The Survival Kinase Mirk/dyrk1B Is Activated through Rac1-MKK3 Signaling*

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The serine/threonine kinase Mirk/dyrk1B is activated in several solid tumors where it mediates cell survival, but the mechanism by which Mirk is activated in tumors is unknown. We now demonstrate that Mirk is activated as a kinase by signaling from Rac1 to the mitogen-activated protein kinase kinase MKK3. Rac is a Ras superfamily GTPase that, when activated, functions downstream of Ras oncoproteins to promote cell survival, transformation, and membrane ruffling. The constitutively active mutant Rac1QL activated Mirk in several cell types through MKK3, which in turn activated Mirk by phosphorylation. Dominant negative Rac1, dominant negative MKK3, and knockdown of MKK3 by RNA interference inhibited the kinase activity of co-expressed Mirk. E-cadherin ligation in confluent Madin-Darby canine kidney (MDCK) epithelial cells is known to transiently activate Rac1. Mirk was activated by endogenous Rac1 following E-cadherin ligation in confluent MDCK epithelial cells, whereas treatment of confluent MDCK cells with an Rac1 inhibitor decreased Mirk activity. Disruption of cadherin ligation by EGTA or prevention of cadherin ligation by maintenance of cells at subconfluent density blocked activation of Mirk. Engagement of cadherin molecules on subconfluent cells by an E-cadherin/Fc chimeric molecule transiently activated both Rac1 and Mirk with a similar time course. Rac activity is up-regulated in many human tumors and mediates survival signals, which enable tumor cells to evade apoptosis. This study characterizes a new anti-apoptotic signaling pathway that connects Rac1 with a novel downstream effector, Mirk kinase, which has recently been demonstrated to mediate survival in human tumors.

Mirk/dyrk1B is an arginine-directed serine/threonine protein kinase that is up-regulated and activated in some solid tumors. Mirk has anti-apoptotic functions and mediates cell survival in pancreatic ductal adenocarcinoma, rhabdomyosarcomas, and HeLa cells (1). The mechanism of activation of Mirk in tumors is unknown. However, Mirk is widely expressed in normal tissues at low levels (2, 3) where it has a role in mediating survival in human tumors.

**EXPERIMENTAL PROCEDURES**

Materials—Antibody to Rac1 and the PAK-1 PBD-agarose for GST pull-down experiments were from Upstate Biotechnology, and antibodies to GST and to the His epitope tag were from Santa Cruz Biotechnology. Rabbit polyclonal antibody to a unique sequence at the C terminus of Mirk was raised as described previously (3). Polyvinylidene difluoride transfer paper Immobilon-P was purchased from Millipore. PLUS reagents were obtained from Origene. All other reagents were purchased from Sigma.

Cell Culture—NIH3T3 cells, 293T cells, and MDCK cells were maintained in Dulbecco’s modified Eagle’s medium containing 7% fetal bovine serum and modified and supplemented as described (5). For density experiments on epithelial cells, MDCK cells were trypsinized, extensively triturated to generate a maximally dispersed cell suspension, and plated in the presence of serum.

Plasmids—pcDNA3.1-HisA-Mirk and the kinase-inactive mutants pcDNA3.1-HisA-YF-Mirk and pcDNA3.1-HisA-KR-Mirk had been previously generated (5), as had FLAG-p27 and FLAG-p27-S10A (4). All other Mirk expression plasmids and Mirk promoter constructs were...
prepared by Dr. Xiaobing Deng (11). The (β-28)-3-luciferase plasmid encoding three tandem repeats of the β-fibrinogen HNF1α binding domain in front of a TATA box promoter and a luciferase reporter gene, the expression plasmid pH5-DCoH, and the expression plasmid pH5-HNF1α were the kind gifts of Dr. G. Crabtree, Stanford University. FLAG-MKK3E and -MKK6E, each in pcDNA3, were the kind gifts of Dr. J. Han, Scripps Institute. Constitutively active Cdc42-QL, Rac1-QL, and RhoA-QL were obtained from S. Gudkina, National Institutes of Health.

Transient Transfections—MDCK cells were transiently transfected by adding a complex of Lipofectamine (2–4 μg/μg of DNA) in serum-free media for 24 h. For reporter gene assays, NIH3T3, 293T, and MDCK cells were seeded the day before transfection at 0.9 × 10⁶/well and allowed to grow overnight in 7% serum-containing media. Cells were transfected by incubating with a complex of PLUS reagent (3 μg/μg DNA) and Lipofectamine (2 μg/μg DNA) in serum-free media for 18–24 h in a CO₂ incubator. The amount of total DNA used was kept constant by the addition of empty vector DNA, and luciferase activities were calibrated by co-transfected β-galactosidase activity to normalize the transfection efficiency. These assays were carried out in triplicate, and the data shown are representative of three independent experiments.

Immunoprecipitations—Cells in multiple 60- or 100-mm dishes were transfected with 1 μg of DNA for Mirk or the pHAsA vector and allowed to express for 24 h, and then each dish was lysed in 0.25 or 0.5 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 200 μM sodium orthovanadate, and 20 mM sodium fluoride. An aliquot of total lystate of 500 μg was immunoprecipitated with 5 μL of anti-Mirk rabbit polyclonal antibody overnight at 4°C; the complexes were then collected by the addition of 20 μl of protein A-agarose, incubated for 1 h at 4°C, washed three times with lysis buffer, and separated by SDS-PAGE.

E-cadherin/Fc Assay—Suspended cells were plated at a 1:10 dilution for low density cultures. Cells were allowed to recover for 24 h. The cells were incubated at various time points at 37°C with 1 μg/ml of the murine recombinant E-cadherin/Fc chimeric protein (Sigma).

Calcium Blocking Assay—Suspended cells were plated either at one-tenth confluent density or at confluent density. Cells were allowed to recover for 24 h. The cells were incubated with 4 μmol EGTA for various time points at 37°C.

In Vitro Kinase Assay—The kinase activity of Mirk was tested with the myelin basic protein (MBP) from Upstate Biotechnology, or with recombinant GST-p27 prepared as described (4). The immunoprecipitates were washed three times with lysis buffer and three times with kinase buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.5 mM dithiothreitol, and 1 mM EGTA), incubated for 30 min at 30°C with 20 μl of kinase buffer containing 10 μM unlabeled ATP plus 2.5 μCi of [γ-³²P]ATP and 1 μg of MBP as substrate, and then analyzed by PAGE and autoradiography.

Immunodetection—Following treatment as indicated and washing with cold phosphate-buffered saline, cells were lysed in lysis buffer. Lysates were pelleted in a microcentrifuge for 15 min to remove insoluble material. Depending upon the experiment, 10–50 μg of cell lysate was blotted onto polyvinylidene difluoride membranes after separation by SDS-PAGE. The blots were blocked in 5% milk in TBST for 1 h at room temperature and incubated for 1 h at room temperature with primary antibody in TBST buffer-3% milk, and proteins were subsequently detected by enhanced chemiluminescence. All Mirk blots used an affinity-purified polyclonal antibody directed to the unique C terminus of Mirk. Band density in autoradiograms was measured using a Lacie Silverscanner and Silverscanner III software and analyzed by the IP LabGel program.

Affinity Precipitation of GTP-bound Rac1—The activation state of Rac1 was assessed by the ability of GTP-Rac1 to bind to the GST-Rac-binding domain of PAK. The GST-PBD construct consists of amino acids 67–150 of human PAK-1 bound to glutathione-agarose (Kit 17-283, Upstate Biotechnology). Briefly, the cells were lysed with ice-cold lysis/wash buffer. The lysates were incubated for 1 h with 5–10 μl of PAK-1 PBD-agarose, followed by three washes. The GTP-bound forms of Rac1 associated with GST-PAK1 PBD were quantified by Western blot analysis using a monoclonal antibody against Rac1 (1:1000 dilution).

In Vivo Labeling and Immunoprecipitations—Cells in multiple 60-mm dishes were transfected with MKK3E, pcDNA3.1-HisA-Mirk, or the kinase-inactive mutant constructs pcDNA3.1-HisA-YF-Mirk or pcDNA3.1-HisA-KR-Mirk as noted for 4 h, then switched to growth media for 19 h. Cells were then switched to phosphate-free media for 1 h, and then incubated with 400 μCi of [³²P]orthophosphate for 4 h in 2 ml of reduced phosphate medium (75% phosphate-free Dulbecco’s modified Eagle’s medium, 25% Dulbecco’s modified Eagle’s medium). Cells were washed twice and then lysed in 0.5 ml of buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, Roche Complete Protease Inhibitor Mixture, and Sigma Phosphatase Inhibitor Mixture I and II (lysis buffer). Lysates were pre-cleared by rocking incubation at 4°C with 50 μl of protein A-agarose (Santa Cruz Biotechnology) for 1 h and then pelleted in a microcentrifuge at 10,000 × g for 20 min to remove insoluble material. An aliquot of total lystate of 500 μg was immunoprecipitated with 5 μl of anti-Mirk rabbit polyclonal antibody overnight at 4°C; the complexes were then collected by the addition of 20 μl of protein A-agarose, incubated for 1 h at 4°C, washed three times with lysis buffer, and separated by SDS-PAGE.

Two-dimensional Phosphopeptide Analysis—Trypsin cleavage and two-dimensional analysis of Mirk phosphopeptides was carried out as described (12). Following immunoprecipitation of phosphoabeled proteins, the substrate was resolved by SDS-PAGE and transferred to a nitrocellulose membrane, and the protein band was identified by autoradiography and excised from the membrane. Proteins were digested with sequencing grade trypsin in 200 μl of freshly prepared 50 mM NH₄HCO₃ overnight at 37°C, and the polypeptides were spotted onto 20- × 20-cm thin-layer cellulose plates and separated in the first dimension by electrophoresis for 45 min at 1000 V on an HTLE-7002 electrophoresis system (CBS Scientific) using pH 1.9 buffer (2.2% formic acid, 7.8% acetic acid). Peptides were separated in the second dimension by chromatography for 9 h in phosphochromatography buffer (38% n-butanol, 25% pyridine, and 7.5% acetic acid). Dried plates were exposed to Kodak Biomax MS film for 2–8 days.

RESULTS
The Rho GTPase Rac1 Is Upstream of Mirk in a Signaling Pathway, whereas Other Rho Family Members, Cdc42 and RhoA, Induce Expression of Mirk—We tested the hypothesis that Mirk was activated in vivo by signaling initiated by endogenous activated Rac1. We co-expressed Mirk in 293T cells with increasing concentrations of dominant negative N17Rac1, and then measured the activity of immunoprecipitated Mirk using in vitro kinase reactions with MBP as the substrate (Fig. 1A). Dominant negative N17Rac1 blocked the activation of Mirk in a dose-dependent manner from 6- to 13-fold. Conversely, co-expression of Mirk in 293T cells with increasing concentrations of constitutively active mutant Rac1 (Rac1QL) increased Mirk activity ~4-fold in multi-
Mirk Kinase Activated by Rac1-MKK3 Signaling

FIGURE 1. The Rho family GTPase Rac1 is an upstream activator of Mirk kinase in 293T epithelial cells. A, co-expression with dominant negative N17Rac1 decreases Mirk kinase activity. A His-Mirk construct was co-expressed in 293T cells together with increasing concentrations of 1–5 μg of DNA of N17Rac1, and allowed to express for 18 h. Cells were lysed and Mirk was immunoprecipitated with affinity-purified antibody raised to the C terminus of Mirk. A non-transfected control culture was immunoprecipitated with preimmune serum (IgG lane). Mirk activity on exogenous MBP was determined by an in vitro kinase assay. Western blotting was performed for immunoprecipitated Mirk. Quantitation of Mirk activity normalized to the amount of immunoprecipitated Mirk is shown below the lines. One of duplicate studies is shown. B, co-expression with activated Rac1 increases Mirk kinase activity. A His-Mirk construct was co-expressed in 293T cells either alone or together with increasing concentrations of 1–5 μg DNA of constitutively activated Rac1QL, and allowed to express for 18 h. Cells were lysed, and Mirk was immunoprecipitated with affinity-purified antibody raised to the C terminus of Mirk. A non-transfected control culture was immunoprecipitated with preimmune serum (IgG lane). Mirk activity on exogenous MBP was determined by an in vitro kinase assay. Western blotting was performed for immunoprecipitated Mirk. Quantitation of Mirk activity normalized to the amount of immunoprecipitated Mirk is shown below the lanes. One of three experiments with similar results is shown. C, Cdc42 and RhoA, but not Rac1, increase the abundance of Mirk. Constitutively activated constructs of Rac1QL (Rac), Cdc42QL (42), and RhoAQL (Rho) were expressed in HD6 colon carcinoma cells, and the protein levels of endogenous Mirk and actin were assayed by Western blot. The normalized ratio of Mirk/actin levels is listed below the appropriate lane. D, neither Rac1 nor Cdc42 alter the stability of Mirk protein. Constitutively activated constructs of Rac1QL, Cdc42QL, or vector control were transiently transfected into 293T cells together with His-Mirk. After 24 h of expression, 20 μg/ml cycloheximide was added to induce translation arrest, and the amount of Mirk protein was determined at intervals from 0 to 6 h later by Western blotting compared to actin. E, the increase in Mirk activity induced by Rac1 is not caused by an increase in Mirk abundance, because Rac1 does not activate the Mirk promoter. A 364-bp Mirk promoter-luciferase reporter construct was transiently co-transfected into 293T epithelial cells with expression plasmids for constitutively active Cdc42QL, RhoAQL, or Rac1QL. Mean ± S.E. of the luciferase activity was normalized against the activity of a co-transfected β-galactosidase encoding expression plasmid. F, constitutively active Rac1QL enhances Mirk’s transcriptional transactivation of HNF1α. The β-fibrinogen reporter construct (β-28)-Luc, consisting of three copies of the HNF1 binding element, was co-transfected in transient transfection assays in 293T epithelial cells with expression plasmids for Mirk (M), HNF1 (H), constitutively active Rac1QL (RacQL), wild-type p38 (p38), and two kinase-inactive Mirk mutants (M-YF and Δ44-M) as shown. Constitutively activated Cdc42QL (42QL) was also co-transfected with HNF1 and Mirk. Data shown is the mean ± S.E. of triplicate measurements, normalized for co-transfected β-galactosidase, and is representative of three separate experiments.

ple experiments (Fig. 1B). As a control we measured the protein level of Mirk in cells transfected with the constitutively active constructs Rac1QL, Cdc42QL, and RhoAQL (Fig. 1C). Cdc42QL and RhoAQL increased Mirk protein levels 2-fold, whereas Rac1QL induced at best a marginal 10% increase, indicating that the increase in Mirk activity seen in Fig. 1B was due to activation of Mirk. Possibly Rac1QL or Cdc42QL altered the stability of Mirk protein. Both of these constructs were transfected into 293T cells and allowed to express, and the stability of co-transfected His-Mirk was then tested by translation arrest by cycloheximide, followed by measurement of Mirk levels by Western blotting. Neither activated rho protein altered the stability of Mirk protein (Fig. 1D). We then speculated that the increase in Mirk protein seen when Cdc42QL or RhoAQL were expressed was due to transcriptional upregulation of Mirk. In an earlier study, we had found that Cdc42 and RhoA induce the transcription of Mirk 8-fold in C2C12 myoblasts, whereas Rac1 had less effect (11). A Mirk promoter construct coupled to the luciferase reporter gene was co-expressed with constitutively active Rho proteins in 293T cells. The constitutively active constructs RhoAQL or Cdc42QL induced an average 3-fold increase in Mirk promoter reporter activity (Fig. 1E). Thus activated RhoA and Cdc42 function in
vivo to increase transcription of the Mirk gene, as had been seen in C2C12 myoblasts. In sharp contrast, no increase in Mirk promoter reporter activity was induced by co-expression with constitutively active Rac1QL. Thus Rac1 does not increase Mirk protein level, but instead Rac1 is upstream of Mirk in a signaling cascade.

In addition to these direct kinase assays, we used reporter studies to assay the activation of Mirk by signaling from Rac1. In our earlier studies, signaling from MKK3 to Mirk had been shown to mediate the transcriptional activation of HNF1α (5). We hypothesized that exogenously expressed constitutively active mutant Rac1 would act as an upstream activator of endogenous MKK3 in 293T epithelial cells and thus would activate Mirk in reporter assays. The effects of co-transfected constitutively activated Rac1QL on Mirk transcriptional activation of HNF1α were determined using the β-fibrinogen promoter reporter construct (β-28)-Luc in transient transfection experiments. Rac1QL activated Mirk to enhance HNF1α-dependent reporter activity (Fig. 1A). Little activation was seen when Rac1QL and HNF1α were co-transfected without Mirk or with either of two kinase-inactive forms of Mirk, Mirk-YF, doubly mutated at the activation domain, or Mirk-ΔN, deleted of amino acids 1–110 (3, 11). Although Rac1 activates p38 MAPK (13, 14), p38 was not able to substitute for Mirk in activation of HNF1α (Fig. 1F).

We also tested whether Cdc42QL would activate Mirk in this assay. Cdc42QL increased Mirk activity 6-fold (Fig. 1F). However, Cdc42QL increased Mirk transcription nearly as much, 4-fold (Fig. 1E). Possibly CDC42QL can induce a slight activation of Mirk in addition to its transcriptional up-regulation of Mirk. However, Rac1 is the more potent activator, is upstream of Mirk in a signaling pathway, and increases Mirk activity without increasing Mirk abundance.

We repeated the reporter activation studies in both NIH3T3 cells (Fig. 2A) and MDCK cells (Fig. 2B) and found similar results. Rac1QL activated Mirk 3- to 4-fold to enhance HNF1α-dependent reporter activity. Similarly, the constitutively active constructs RhoAQL or Cdc42QL induced an average 3-fold increase in Mirk promoter reporter activity in NIH3T3 cells (Fig. 2C) and MDCK cells (Fig. 2D), while Rac1QL was ineffective. Therefore, Rac1 is upstream of Mirk in a signaling pathway and increases Mirk activity without increasing Mirk abundance.

Activation of Endogenous Rac by Homophilic E-cadherin Ligation in MDCK Cells Results in Activation of Mirk in Vivo, whereas Dissociation of Cell-to-Cell Adhesion by EGTA Decreases Mirk Activity—Rac has been shown by others to be activated by E-cadherin ligation when
MDCK cells become confluent (7). We tested the hypothesis that Mirk would be activated by signaling cascades initiated by ligation of cell-surface cadherins when MDCK epithelial cells were grown to confluence. Mirk expression is limited in non-transformed MDCK cells, so exogenous Mirk was studied initially. A His-epitope-tagged Mirk expression plasmid was transiently transfected into MDCK cells, which were then plated into multiple parallel dishes, which became confluent by overnight culture (Fig. 3A). One confluent plate was harvested, and the others were gently trypsinized, replated without dilution, and then incubated for 6 h to 4 days before lysis. Mirk was immunoprecipitated from these cultures, and its activity was determined by \textit{in vitro} kinase assays on MBP. Mirk activity was high in the confluent culture but was almost completely lost in cells that had been trypsinized and replated for 6 h. The activity of Mirk increased after 12 h, returned to control levels by 24 h, and then decreased to less than half at 48 h. The activity of Mirk increased after 12 h, returned to control levels by 24 h, and then decreased to less than half at 48 h. The activity of Mirk increased after 12 h, returned to control levels by 24 h, and then decreased to less than half at 48 h.

We next tested whether the sharp decline in Mirk activity was due to the loss of cell-to-cell contact in trypsinized cultures and whether the recovery of Mirk activity was due to restoration of cadherin-cadherin interactions. A His-epitope-tagged Mirk expression plasmid was transiently transfected into MDCK cells, which were then plated at either subconfluent or confluent cell densities, cultured for 24 h, and then treated with 4 mM EGTA for 30 min or left untreated. The amount of GTP-Rac1 (upper lanes) was determined by GST-pull-down assays with GST-PAK1 binding domain-conjugated to agarose, followed by SDS-PAGE and Western blotting for Rac1. The total amount of Rac1 in the cell lysates is shown in the lower lanes. The percentage of activated GTP-Rac1/totall Rac1 is listed below each lane. The cell-permeable Rac1 GDP/GTP exchange inhibitor NSC23766 inhibits Mirk kinase activated by growth to confluence. A His-epitope-tagged Mirk expression plasmid was transiently transfected into subconfluent MDCK cells and allowed to express for 18 h. Cells were then plated at either subconfluent or confluent cell densities, cultured for 24 h, and then treated with 4 mM EGTA for 10 min or left untreated. Cells were lysed in RIPA buffer, Mirk immunoprecipitated, and Mirk activity on exogenous MBP determined by GST-pull-down assays with GST-PAK1 binding domain-conjugated to agarose, followed by SDS-PAGE and Western blotting for Rac1. The total amount of Rac1 in the cell lysates is shown in the lower lanes. The percentage of activated GTP-Rac1/totall Rac1 is listed below each lane. The cell-permeable Rac1 GDP/GTP exchange inhibitor NSC23766 inhibits Mirk kinase activated by growth to confluence.
Ligating Cadherin Receptors on the Surface of Subconfluent MDCK Cells Transiently Activate Both Mirk and Rac with the Same Time Course—We next tested the hypothesis that ligation of cadherin receptors on MDCK cells, not growth to confluence, was sufficient to activate Mirk. Subconfluent MDCK cells were transfected with the His-epitope-tagged Mirk construct for 18 h, then plated at subconfluent density. After 24 h, the subconfluent cells were treated with 1 μg/ml of a soluble chimeric E-cadherin-Fc protein. This recombinant protein consists of the extracellular domain of the mouse E-cadherin amino acids 1–709 fused by a polypeptide linker to the Fc region of human IgG1. Mirk was immunoprecipitated, and Mirk kinase activity was assayed at 0–30 min after treatment with the soluble E-cadherin-Fc protein. Mirk was activated at the 10-min point with less activation observed after 30 min, whereas Western blotting for the His-epitope demonstrated equal expression of His-Mirk and equal immunoprecipitation at all time points (Fig. 5A, 10-min point normalized as 100% activity). Parallel cultures were then assessed for the activation of Rac, by assaying for GTP-Rac bound to the Pak-1 binding domain conjugated to agarose. In parallel to the Mirk activation time course, Rac1 activation was maximal 10 min after treatment with the E-cadherin-Fc fragment, then declined (Fig. 5B). When Chinese hamster ovary cells expressing C-cadherin were plated on dishes coated with the extracellular domain of C-cadherin, a similar decline in Rac1 activation after 30 min was seen and preceded a strong rise in Rac1 activity at 60 min (7). In our study, the extracellular domains of the chimeric E-cadherin/Fc protein transiently engaged the E-cadherin molecules on the MDCK cell surface, which were largely unligated because of the low cell density of the cultures. Cadherin engagement by the E-cadherin/Fc chimera protein activated both Rac1 and Mirk kinase in a similar time course and confirmed that Rac1 activation led to activation of Mirk in vivo.

Rac Activation of Mirk Is Mediated through MKK3—Recently we demonstrated that Mirk protein kinase functions as a transcriptional activator of HNF1α and that Mirk can be stimulated as an activator by co-expression with the MAPK kinase MKK3 (5). We next tested
MKK3A blocks Mirk activation by Rac1

**FIGURE 6.** Rac activation of Mirk is mediated through MKK3: MKK3A studies. A, MKK3A blocks Mirk activation by Rac1. The β-fibrinogen reporter construct (β-28)-Luc, consisting of three copies of the HNF1 binding element, was co-transfected in transient transfection assays in 293T cells, with expression plasmids for Mirk (M), HNF1 (H), the constitutively active MKK3E (M3E), constitutively active Rac1QL (R), and the dominant negative MKK3A (A), as noted. Data shown is the mean ± S.E. of triplicate measurements, normalized for co-transfected β-galactosidase, and is representative of two separate experiments. The S.E. is only shown if it is >5%. B, A His-epitope-tagged Mirk expression plasmid was transiently co-transfected into subconfluent 293T cells together with increasing concentrations of constitutively active MKK3E or increasing concentrations of the dominant negative MKK3AA, and allowed to express for 18 h. Cells were lysed in RIPA buffer, Mirk was immunoprecipitated, and Mirk activity on exogenous MBP was determined by an *in vitro* kinase assay. Western blotting of the immunoprecipitates for the His epitope shows equal expression of Mirk. One of duplicate experiments with similar results is shown.

Immune complex kinase assays were then employed to show the effect of MKK3 knockdown by RNA interference on Mirk activation by Rac1. In these experiments, MKK3 knockdown was initiated by transfection of three concentrations of RNA interference-encoding pRS plasmid, and knockdown was allowed to proceed for 72 h before analysis of Mirk activity. MKK3 knockdown caused up to a 4- to 20-fold decrease in Mirk kinase activity in the absence of transfected active Rac1 (Fig. 7A). Note that the greatest effect was seen with 1–3 μg of the pRS plasmid (Fig. 7A, *last lanes*). These concentrations of plasmid decreased MKK3 protein levels only ~2-fold, but this reduction was sufficient to markedly inhibit Mirk activity. Note that, in these experiments, Mirk was activated by exogenous upstream activators, so these conditions most closely mimic the *in vivo* signaling milieu. However, when exogenous Rac1 was transfected into 293T cells following MKK3 knockdown, the decrease in Mirk activity that was observed was only 4-fold, although MKK3 levels were decreased up to 10-fold (Fig. 7B). It is possible that constitutively activated exogenous Rac1QL activates Mirk through more intermediates than MKK3. In these experiments, constitutively active MKK3E was used as a positive control and increased Mirk activity 2-fold, whereas exogenous Rac1 activated Mirk 3-fold. These data taken together demonstrate that Rac1 is an upstream activator of Mirk *in vivo*, and one of its mediators is MKK3.

**Co-expression of Activated MKK3 (MKK3E) Increases the Phosphorylation of Mirk**—In an earlier study, we demonstrated that the MAPK kinase MKK3 binds to Mirk/dyrk1B and activates it *in vivo* (5). We next sought to determine whether MKK3 would activate Mirk through phosphorylation by analyzing Mirk phosphopeptides following co-expression of Mirk and MKK3. The constitutively activated form of MKK3, MKK3E, was co-transfected with either wild-type Mirk, kinase-inactive Mirk doubly mutated at the activation domain (Y271F/Y273F) or
kinase-inactive Mirk mutated at the ATP-binding site (K140R), allowed to express, and then pulse-labeled with $^{32}$Porthophosphate. Equal amounts of the wild-type and mutant Mirk forms were immunoprecipitated, as demonstrated by Western blotting, and then analyzed by autoradiography following SDS-PAGE (Fig. 8A). MKK3E caused a doubling of $^{32}$Porthophosphate incorporation into wild-type Mirk, over a high background due to autophosphorylation and phosphorylation by various endogenous kinases. However, there was almost no $^{32}$P incorporation when either kinase-inactive Mirk form was expressed in the absence of MKK3E. Furthermore, co-expression of MKK3E increased $^{32}$P incorporation into kinase-inactive Mirk forms 4- to 5-fold when it activated Mirk.

We next performed peptide mapping studies of phosphorylated Mirk (Fig. 8B). In vivo phosphorylation of sites in Mirk would include both the sites of autophosphorylation and sites of phosphorylation by other kinases. Mirk has been shown to autophosphorylate in vivo in a manner dependent on the presence of tyrosines 271 and 273 within its activation domain (3). The sites of autophosphorylation of Dyrk1A, another member of the Mirk/dyrk/minibrain family of related protein kinases, are the second tyrosine in the activation domain, and a tyrosine and a serine in the non-catalytic N-terminal domain (17). All of these sites are conserved in Mirk and would be found on separate peptides released from Mirk by digestion with trypsin. Mirk has three potential MAPK sites within its non-conserved C terminus and is a substrate of all three classes of MAPK in vitro (3). Three potential sites for protein kinase C and glycogen synthase kinase 3B were also found in Mirk, so a minimum of six to nine phosphorylated tryptic fragments was expected to be derived from Mirk expressed in vivo.

Peptide mapping of in vivo expressed Mirk demonstrated seven phospholabeled peptides, designated “a–g” whereas no phospholabeled peptides were detected in the tryptic digest of a similar amount of kinase-inactive doubly mutated Y271F/Y273F-Mirk, which cannot autophosphorylate (Fig. 8B, right panels) (3) or in the tryptic digest of another autophosphorylation deficient mutant, K140R (data not shown). These data suggested that autophosphorylation of Mirk was essential for Mirk to alter its conformation in vivo so that it could be effectively phosphorylated by other kinases. Co-expression of Mirk and MKK3E led to an increase in phosphorylation of each of the seven phosphopeptides while co-expression of MKK3E with kinase-inactive, autophosphorylation-defective YF-Mirk (Fig. 8B, lower left panel) or KR-Mirk (not shown) resulted in only three phosphorylated Mirk tryptic peptides: a, b, and g. By analysis of the probable mobility of the tryptic fragments using Mobility Plot 7 (IGH/Cell Biology Unit), we conclude...
that fragments d, e, and f include the YQY activation domain and the two autophosphorylation sites. These data, taken together, indicate that MKK3 activates Mirk by increasing the phosphorylation of Mirk.

**DISCUSSION**

The Rac GTPase transduces signals to the nucleus from the cell surface in response to growth factor stimulation, integrin ligation, or E-cadherin ligation. Activated Rac1 induces a series of functional responses, including reorganization of the actin cytoskeleton during cell migration, transformation to malignancy, and generation of superoxides. Rac activity is up-regulated in many human tumors either by overexpression of the protein (18) or by altered regulation of function (19, 20). Rac1 is a downstream effector of oncogenic Ras proteins (21) and oncogenic Src (22). Recently, inactivation of Rac1 by caspase-mediated cleavage was shown to promote apoptosis in human lymphoma cells, suggesting that Rac1 mediates survival signals that aid cancer cells in overcoming the cytotoxic effects of chemotherapy (23). Rac1 was then shown to inhibit apoptosis in human BL-41 Burkitt’s lymphoma cells by stimulating phosphorylation of the Bcl-2 family member Bad, thereby inhibiting etoposide-induced caspase activation and apoptosis (24). Rac has also been shown to mediate anchorage-independent survival of breast cancers after ligation of α6β4 integrin (25). CD98 cell-surface proteins interact with integrins through CD98hc, their type II transmembrane heavy chain, and mediate integrin signaling leading to Rac1 activation and survival of embryonic stem cells (26). Therefore, Rac1 initiates anti-apoptotic signaling in several cancers. We have recently shown that Mirk functions as a survival kinase in rhabdomyosarcoma cells as well as skeletal myoblasts (27) and pancreatic ductal adenocarcinomas. Others have shown that Mirk/Dyrk1B is a survival kinase in HeLa cervical carcinoma cells (1). In the current study, we have connected the GTPase Rac1, which is overexpressed or activated in many human tumors with a novel downstream effector, Mirk kinase, which has demonstrated survival capacities in human tumors.

In this study, we have identified endogenous Mirk as an in vivo downstream signaling target of endogenous Rac1 activated by E-cadherin ligation, or ectopic Rac1 constitutively activated by mutation. We also have shown that a Rac-activated MAPK kinase, MKK3, activates Mirk in both in vitro kinase assays and in Rac-responsive promoter reporter assays. Conversely, the dominant negative MKK3 mutant, MKK3A, and knockdown of endogenous MKK3 by RNA interference inhibited Mirk activation by Rac1. Co-expression of constitutively activated MKK3 leads to enhanced phosphorylation of Mirk and increases the phosphorylation of certain tryptic peptides as shown by two-dimensional mapping studies. Rac has been previously shown to activate other kinases, such as p70 S6 kinase (28) and p38 MAPK. The p38 signaling cascade is activated in response to osmotic shock when the scaffold protein OSM links actin to both Rac and the p38 upstream activator proteins MEKK3 and MKK3 (8). Mirk itself is activated by the p38 activator MKK3 (5), and p38 and Mirk compete for access to this activator (6). Possibly another scaffold protein links Rac with MKK3 and Mirk.

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