). Ras attachment to the membrane is dictated through its COOH-terminal CAAX sequence, which undergoes three post-translational modifications (Zhang and Casey, 1996). The first modification is farnesylation, in which a farnesyl (C15 isoprenoid) moiety is covalently attached to the cysteine residue of the CAAX motif. After farnesylation, AAX

residues are cleaved and Ras undergoes COOH-terminal methyl esterification. The farnesylated Ras proteins also use other anchoring signals to enhance their attachment to the membrane; e.g., palmitoylation of an upstream cysteine or existence of a polybasic sequence (Hancock et al., 1990). Since farnesylation is obligatory for Ras oncogenicity (Kato et al., 1992), farnesyltransferase inhibitors (FTIs)¹ were sought as a strategy to block Ras-mediated signal transduction and Ras-induced tumorigenesis. Indeed, FTIs have been shown to block Ras attachment to the membrane and to reverse Ras-dependent transformation and suppress anchorage-independent cell growth. Furthermore, using xenograft or transgenic mouse models, FTIs were shown to prevent tumor growth and elicit tumor regression, in the absence of detectable toxic side effects (reviewed in Prendergast, 2000). We have recently reported on a novel FTI, HR12 [cysteine-*N*(methyl)valine-*N*(cyclohexyl)glycine-methionine-*O*-methyl-ester], which is selec-

Address correspondence to Alexander Levitzki, Department of Biological Chemistry, The Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel. Tel.: 972-2-6585404. Fax: 972-2-6512958. E-mail: levitzki@vms.huji.ac.il

¹Abbreviations used in this paper: Erk, extracellular-signal regulated kinase; FTI, farnesyltransferase inhibitor; Jnk, Jun NH₂-terminal kinase; MAPK, mitogen activated protein kinase; Mek, MAPK kinase; PI-3'kinase, phosphatidylinositol 3-kinase; PKB, protein kinase-B/c-Akt.

tive and potent (Reuveni et al., 1997). Here, we show for the first time the biochemical effects of HR12, and its biological effects on cell-adhesion and cytoskeletal reorganization in *ras*-transformed cells.

Transformed cells often show altered patterns of cytoskeletal protein expression, and commonly display a disorganized actin cytoskeleton. This phenotype is associated with the poor adhesiveness of transformed cells, their enhanced motility, and ability to grow in an anchorage-independent fashion (Hunter, 1997; Behrens, 1999; Christofori and Semb, 1999). In particular, transformation of cells by constitutively activated Ras results in the loss of adherens junctions and stress fibers (Izawa et al., 1998; Potempa and Ridley, 1998). Application of FTI to *ras*-transformed fibroblasts was reported to induce stress-fiber formation and increase cell spreading (Prendergast et al., 1994). In the present study, we show a major and pleiotropic phenotypic reversion of Rat1/*ras* cells induced by HR12, including: (a) a dramatic increase in the stress fiber organization, (b) similar increases in focal contact formation and tyrosine phosphorylation, (c) increases in the levels of cadherin and β -catenin, and (d) assembly of cadherin- and catenin-rich adherens junctions. Both cell-matrix and cell-cell adhesions play important roles in growth control (St. Croix et al., 1998; Levenberg et al., 1999) and tumorigenesis (Perl et al., 1998; Christofori and Semb, 1999). In this report, FTI is shown, for the first time, to induce a marked increase in cadherin and β -catenin levels and recovery of adherens junctions, suggesting a new mechanism for FTI-mediated phenotypic reversion and growth inhibition.

We show that the extracellular-signal regulated kinase (Erk) pathways is inhibited by HR12 treatment. We further show that the inhibition of mitogen-activated protein kinase (MAPK) kinase (Mek) induces morphological reversion of Rat1/*ras* cells indistinguishable from that of HR12. The expression of constitutively active Mek in Rat1/*ras* cells prevents HR12-induced cytoskeletal recovery, suggesting that the Mek/Erk pathway plays a major role in the Ras-induced oncogenic phenotype.

Materials and Methods

Materials

HR12 was synthesized, followed by semipreparative RPHPLC to >90% purity, as described (Reuveni et al., 1997). The molecular weight of the pure product was determined by mass spectroscopy (518 D). Its k' value was 5.2, and its residue configuration was verified (CAT). PD98059, LY294002, and SB203580 were obtained from Calbiochem. MAPKK1(Δ N3) cDNA was a generous gift from N.G. Ahn (University of California at San Diego, La Jolla, CA). Dominant-negative SEK (SEK-AL) cDNA was generously obtained from B. Zanke (University of Toronto, Toronto, Ontario, Canada).

Antibodies

Immunocytochemistry. Anti- β -catenin and anti-paxillin mAb were from Transduction Laboratories (dilution 1:20 in PBS), anti-vinculin and anti-phosphotyrosine (PT-66) mAb were from Sigma-Aldrich (dilution 1:20 and 1:30 in PBS). Secondary antibody Cy3-conjugated goat anti-mouse IgG was from Jackson ImmunoResearch Laboratories (dilution 1:80).

Immunoblotting. Anti-pan-cadherin mAb (CH-19; dilution 1:2,000) and anti-diphospho(Thr183/Tyr185) ERK-1&2 mAb (dilution 1:10,000) were from Sigma-Aldrich. Polyclonal anti-ERK-2 (C-14; dilution 1:10,000), polyclonal anti-p38 (C-20; dilution 1:2,000), polyclonal anti-Akt1/2 (H-136; dilution 1:1,000), polyclonal anti-Jnk1 (C-17; dilution 1:2,000) were from Transduction Laboratories. Polyclonal anti-diphospho(Thr180/

Tyr182)-p38 MAPK (dilution 1:500), polyclonal antiphospho(Thr308)-Akt (dilution 1:1,000), and polyclonal anti-diphospho(Thr183/Tyr185)-SAPK/Jnk (dilution 1:500) were from New England Biolabs, Inc. Anti-Ras antibody was produced from Hybridoma Y13-259. HRP-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (dilution 1:10,000).

Cell Culture and Transfection

Rat1 fibroblasts were obtained from M. Oren (Weizmann Institute, Rehovot, Israel). Ha-*ras*^{V12}-transformed Rat1 (Rat1/*ras*) cells were constructed in our laboratory by J. Axelrod, who transformed Rat1 cells with EJ Ras 6.6 DNA from R. Weinberg (Whitehead Institute, Cambridge, MA). NIH3T3, Ha-*ras*^{V12}-transformed NIH3T3 (NIH3T3v-*ras*) and myristoylated-*ras*-transformed NIH3T3 (NIH3T3myr-*ras*) were obtained from D.R. Lowy (National Cancer Institute, Bethesda, MD). All cells were grown in DME supplemented with 10% FCS (Bet-Haemek). All treatments described in this report were performed in DME containing 10% FCS. Transient transfection of Rat1/*ras* and Rat1 cells was performed by electroporation as follows. 5×10^6 cells were suspended in 0.3 ml of serum-free medium and mixed with 5–7 μ g of plasmid expressing MAPKK1(Δ N3) or SEK-AL and 5 μ g plasmid expressing GFP, at room temperature, in a 0.4 cm cuvette (Bio-Rad Laboratories). The mixture was electroporated at 220 V, 960 μ F and, after a 5-min incubation, the transfected cells were introduced into DME containing 10% FCS and seeded onto coverslips.

Immunostaining

Cells were plated on coverslips in DME containing 10% FCS and maintained at 37°C with 5% CO₂, 24 h after seeding, the medium was replaced with fresh medium containing 20 μ M HR12, 50 μ M PD98059, 10 μ M LY294002, 10 μ M SB203580, or 0.05% DMSO (veh). The medium with the inhibitor was replaced every 24 h where applicable. Fixation was performed at 37°C, as follows. Cells were washed once with PBS, fixed, and permeabilized in a solution containing 3% paraformaldehyde, 50 mM MES buffer, pH 6, 0.5% Triton X-100, and 5 mM CaCl₂, for 30 s, followed by a 1-h incubation in the same solution without Triton X-100. The fixed cells were incubated with specific antibodies at the appropriate dilutions (see above) for 30 min at room temperature. To stain actin, the fixed cells were incubated with TRITC-conjugated phalloidin (Sigma-Aldrich; dilution 1:500 in PBS). After staining, coverslips were mounted in GEL/MOUNT™ (Biomedica Corp.) or Elvanol.

Fluorescent images were recorded with an Axiophot microscope (Carl Zeiss, Inc.) equipped for fluorescence using $\times 66/1.4$ or $\times 100/1.3$ objectives.

Immunoblotting

For detecting adhesion molecule levels and phospho-enzyme status, cells were lysed in sample buffer containing 50 mM TRIS-HCl, pH 6.8, 5% 2-mercaptoethanol, 3% sodium dodecyl sulfate, 0.5 mg/ml bromophenol-blue and phosphatase inhibitors, followed by a 10-min incubation at 100°C. Detergent-soluble and -insoluble fractions were obtained by lysing the attached cells on the plate in Triton X-100 buffer (50 mM Mes, pH 6.0, 0.5% Triton X-100, 2.5 mM EGTA, 5 mM magnesium acetate, 0.4 mM AEBSEF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM Na₂VO₄, 20 mM NaF, 5 mM sodium pyrophosphate) with gentle agitation for 2 min at room temperature. The lysates were collected, centrifuged for 2 min at 20,000 g, and the supernatants were saved (Triton-soluble fractions). The remaining material on the plate was washed and dissolved in sample buffer to give the Triton X-100-insoluble fraction. Aliquots of cell extracts containing equal amounts of protein were resolved by 10% SDS-PAGE and electroblotted onto nitrocellulose filters. The membranes were blocked with TBS-tween containing 5% low-fat milk (blocking solution), incubated with primary antibodies overnight at 4°C, and then with HRP-conjugated secondary antibodies for 75 min at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence.

Results

HR12 Blocks the Effects of *ras* Transformation on Cell-Matrix Adhesion, Cell-Cell Contacts, and Actin Reorganization in Rat1 Cells

We examined the effect of the Ras-FTI, HR12, on Rat1/*ras* cells (fibroblasts stably transformed with Ha-*ras*^{V12}).

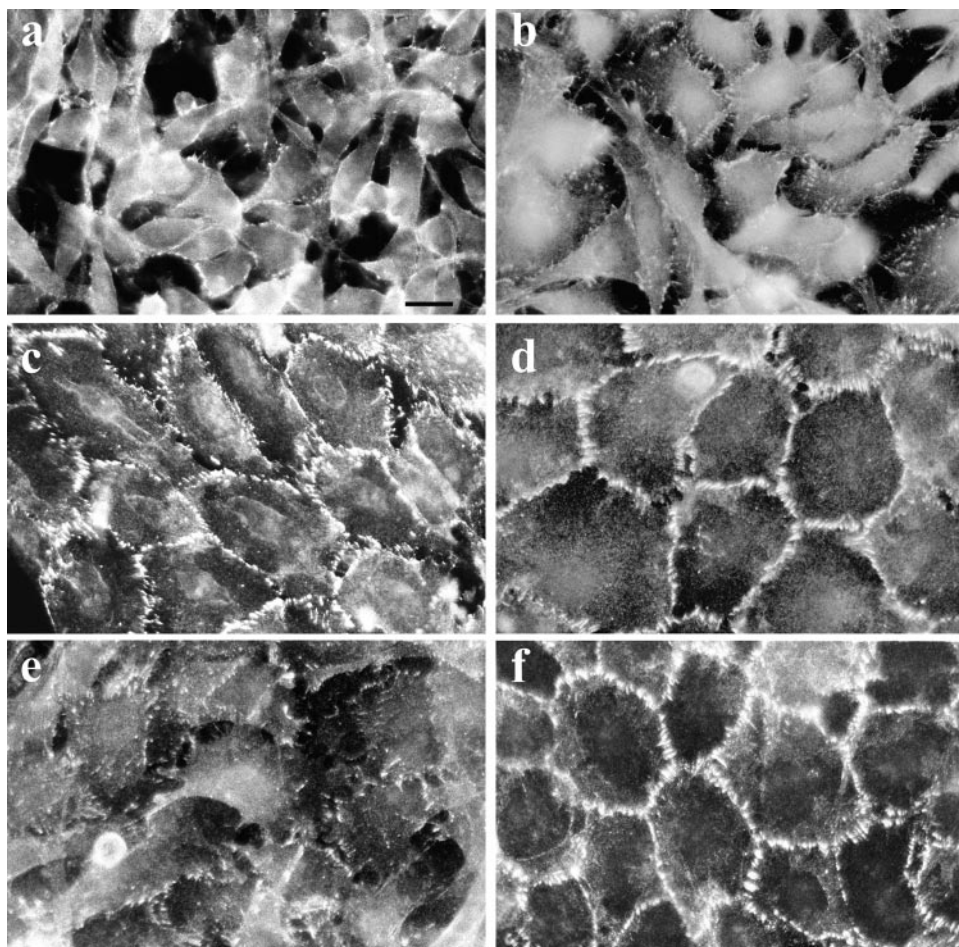


Figure 1. HR12 treatment induces adherens junction organization in *ras*-transformed Rat1 cells. Rat1/*ras* cells grown in medium containing 10% FCS were exposed to 20 μ M HR12, fixed, and stained with anti- β -catenin antibody and Cy3-conjugated anti-mouse antibody. (a) No treatment; (b–d) time course of exposure to HR12: (b) 24 h, (c) 48 h, and (d) 72 h. (e) Cells were exposed to HR12 for 48 h, washed, and incubated in medium containing 10% FCS 24 h longer, before fixation. (f) Untransformed Rat1 cells are shown for comparison. Bar, 10 μ m.

Figs. 1–3 show a comparison of nontransformed Rat1 cells, Rat1/*ras* cells, and HR12-treated Rat1/*ras* cells (48 h, 20 μ M). Transformation of Rat1 cells with activated Ha-*ras* resulted in the disruption of β -catenin and vinculin-containing cell–cell contacts (Figs. 1 and 3). Stress fibers, labeled by TRITC-phalloidin, were also disrupted (Fig. 2). Cell–matrix adhesions, observed by labeling paxillin, phospho-tyrosine, and vinculin, were lost (Fig. 3).

The effects of HR12 were seen within 24 h of treatment (Fig. 1 b), as manifested by considerable cell spreading and the acquisition of a more epithelial morphology. These effects were evident with as little as 1 μ M HR12 (data not shown). At 24 h, β -catenin distribution was still largely diffuse, with occasional dot-like cell–cell adhesions (Fig. 1 b), but, by 48 h, most of the β -catenin was associated with extensive arrays of adherens junctions (Fig. 1 c) and the diffuse cytoplasmic labeling was markedly reduced. By 72 h, the junctions were labeled even more extensively (Fig. 1 d), and were essentially indistinguishable from those of control Rat1 cells (Fig. 1 f). Upon removal of HR12 for 24 h (after a 48-h incubation with the drug), cell–cell adhesion markedly deteriorated, leaving only a few sporadic junctions (Fig. 1 e).

In Rat1 cells, F-actin was engaged as a dense web of conspicuous stress-fibers (Fig. 2 f). These actin bundles were assembled into either parallel arrays terminating in cell–substrate focal contacts or circumferential arrays running along the intercellular junctions. This filament network was disrupted in Rat1/*ras* cells. Rat1/*ras* cells

appeared elongated, with numerous F-actin-containing protrusions and ruffles, but essentially no actin bundles (Fig. 2 a). Within 24 h in the presence of HR12, the cells flattened, developing parallel arrays of large bundles. At 48 h and later, circumferential bundles also became apparent (Fig. 2, c and d). Removal of HR12 for 24 h, after a 48-h treatment, resulted in the loss of organized actin bundles and the appearance of a more diffuse pattern (Fig. 2 e).

Immunofluorescence labeling with anti-paxillin, anti-phosphotyrosine, and anti-vinculin showed that Rat1 cells contained prominent focal adhesions (Fig. 3). Rat1/*ras* cells had far fewer and smaller focal adhesions and diffuse staining patterns. The addition of HR12 to Rat1/*ras* cells for 48 h or longer resulted in complete restoration of focal adhesions, as defined by labeling with the three antibodies. In fact, often the labeling patterns obtained in HR12-treated cells were more extensive than those found in untreated Rat1 controls.

In parallel to monitoring the localization and assembly of the adhesion molecules, we measured their levels in the cells using quantitative immunoblotting. Rat1/*ras* cells were treated with HR12 at various concentrations for 48 h, and then lysed in sample buffer. Cell lysates were then subjected to immunoblotting with antibodies to β -catenin, pan-cadherin, and phosphotyrosine. Fig. 4 shows an HR12 dose-dependent increase in β -catenin and cadherin levels. The antibody against phosphotyrosine (PY20) recognizes two major bands (125 and 135 kD), whose intensities increase 5- and 10-fold respectively. The lower band corre-

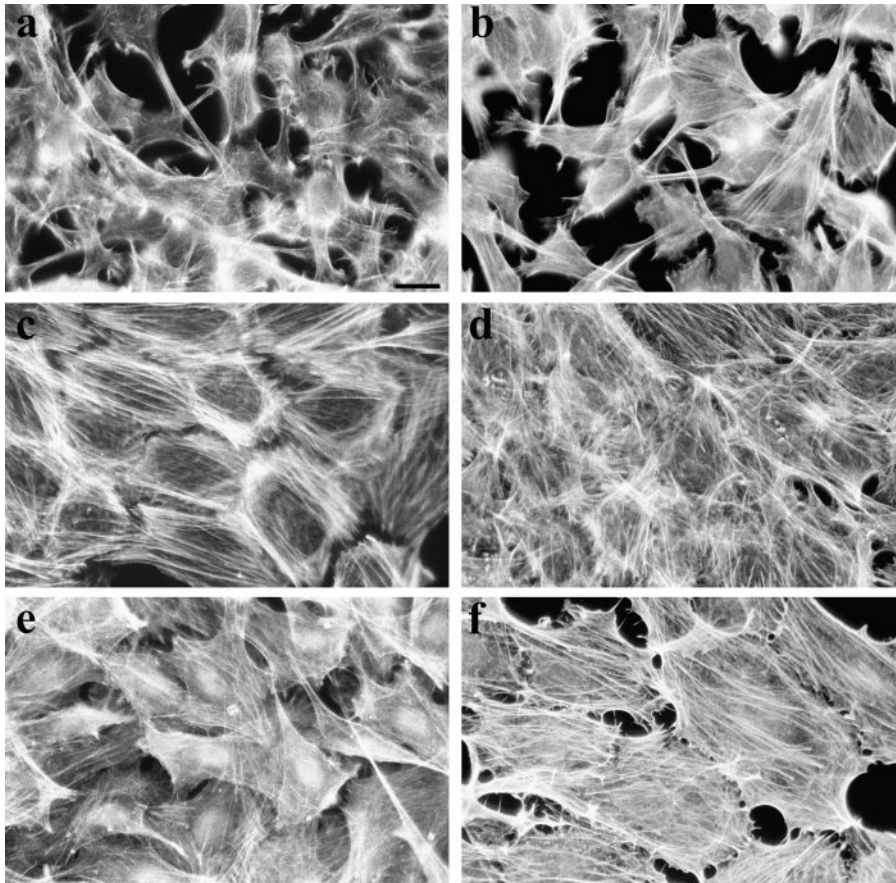


Figure 2. HR12-treatment induces stress-fiber formation in Rat1/ras cells. Rat1/ras cells were treated as in Fig. 1 and stained with TRITC-labeled phalloidin. (a) No treatment, (b) 24 h, (c) 48 h, (d) 72 h, and (e) 48 h exposure to HR12, followed by 24 h without the inhibitor. (f) Untransformed Rat1 cells. Bar, 10 μm .

lates well with the band recognized by anti-focal-adhesion-kinase antibody (data not shown). This is in agreement with the immunofluorescence data, which showed intense staining of focal adhesions in Rat1/ras cells treated with HR12, using antiphosphotyrosine antibody (Fig. 3).

Actin exists in a dynamic equilibrium between a Triton-soluble pool and Triton-insoluble cytoskeletal filaments. Fig. 2 shows that HR12 triggers dramatic assembly of actin into stress fiber networks in Rat1/ras cells. In parallel, we show the translocation of actin from the Triton-soluble into the Triton-insoluble fraction of Rat1/ras cells treated with increasing concentrations of HR12 (Fig. 4, bottom).

HR12 Treatment of Rat1/ras Cells Inhibits Ras Processing and Erk Phosphorylation

We next examined the effects of HR12 on the pathways downstream to Ras, which might be responsible for the morphological changes induced by HR12. These cascades include MAPK pathways and the PI-3'kinase pathway. Rat1/ras cells treated with HR12 for 48 h were lysed and immunoblotted with anti-Ras antibody. Unprocessed Ras migrates more slowly than its processed form in SDS-PAGE. Fig. 5 shows the shift of Ras towards its unprocessed form with increasing concentrations of HR12, with an IC_{50} of 1 μM . The same blots were reacted with antibodies recognizing the activated forms of MAPKs (anti-double-phosphorylated Erk, Jnk, or p38), or the activated form of PKB (antiphospho-Thr308-Akt). The total level of each enzyme was determined using antibodies that recognize both phosphorylated and nonphosphorylated forms of the enzyme. Enzyme activation was quantified by deter-

mining the ratio between the level of the phosphorylated enzyme and its total level. HR12 induced a dose-dependent decrease in Erk and Jnk activation and a dose-dependent increase in p38 and PKB activation (Fig. 5).

The inhibition of Erk1&2 phosphorylation induced by HR12 exhibited an IC_{50} value of 1 μM (Fig. 5), comparable with the IC_{50} for the inhibition of Ras processing (Fig. 5). The high apparent level of phosphorylation of Erk2 in untreated Rat1/ras cells was probably due to the very low expression of Erk2 in these cells. We noticed that the Erk2 level was inversely correlated with its activation state (Figs. 5 and 6). Comparison of the phosphorylation state of Erk1&2 in nontransformed Rat1 cells to that in Rat1/ras cells, showed that transformation with active Ras dramatically induced Erk1&2 phosphorylation (5- and 35-fold, respectively), while treatment with HR12 brought Erk-phosphorylation back to the level that exists in Rat1 cells.

The kinetics of the decline in Erk phosphorylation in response to HR12 treatment corresponded to the kinetics of the increase in the levels of nonfarnesylated Ras (Fig. 6). For the first 15 h of HR12 treatment, there was a gradual decrease in Erk activation, which closely correlated with the inhibition of Ras farnesylation by HR12. At 15 h of HR12 treatment, most of the Ras population was unprocessed and the inhibitory effect on Erk phosphorylation was maximal. Unprocessed Ras continued to accumulate after 15 h of treatment with HR12. However, the phosphorylation level of Erk was already at its lowest value after 15 h of exposure. The amount of active cytosolic Ras accumulated at 15 h would appear to be sufficient to titrate the Raf molecules in the cell, thus achieving the maximal in-

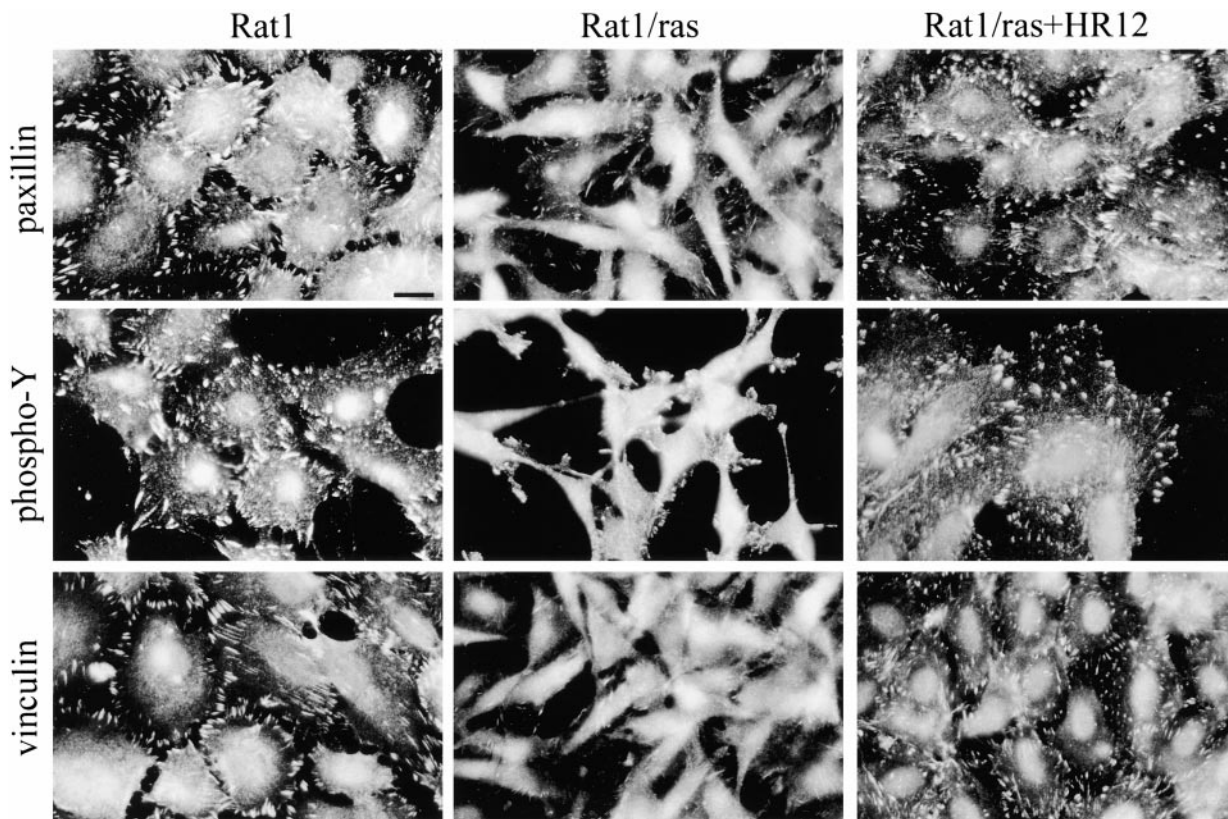


Figure 3. HR12 leads to morphological reversion of *ras*-transformed cells and increases dramatically cell–matrix adhesion and cell–cell contacts. Rat1 and Rat1/ras cells are compared with Rat1/ras cells that were exposed to 20 μ M HR12 for 48 h. The fixed cells were stained with anti–paxillin, anti–vinculin, or antiphosphotyrosine mAb, followed by Cy3-conjugated anti–mouse antibody. Bar, 10 μ m.

hibitory effect on Mek activation (Lerner et al., 1995). In summary, the Raf/Mek/Erk pathway seems to be dramatically affected by HR12.

Mek Inhibition by PD98059 Causes Rat1/ras Morphological Reversion, Induction of Cell–Cell and Cell–Matrix Adhesion and Stress Fiber Formation

To test whether the Mek/Erk pathway mediates the Ras effect on the cytoskeleton and cell adhesion, we specifically inhibited the Mek/Erk cascade. Fig. 7 shows that a 24-h incubation with the Mek inhibitor, PD98059, induced phenotypic reversion almost indistinguishable from that induced by HR12. The cells became spread and flattened. β -catenin-containing adherens junctions were assembled, stress fibers were formed, and vinculin-labeled cell–matrix contacts were reestablished. The morphology of Rat1/ras cells treated with either HR12 or PD98059 was indistinguishable from that of parental Rat1 cells. These findings suggest that the Ras–Mek–Erk pathway plays a pivotal role in the regulation of the cytoskeleton and the cell adhesions.

Activated Mek1 Disrupts Adherens Junctions and Stress Fiber Formation

To further characterize the role of the Ras/Mek/Erk pathway in the regulation of adherens junctions, cell–matrix contacts, and stress fibers, we examined the morphological consequences of the expression of an activated form of Mek1 in Rat1/ras treated with HR12 or in untreated Rat1 cells. Rat1/ras and Rat1 cells were transiently cotrans-

fectured with plasmids encoding GFP and constitutively active MEK [MAPKK1(Δ N3)] (Mansour et al., 1994). 24 h later, Rat1/ras cells were treated with 20 μ M HR12 for 48 h. Cells were then fixed and stained with anti– β -catenin or phalloidin (Fig. 8). Examination of the transfected cells (identified by GFP expression) revealed a loss or reduction in β -catenin-containing adherens junctions. This effect was most prominent when the neighboring cells were transfected as well (Fig. 8). Labeling of actin indicated that MAPKK1(Δ N3)-transfected cells lost stress fibers (compared with nontransfected neighboring cells). These effects were not observed when the empty vector was transfected into the cells. Thus, overexpressing activated Mek itself is sufficient to disrupt cell–cell contacts and stress fibers.

The Effect of HR12 on PI-3'kinase and PKB

We used the selective inhibitor of PI-3'kinase, LY294002, to examine the role, if any, of PI-3'kinase in transducing the Ras effect on cell adhesion and stress-fiber formation. Rat1/ras cells were exposed to 10 μ M LY294002 for 48 h in DME containing 10% FCS, fixed, and stained with anti– β -catenin, anti–vinculin, and TRITC-phalloidin, as described in Materials and Methods. LY294002 treatment was found to have no effect on cell–cell contact, cell–matrix adhesion, or stress-fiber formation in Rat1/ras cells (Fig. 7). Surprisingly, PKB, a well-established downstream target of PI-3'kinase (Alessi and Cohen, 1998; Kandel and Hay, 1999), was activated after HR12 treatment of Rat1/ras cells, rather than inhibited (Fig. 5). The elevation in

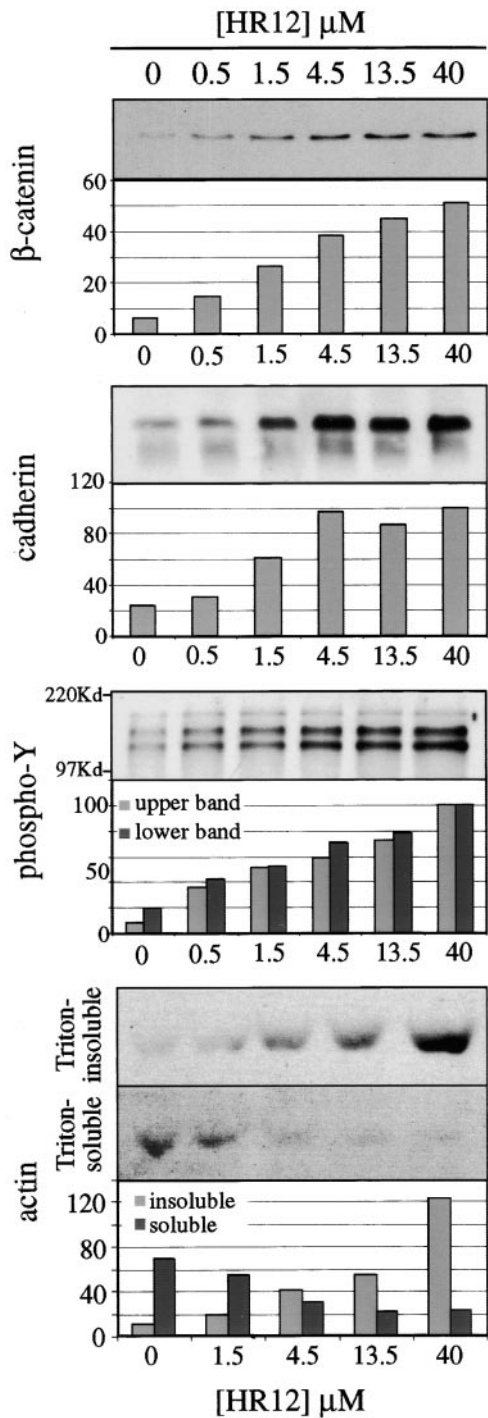


Figure 4. HR12 treatment of Rat1/ras cells causes elevation of adhesion molecule levels in a dose-dependent manner. Whole-cell lysates were immunoblotted with anti- β -catenin or anti-pan-cadherin or antiphosphotyrosine (pY20) mAb. Actin levels in the Triton X-100 insoluble and soluble fractions were detected by polyclonal anti-actin antibody. The levels of β -catenin, cadherin, phosphotyrosine, and actin were quantified using the NIH-IMAGE 1.61 program.

PKB phosphorylation might be induced by the cadherin- or integrin-mediated signaling pathways as a secondary effect of HR12 (see Discussion). Our findings suggest that PKB phosphorylation might be induced by the cadherin- or integrin-mediated signaling pathways as a secondary effect of HR12 (see Discussion). Our findings suggest that PI-3'kinase does not play a role in the organization of adherens junctions, cell-matrix contacts, or stress fibers induced by HR12 in Rat1/ras cells.

Differential Effect of HR12 on Jnk and p38

We also examined the possible role of the two other MAPK pathways in mediating Ras signaling to the cytoskeleton. Fig. 5 shows that Jnk phosphorylation is inhibited by HR12 in a dose-dependent manner. Jnk phosphorylation in Rat1 cells is lower (2.5-fold) than in Rat1/ras cells. The difference between Erk phosphorylation in Rat1 versus Rat1/ras cells was much more striking (see above). The relevance of Jnk inhibition to the morphological effects of HR12 was examined using a plasmid-expressing dominant-negative SEK (SEK-AL) (Zanke et al., 1996a,b). Sek is the direct activator of Jnk. To detect transfected cells, we cotransfected the SEK-AL plasmid with a plasmid expressing GFP. Adherens junctions were labeled using anti- β -catenin antibodies, and stress fibers were visualized by TRITC-phalloidin. No effect of the dominant-interfering SEK on adherens junctions and stress-fiber formation was detected (data not shown).

In many biological systems, and in particular in fibroblasts grown in culture, p38 and Jnk are often coactivated. Yet we saw a differential effect of HR12 on these two pathways. As seen in Fig. 5, p38 phosphorylation levels increased up to 10-fold in Rat1/ras cells treated with HR12, whereas Jnk phosphorylation levels dropped, as discussed above. Phosphorylation of p38 in Rat1 cells was fivefold stronger than in Rat1/ras cells. The HR12-induced increase in p38 phosphorylation in Rat1/ras cells was dose dependent: at 4.5 μ M HR12, the p38 phosphorylation level was similar to that in nontransformed Rat1 cells. To examine the possible relevance of p38 activation to the changes in the morphological features induced by HR12, we tested whether SB203580, an inhibitor of p38 activity, antagonizes the effect of HR12 on actin reorganization. When Rat1/ras cells were treated with 20 μ M HR12 and 10 μ M SB203580, or Rat1 cells with 10 μ M SB203580, some interference with stress fiber formation was noted (data not shown). Since the specificity of the inhibitor SB203580 is controversial, the question of the relevance of p38 remains unresolved (see Discussion).

Phenotypic Reversion Induced by HR12 Results from Ras Inhibition Rather than Inhibition of Other Farnesylated Proteins

To test whether the cytoskeletal effects of HR12 described here were due to the inhibition of Ras farnesylation, rather than inhibition of some other protein(s), we investigated the effect of HR12 on cells transformed with myristoylated oncogenic-ras (myr-ras). The anchorage of myristoylated-ras does not depend on farnesyltransferase activity. The morphology of fibroblasts transformed by myr-ras is therefore expected to not be affected by HR12, if the phenomena described result exclu-

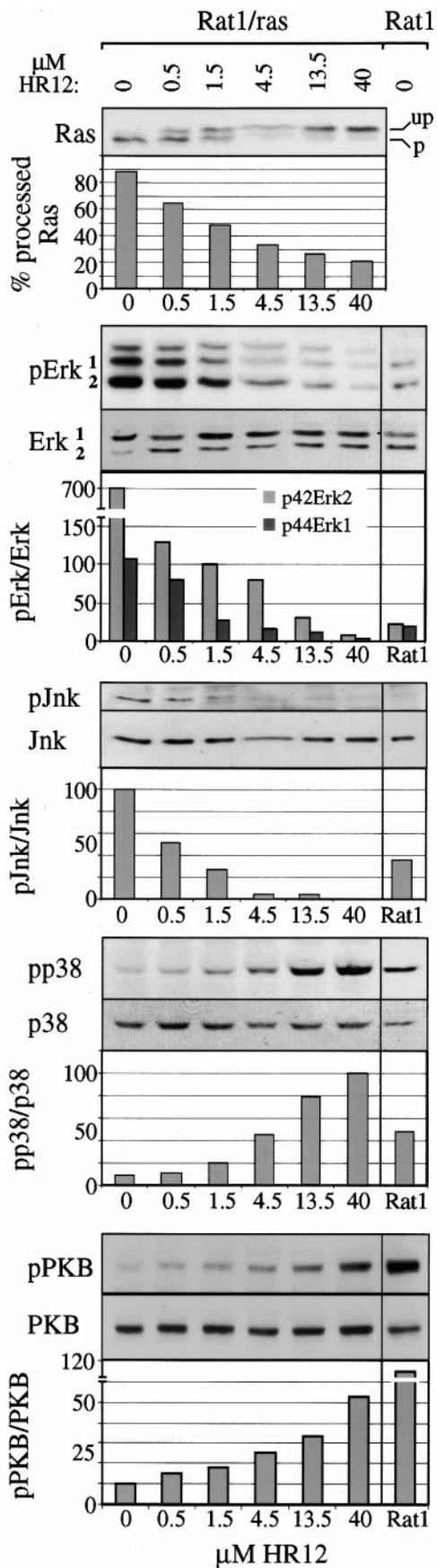


Figure 5. HR12 treatment of Rat1/ras cells inhibits Ras processing and affects the phosphorylation levels of MAPKs and PKB in a dose-dependent manner. Rat1/ras cells were exposed to the indi-

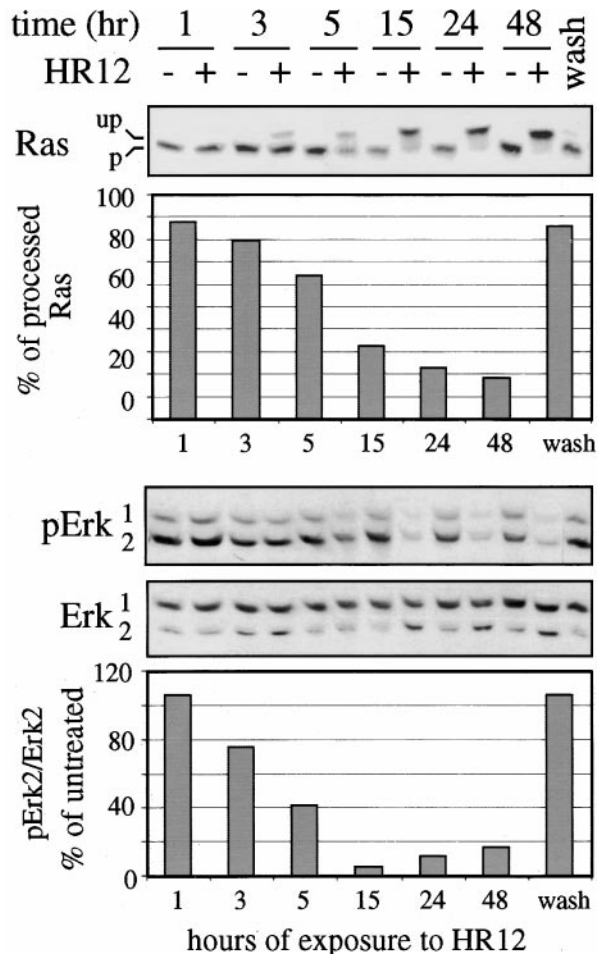


Figure 6. Correlation between the inhibition of Ras processing and inactivation of Erk. Rat1/ras cells grown in DME containing 10% FCS were treated with 20 μ M HR12 for the indicated time periods, or exposed to 20 μ M HR12 for 48 h, washed, and incubated without the inhibitor 24 h longer, before lysis (wash). Lysates were immunoblotted with anti-Ras, antiphosphorylated Erk (pErk), and anti-Erk2 (Erk) antibodies.

sively from the inhibition of Ras farnesylation. If, however, other farnesylated proteins downstream of Ras; e.g., RhoB (Lebowitz and Prendergast, 1998), are responsible for the phenotype, one would expect HR12 to be inhibitory. We treated v-Ha-ras- and myr-ras-transformed NIH3T3 fibroblasts with 20 μ M HR12 for 48 h. Cells were stained with anti-vinculin antibody. HR12 treatment restored focal contacts in NIH3T3v-ras, but had no effect on vinculin distribution in NIH3T3myr-ras (Fig. 9 a).

Since our findings point to the Mek/Erk pathway as a transducer of the Ras effect on the cell morphology and

cated concentrations of HR12 for 48 h, lysed, and immunoblotted with anti-Ras to allow dose-dependent inhibition of Ras processing. (up) Unprocessed Ras, (p) processed Ras. In parallel, blots were labeled with antiphosphorylated Erk (pErk), anti-Erk2 (Erk), antiphosphorylated-p38 (pp38), anti-p38 (p38), antiphospho-Jnk (pJnk), anti-Jnk (Jnk), antiphospho-PKB/Akt (pPKB), and anti-PKB/Akt (PKB). In each case, the level of phosphoenzyme was normalized to the level of total enzyme. For comparison, phosphoenzyme levels in Rat1 cells are shown (right lane).

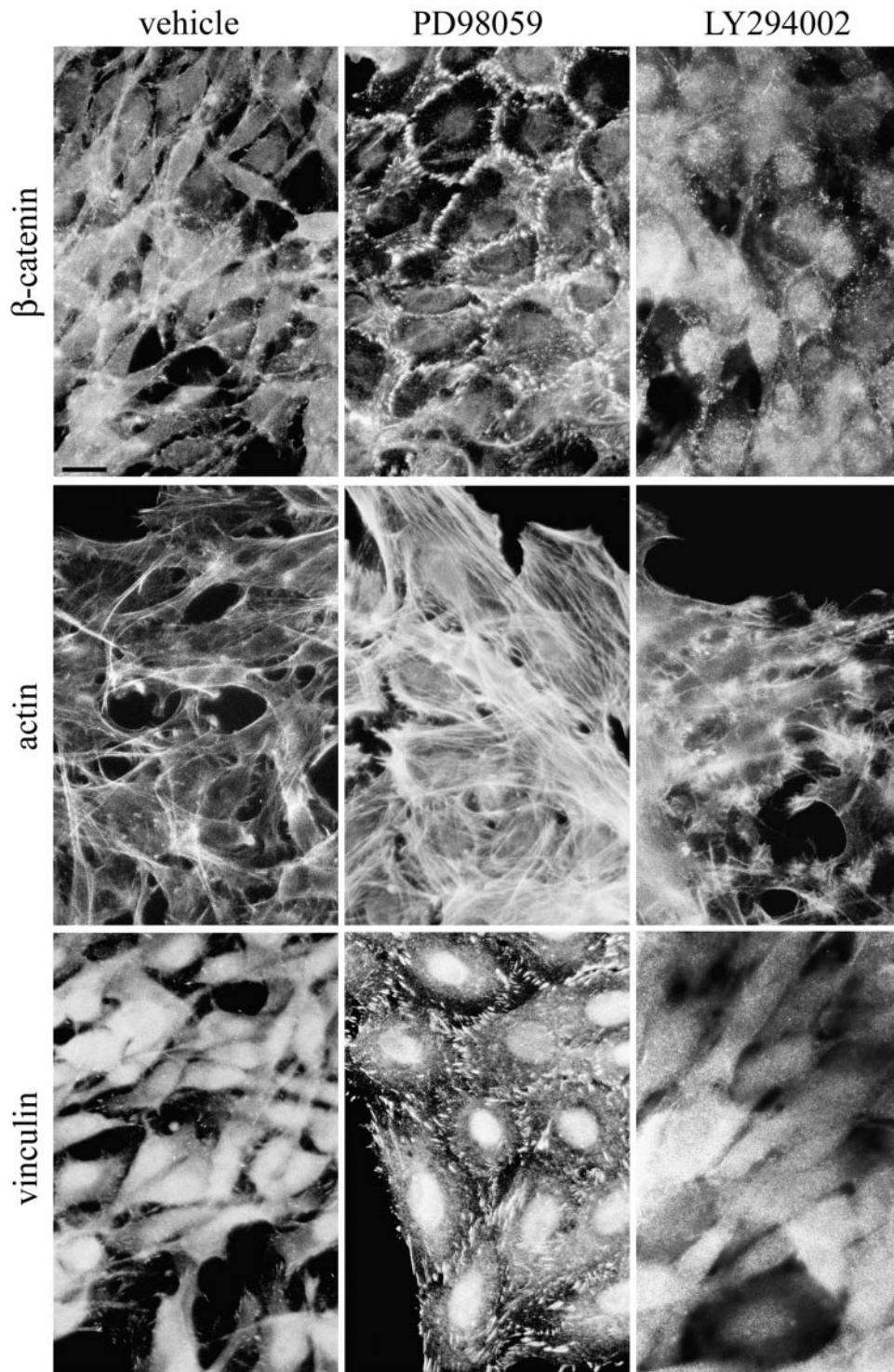


Figure 7. Inhibition of Mek/Erk pathway by PD98059 causes morphological reversion of ras-transformed Rat1 cells. Rat1/ras grown in DME containing 10% FCS were treated for 24 h with 50 μ M PD98059 or 0.05% DMSO (veh), and fixed. β -Catenin, actin, or vinculin were labeled as described in Materials and Methods. Bar, 10 μ m.

adhesion, we also examined ERK phosphorylation levels in these cells. Cell lysates from both cell types, with and without HR12 treatment, were immunoblotted with antiphospho-Erk antibody. Blots were then stripped and reacted with anti-Erk2 antibody. HR12 inhibited Erk phosphorylation in NIH3T3v-ras, but did not inhibit Erk phosphorylation in NIH3T3myr-ras (Fig. 9 b). Both cell lines exhibited enhanced Erk phosphorylation, compared with the parental nontransformed NIH3T3.

Discussion

Among the most striking manifestations of the transformed phenotype are the severe disruption of the actin cytoskeleton and the loss of cell–matrix and cell–cell adhesions (Behrens et al., 1992; Hunter, 1997). These cellular changes contribute directly to the anaplastic appearance of tumors in vivo and to their deregulated growth, invasive properties, and metastatic potential (Birchmeier et al., 1993; Christofori and Semb, 1999). It is therefore impor-

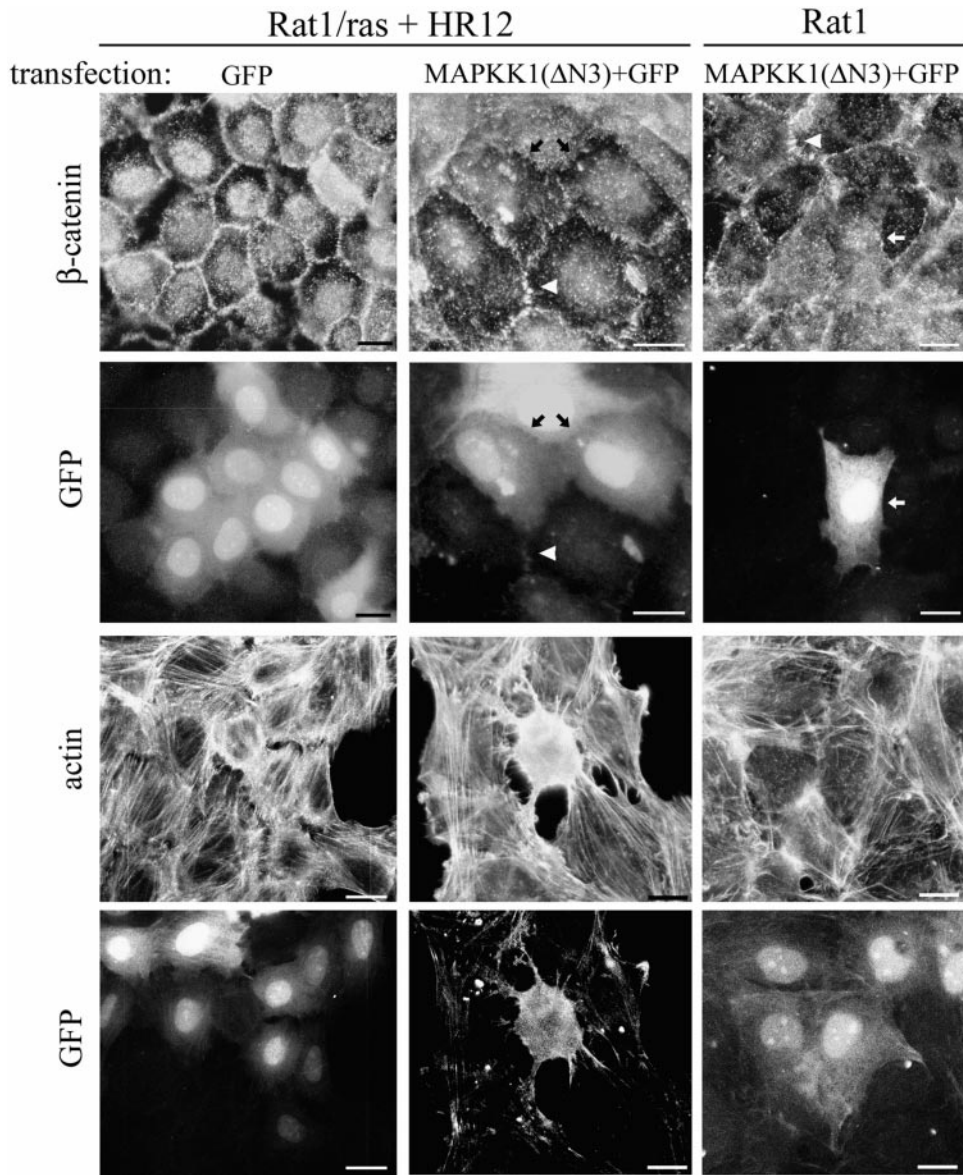


Figure 8. Activated Mek disrupts adherens junctions and stress fibers. Rat1/ras (center) and Rat1 (right) cells were transiently cotransfected with vectors carrying GFP and activated MEK [MAPKK1(Δ N3)]. 24 h after transfection, the Rat1/ras cells were treated with 20 μ M HR12 for 48 h. Cells were fixed and stained with anti- β -catenin or phalloidin. In cells that showed GFP fluorescence, indicating that they had been transfected, stress fibers and adherens junctions were disrupted. Arrowheads indicate β -catenin-labeled adherens junctions in the nontransfected cells, while arrows indicate their loss in the MAPKK1(Δ N3)-transfected cells. In Rat1/ras cells transfected with the GFP-vector alone and treated with HR12 (left), the stress fibers and adherens junctions remained intact. Bar, 10 μ m.

tant to determine the molecular mechanisms responsible for these cellular changes.

Oncogenic *ras*, which is one of the most powerful oncogenes, is frequently associated with human cancer and has been shown to dramatically affect the cytoskeleton. Activated Ras affects actin organization (Lombardi et al., 1990; Moustakas and Stournaras, 1999), assembly of focal adhesions, and the formation of adherens junctions (Izawa et al., 1998; Hegland et al., 1999) and tight junctions (Yamamoto et al., 1997; Chen et al., 2000a). Farnesyltransferase inhibitors have been shown to induce morphological reversion of *ras*-transformed fibroblasts (Rat1/ras), and to restore stress-fiber formation (Prendergast et al., 1994). Here we show, for the first time, that application of a novel FTI, HR12 (Reuveni et al., 1997), to Rat1/ras cells induces complete recovery of cell–cell adherens junctions, with a concomitant rise in the levels of the junctional protein components, cadherin and β -catenin. Furthermore, HR12 induces assembly of vinculin, paxillin, and phosphotyrosine-containing focal contacts, and restores normal stress fibers. HR12 also inhib-

its potentially the invasive growth of Rat1/ras cells (data not shown). Loss of functional cell–cell junction complexes is a critical step in the progression of cancer from noninvasive to invasive (Birchmeier et al., 1995; Christofori and Semb, 1999), and is detected in the progress of most human cancers (Behrens, 1999). Moreover, it has been shown that E-cadherin plays a role in the suppression of tumor invasion in cultured cells and in mouse models (Christofori and Semb, 1999). E-cadherin also functions in the suppression of cell growth (St. Croix et al., 1998; Levenberg et al., 1999), consistent with our unpublished observations that HR12 inhibits growth of Rat1/ras cells, with minimal effects on non-transformed Rat1 cells. We propose that the elevation in cadherin levels and recovery of adherens junctions induced by HR12 may be the mechanism whereby FTI suppresses invasion and induces growth arrest.

Being a pivotal regulator of many signaling pathways, Ras could, in principle, exert its cytoskeletal and junctional effects via several distinct pathways. In this study, we have examined the effect of HR12 on the main path-

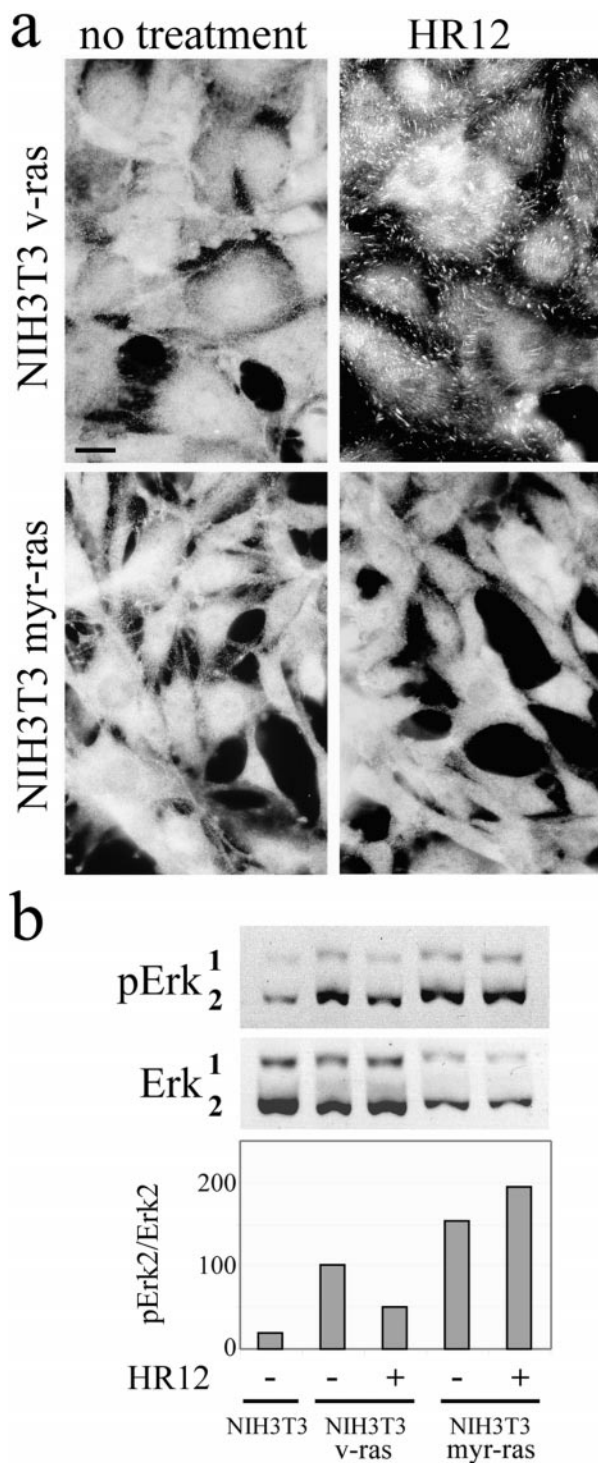


Figure 9. Phenotypic reversion by HR12 depends on inhibition of Ras function. (a) HR12 treatment increases cell adhesion of *v-ras*-transformed NIH3T3 cells, but not of myristoylated-*ras*-transformed cells. After a 48-h treatment with 20 μ M HR12 in DME + 10% FCS, the cells were fixed and stained with anti-vinculin mAb followed by Cy3-conjugated anti-mouse antibody. Bar, 10 μ m. (b) Erk phosphorylation is inhibited by HR12 in NIH3T3v-ras, but not in NIH3T3myr-ras. Cell lysates were immunoblotted with antiphosphorylated-Erk mAb (pErk). The blot was stripped and reacted with polyclonal anti-Erk2 antibody. Phospho-Erk levels were normalized to total Erk levels.

ways downstream of Ras, namely the MAP-kinase cascades and the PI-3'kinase pathway. To assign which of these pathways mediates the effects of Ras on the cytoskeleton and cell adhesions, we examined the effects of selective inhibitors.

The Role of the Ras to Erk Pathway

Our results point to the Mek/Erk pathway as the main route mediating the recovery of adherens junctions, focal adhesion loci, and stress fibers induced by Ras inhibition in Rat1/ras cells. Several lines of evidence support this notion. First, using Ha-*ras*-transformed Rat1 fibroblasts, we show that inhibition of Ras farnesylation by the farnesyltransferase inhibitor HR12 results in a dose-dependent decrease in Erk activation, which parallels the dose-dependent inhibition of Ras farnesylation (Fig. 5) and the morphological changes (data not shown). Second, the kinetics of soluble Ras accumulation induced by HR12 treatment and the kinetics of Mek/Erk inactivation are identical (Fig. 6) and precede the observed morphological changes. The Mek inhibition can most probably be accounted for by the titration of Raf by the soluble, unprocessed oncogenic-Ras protein (Lerner et al., 1995). Third, the HR12-induced morphological reversion of Rat1/ras cells to the untransformed phenotype characteristic of the parental Rat1 cells is fully mimicked by the selective Mek inhibitor PD98059 (Fig. 7). We show identical effects of HR12 and PD98059 treatments on the reformation of cell-cell contacts, cell-matrix adhesions, and actin reorganization (Fig. 7). Finally, the finding that a constitutively activated form of Mek, MAPKK1(Δ N3), induces disruption of adherens junctions and reduction of stress-fiber content in nontransformed Rat1 cells as well as in Rat1/ras cells treated with HR12 (Fig. 8), strongly supports the notion that the Ras to Erk pathway is a major pathway through which morphological transformation occurs in these cells. Further support comes from a recent report showing that inhibition of Erk activity in PC12 cells and in *ras*-transformed MDCK cells resulted in increased synthesis of cadherin and β -catenin (Lu et al., 1998; Chen et al., 2000a). Taken together, these findings suggest that the level of adherens junction proteins is regulated by the Ras to Erk pathway in a variety of cell types. The Ras to Erk pathway also seems to be involved in stress-fiber formation. Although it was reported that stress-fiber formation is regulated by Rho (Ridley and Hall, 1992; Zohn et al., 1998), we and others (Reszka et al., 1997; Plattner et al., 1999) find the Mek/Erk pathway to be the major regulator of these structures. Moreover, the Mek/Erk pathway is reported to have an essential role in metastasis and morphogenesis in epithelial cells (Montesano et al., 1999; Sebolt-Leopold et al., 1999). In particular, of the known Ras effectors, only Raf was able to induce metastasis (Webb et al., 1998). The molecular mechanism by which Raf mediates tumor invasion and metastasis may involve the key role of the Raf/Mek/Erk pathway in the regulation of cell adhesion and stress-fiber formation.

The mechanism by which Mek or Erk delivers the signal to the cytoskeletal components is still unknown. We detect initial actin cytoskeleton reorganization \sim 6 h after Mek is inhibited, and full recovery is attained within 15 h of treat-

ment with PD98059. The establishment of adherens junctions, however, has a delayed time course. It is first detected after 10 h of treatment and complete structures develop 19 h after Mek is inhibited (data not shown). These relatively slow time courses suggest that the induction of these structures is not delivered by a direct signal, but rather involves a novel gene expression induced by the inhibition of the Ras to Erk pathway. The increases in the levels of the junction components, β -catenin and cadherin, subsequent to HR12 treatment (Fig. 4) support this notion. One of the known substrates of Erk, which might serve as a potential mediator of Erk signaling to the cytoskeleton, is the transcription factor Ets-1/2 (McCarthy et al., 1997). Recent reports have shown that expression of dominant-negative Ets leads to increased spreading of the cell, cytoskeletal organization, and enhanced cell-matrix adhesion (Mattot et al., 2000), as does Erk inhibition in our case. Whether Erk signals directly by phosphorylating cytoskeletal and junctional components, or indirectly by controlling their protein levels through transcription, translation, or degradation remains to be seen.

The Role of Jnk and p38

Jnk activation in Rat1/ras cells is much lower than Erk1&2 activation, but is inhibited in a dose-dependent manner by HR12 (Fig. 5). Since the overexpression of SEK-AL, a dominant negative Jnk activator, has no attenuating effect (data not shown), we believe that the involvement of Jnk is minimal or nonexistent. Although Jnk and p38 are often coactivated, we find that treatment of Rat1/ras cells by HR12 results in a dose-dependent increase in the activity of p38 (Fig. 5). Application of the selective p38 kinase inhibitor SB203580 partially antagonizes the effect of HR12 (data not shown). Since SB203580 was reported recently to activate Raf-1 (Hall-Jackson et al., 1999; Kalmes et al., 1999), and since we show that blocking Mek activity in Rat1/ras cells is sufficient to induce stress fiber formation (Fig. 7), the effect of SB203580 may also be accounted for by reactivation of the Mek/Erk pathway downstream of Raf-1. However, Hall-Jackson et al. (1999) and Kalmes et al. (1999) saw no stimulation of Mek or Erk, although Raf-1 was activated in cells treated with 10 or 25 μ M SB203580. Another side effect of SB203580, reported recently, is inhibition of PKB phosphorylation (Lali et al., 2000), which we found also in Rat1/ras cells (data not shown). We find, however, that PKB activation has no role in the HR12 effect on the cytoskeleton (Fig. 7), so this inhibition seems irrelevant. A few reports suggest that p38 activation is indeed required for actin reorganization, probably through activation of MAPKAP-kinase-2, a p38 substrate. MAPKAP-kinase-2 in turn phosphorylates HSP27, a modulator of actin polymerization (Huot et al., 1998; Matsumoto et al., 1999; Ono and Han, 2000). In particular, stress-fiber formation was reported to be a consequence of enhanced actin polymerization induced by p38 activation and of assembly of focal adhesions induced by FAK phosphorylation (Rousseau et al., 2000).

We find that p38 is also activated by treatment of Rat1/ras cells with the Mek inhibitor PD98059 (data not shown), suggesting that the activation of p38 lies downstream to Mek inhibition, as suggested also by others (Berra et al.,

1998). To further discriminate between the effects of SB203580 on Raf activation and p38 inhibition, and to explore the potential role for p38 in the pathway leading to stress-fiber induction, we examined whether SB203580 antagonizes the effects of PD98059. An antagonistic effect would rule out Raf activation as the mediator of the SB203580 effect, since Mek is not active in the presence of PD98059. We observed that in the presence of SB203580 the content of stress fibers induced by PD98059 or HR12 is reduced (data not shown), thus the activation of p38, induced by the inhibition of the Ras-Erk pathway, probably does play a role in the morphological reversion induced by HR12. This interesting nuance deserves further attention.

No Role for PI-3'Kinase

PI-3'kinase was reported to be a Ras effector (Rodriguez-Viciana et al., 1994, 1996), mediating signals from Ras to the actin cytoskeleton (Rodriguez-Viciana et al., 1997) and to adherens junctions (Potempa and Ridley, 1998) in a variety of cell lines. If the Ras effects studied here are also transmitted via PI-3'kinase, we would expect the PI-3'kinase inhibitor LY294002 to mimic the effects of HR12. Our finding that LY294002 has no effect on stress fibers and junctions in Rat1/ras cells (Fig. 7), implies that the putative PI-3'kinase inhibition, due to inhibition of Ras farnesylation, cannot account for the effects of HR12 on the cytoskeleton and adhesion.

In fact, application of HR12 to Rat1/ras cells for 48 h results in dose-dependent activation of PKB (Fig. 5), a well-established downstream target of PI-3'kinase (Alessi and Cohen, 1998; Kandel and Hay, 1999). We examined whether PKB activation is responsible for the observed cytoskeletal recovery. In this case, we would expect inhibition of PI-3'kinase/PKB pathway by LY294002 to oppose the effects of HR12. We treated Rat1/ras cells with a combination of HR12 and LY294002, but the effects of HR12 on stress-fiber formation, adherens junction, and focal adhesion assembly were not counteracted by LY294002 (data not shown). Moreover, we observed no activation of PKB during short exposures to HR12 (up to 20 h, data not shown), implying that the phosphorylation of PKB is not a direct consequence of HR12 treatment. We further wondered whether glycogen synthase kinase 3 (GSK3), whose activity is known to be repressed by PKB (Cross et al., 1995; van Weeren et al., 1998), could mediate the HR12-induced increase in β -catenin levels, as activation of GSK3 is known to lead to β -catenin degradation (Ben-Ze'ev and Geiger, 1998). But treatment of Rat1/ras cells with HR12 did not cause increased phosphorylation of GSK3 (data not shown), and GSK3 phosphorylation levels were similar in Rat1/ras and Rat1 cells (data not shown). Thus it seems that signaling pathways emanating from Ras to PI-3'kinase, including PKB, are not involved in the HR12-induced morphological reversion. We believe that the increase in PKB phosphorylation is a secondary effect of the cytoskeletal recovery induced by HR12. The induction of PKB phosphorylation parallels that of cytoskeletal recovery, supporting this contention. Integrin-linked kinase (ILK) and cadherin are both known to be involved in activation of PKB (Delcommenne et al., 1998; Pece et al., 1999). ILK activity may be

reinstated once the integrin fabric is restored due to HR12 treatment, and we have shown (Fig. 1) that cadherin was activated upon HR12 treatment. Thus, both ILK and cadherin are likely to be responsible for the PKB phosphorylation detected in our study. The observed HR12-induced increase in β -catenin levels is probably mediated by PI-3'kinase-independent activation of cadherin (Sadot et al., 1998).

Is the HR12 Effect Mediated Solely by Ras Inhibition?

In this report, we used the FTI, HR12, as a tool for Ras inhibition. A series of studies using other FTIs, L-739,749 (Prendergast et al., 1994; Lebowitz et al., 1995) and L-744,832 (Du et al., 1999), suggests that the cytoskeletal events induced by FTI are not mediated by the inhibition of Ras farnesylation. Rather, these studies suggest that the reduction in the proportion of the farnesylated-RhoB, and the consequent increase in the proportion of geranylgeranylated-RhoB, lead to the morphological effects of their FTIs in Rat1/ras cells (Lebowitz and Prendergast, 1998; Du et al., 1999; Prendergast, 2000). The main arguments in favor of the RhoB theory are: (a) the half life of farnesylated Ras is 24 h, which is too long to mediate the morphological reversion induced by their FTI (18–24 h; Prendergast et al., 1994); (b) NIH3T3myr-ras cells, which are not dependent on farnesylation for Ras function, were sensitive to their FTI in an assay for anchorage-independent growth (Lebowitz et al., 1995); and (c) there is insufficient accumulation of soluble oncogenic Ras to interfere with the functioning of the prenylated Ras (Prendergast et al., 1994). Our data do not support this hypothesis, but rather suggest that inhibition of Ras farnesylation is the main mechanism for the morphological reversion and adhesion changes induced by HR12 in Rat1/ras cells. Moreover, it has recently been reported that both farnesylated and geranylgeranylated-RhoB inhibit transformation and tumorigenesis (Chen et al., 2000b). In contrast to the reports cited above in support of the RhoB theory, we show here: (a) by 15 h, most of Ras population is unprocessed (Fig. 6), which corresponds to the kinetics of the morphological changes (Figs. 1 and 2); (b) NIH3T3myr-ras cells fail to form adhesions in response to HR12, unlike NIH3T3 cells transformed by farnesylation-dependent oncogenic *ras* (Fig. 9); and (c) HR12 treatment leads to the accumulation of high levels of oncogenic Ras in the cytoplasm, followed by potent inhibition of Mek/Erk activation (Figs. 5 and 6). Lerner et al. (1995) have shown that the accumulation of inactive Ras–Raf complexes in the cytoplasm leads to the shut off of the Ras to Mek pathway. Taking together the above data and the essential role of the Ras regulated Raf/Mek/Erk pathway for junction and cytoskeleton organization, discussed earlier, we conclude that the effect of HR12 on the construction of stress fibers, adherens junctions, and focal adhesions is mediated primarily by Ras translocation.

Is the Raf/Mek/Erk Pathway the Sole Pathway Mediating the Ras Effect on Adhesion and the Cytoskeleton?

The findings reported in this study strongly suggest that the morphological reversion and the recovery of the cy-

toskeletal structure induced by the FT inhibitor HR12 are mediated by the inhibition of the Ras to Erk pathway. p38 activation, downstream of Mek inhibition, may also be involved. Other studies claim that Rho, a component downstream to Ras, mediates the same morphological effects in Rat1/ras cells, including induction of adherens junctions, focal adhesions, and stress fibers (Izawa et al., 1998; Du et al., 1999). Others report that Rac or Cdc42 are regulators of adherens junctions (Braga et al., 1997; Fischer and Quinlan, 1998; Kaibuchi et al., 1999). It is difficult to examine the contribution of Rho family members to the HR12 effect on the cytoskeleton, because the effects of Rho activation and inhibition are very rapid (minutes to hours), while the full effects of HR12 are manifested after 48 h of treatment. It has been suggested that combinations of several pathways downstream of Ras control cell–cell adhesion (Potempa and Ridley, 1998) and morphological transformation (Yang et al., 1998). We do not exclude the possibility that other Ras-dependent pathways mediate morphological transformation. Since Ras activates multiple signaling pathways, the relative contributions of each one to a particular phenotype may differ between cell types. Our findings, however, do implicate putative Mek or Erk substrates as mediators of Ras-dependent disruption of the cytoskeleton in Rat1/ras cells.

Our work shows that HR12 is a powerful tool to inhibit oncogenic-Ras signaling. HR12 could serve as an anticancer drug, with particular antimetastatic potential, deriving from its ability to induce the formation of cell–cell junctions.

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