Serine/Threonine Kinase Mirk/Dyrk1B Is an Inhibitor of Epithelial Cell Migration and Is Negatively Regulated by the Met Adaptor Ran-binding Protein M

Yonglong Zou, Seungwhan Lim, Kangmoon Lee, Xiaobing Deng, and Eileen Friedman

From the Pathology Department, Upstate Medical University, Syracuse, New York 13210

Minibrain-related kinase (Mirk)1 is a member of the Dyrk/minibrain family of tyrosine-regulated, arginine-directed serine/threonine protein kinases (2–4) and is identical to Dyrk1B (5). Mirk is highly expressed in normal skeletal muscle cells, testes, and some carcinomas but is only expressed at low levels in other tissues (1). Depletion of endogenous Mirk in skeletal myoblasts by RNA interference prevents myoblast fusion and the induction of various muscle-specific contractile proteins, demonstrating a function for Mirk in muscle cell development (6). Mirk functions as a transcriptional coactivator that is activated by co-expressed MKK3 (7), a MAP kinase kinase that also can activate p38 MAP kinase (8–10). Since p38MAPK also functions in muscle development (11) and p38 sequesters Mirk under certain cellular conditions (12), p38 may control Mirk function.

The transcriptional coactivator function of Mirk/Dyrk1B was discovered by using a yeast two-hybrid screen to identify the strong binding of Mirk to DCoH, the dimerization cofactor of the transcription factor hepatocyte nuclear factor 1α (7). In the current study we have identified a second binding partner of Mirk, RanBPM, and have investigated its effects on the activity of Mirk. RanBPM is a nuclear Ran-binding protein (13) that regulates transcriptional activity of the androgen receptor and the glucocorticoid receptor (14). However, cytoplasmic Ran-BPM has been found to function as an adaptor for the Met tyrosine kinase domain (15). The hepatocyte growth factor (HGF) receptor Met regulates invasive growth, a process that includes cell motility, cell growth, and the formation of tubular structures during morphogenesis (16). There is much evidence that Met functions in tumor progression. Amplification of the met gene or enhanced Met expression have been found in many human cancers, while activating mutations in Met have been identified in human carcinoma metastases (17). The presence of such activating mutations enabled the met gene to confer a motile-invasive phenotype in transfected carcinoma cells (17). The Met receptor is unusual as it must synergize with other molecules to produce its biological outcomes. Met forms a selective complex with α5β1 (18), an integrin already correlated with invasive growth in several studies. The Met-α5β1 complex was found to be essential for Met to mediate neoplastic invasive growth. Following Met activation by HGF, the β1 integrin is tyrosine phosphorylated and recruits the adaptor Shc, amplifying the HGF signal (18). Other proteins function as Met adaptors, including CD44. Splice variants of CD44 have been identified as metastasis-associated proteins (19). A CD44 isoform containing variant exon v6 sequences was found to be strictly required for Met activation by HGF in carcinoma cell lines and in primary cultured keratinocytes (20). CD44, Met, and HGF formed a multimeric complex, enabling activated Met to utilize the cytoplasmic tail of CD44 for signal transfer. Both of these examples demonstrate the essential role of other signaling systems for Met function. Another Met adaptor, Ran-BPM, has been shown to stimulate the Ras/Erk/SRE signaling pathway by recruiting Sos, leading to increased Met-related migration activity (15). In the current study we characterize
the effects of RanBPM on Mirk activity and demonstrate a new function for Mirk, inhibition of epithelial cell migration in an HDF- and RanBPM-modulated manner.

EXPERIMENTAL PROCEDURES

Materials—Rabbit polyclonal antibody to a unique sequence at the C terminus of Mirk was raised as described previously (1). Rabbit polyclonal antibody to full-length recombinant RanBPM protein was raised and purified by absorption to protein A. Antibodies to the FLAG epitope and the HA epitope were purchased from Sigma. Recombinant purified GST-p38 and myelin basic protein (MBP) were from Upstate Biotechnology. Polyclinivirus directed ddiifluoro transfer membrane (Immobilon-P) was from Millipore. PLUS reagents and LipofectAMINE were purchased from Invitrogen, all radioactive materials were purchased from PerkinElmer Life Sciences, and ECL reagents were from Amersham Biosciences. All other reagents were from Sigma.

Yeast Two-hybrid Screening—The bait construct was prepared by subcloning wild-type full-length Mirk cDNA into the pGBK7T7 vector. Analysis of induction of β-galactosidase activity confirmed that Mirk had no transcriptional activity by itself. The yeast host strain AH109 contains three different reporter genes that are tightly controlled by UAS, thus requiring GAL4 binding for expression of the reporter gene. AH109 cells were simultaneously co-transformed with pGBK7T7-Mirk and the pACT2 muscle CDNA library (Clontech) according to the manufacturer’s protocol. The transformants were plated on His–/Leu–/Trp–/5-bromo-4-chloro-3-indolyli-a-o-galactopyranoside (X-a-gal) medium, and blue colonies were selected as positive candidates. The plasmids from candidate colonies were sequenced using the same plasmid subcloning method. Briefly, colonies were grown in 5 ml of selective medium overnight and pelleted. The pellets were resuspended in 200 μl of lysis buffer and then incubated at 37 °C for 20–30 min after brief vortexing. Lysis buffer consisted of a mixture of 5 ml of SCE (1 m sorbitol, 0.1 M sodium citrate, pH 7.6, 0.06 mM EDTA), 60 μl of 100 mM sylmolase (10 mg/ml), and 10 μl of β-mercaptoethanol. Following addition of 400 μl of 0.5% SDS to a solution of 105 cells, the lyses were incubated on ice for 5 min and pelleted in a microcentrifuge, and the supernatants were decanted into fresh tubes and then reclarified by repeated pelleting. The plasmid DNA of each candidate was precipitated and then transformed into Escherichia coli DH5α using electroporation. The sequences of candidate DNAs were analyzed by using an automatic DNA sequencer (Model 3700 BC 11.1.0.0, ABI Prism).

Plasmid Construction—The full-length RanBPM expression vector, pDEBα-T7-His-3B-PBM90 and the empty vector pDEBα were provided by Dr. T. Nishimoto, Kyushu University. The longest RanBPM library clone isolated in the yeast two-hybrid library was 2.7 kb fragment that coded for amino acids 118–729 of RanBPM and additional 3′-untranslated sequences. A FLAG epitope-tagged RanBPM construct was generated. Plasmids pCDNA3.1 (Mirk) and pCDNA3.1 (kinase-inactive YF-Mirk) had been generated previously (1). The deletion mutants ΔN (A1–110), ΔN2 (A1–233), ΔC (A432–629), ΔC7 (aa1–519), ΔC5 (Δ438–629), and ΔC5 (aa1–604) were generated by using selected restriction enzymes, while the leucine zipper quadruple mutant L226V/L233V/L240V/L247V was generated by site-directed mutagenesis using the Gene-Editor system (Promega). The β-(28)-Luciferase plasmid (encoding three tandem repeats of the β-fibrinogen HFN binding domain in a GST pull-down system) was the expression plasmid pB5-DCoE, and the expression plasmid pB5-HFN1 were the gift gifts of Dr. G. Crabtree, Stanford. The plasmids pCDNA-A-H-Dyrk1A wild type and kinase-inactive pCDNA-HA-Dyrk1AKR were the gifts of Dr. J. Mymryk (University of Western Ontario). The SRE-luciferase reporter plasmid was purchased from Clontech.

Cell Culture—Drosophila Sf21 Insect Cells—Mirk and Mirk mutants were produced in vitro by a coupled transcription and translation system (Promega) using 1 μg of plasmid/reaction. Translation took place in the presence of 20 μCi of [35S]methionine. The labeled TVT proteins and the GST-RanBPM fusion were incubated together in binding buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol) overnight at 4 °C, incubated with glutathione-Sepharose 4B beads for 3 h, and then washed six times in binding buffer before analysis by SDS-PAGE and autoradiography.

Expression of Mirk in Sf21 Insect Cells—Mirk was subcloned into pBACEMK (Invitrogen), stable clones in Sf21 cells were selected, and expression was confirmed by Western blotting of cell lysates. The hexahistidine-Mirk fusion protein was purified by a Talon spin column (Clontech) and then subjected to treatment with protein tyrosine phosphatase-β as described earlier (1).

Size Fractionation by FPLC—Cells were lysed in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40 with protease inhibitors (Roche Applied Science Complete) and 1% Triton X-100) overnight at 4 °C, incubated with glutathione-Sepharose 4B beads for 3 h, and then washed six times in binding buffer before analysis by SDS-PAGE and autoradiography.
Identification of RanBPM as a Mirk-interacting Protein

In Vitro Kinase Reactions—GST-Mirk and HA-Dyrk1A, or their kinase-inactive mutants, were incubated together with 10 μg of MBP or ΔHNF1α (1–283) and GST-RanBPM as noted in kinase buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 0.5 mM dithiothreitol with protease and phosphatase inhibitors) containing 10 μM cold ATP plus 2.5 μCi of [32P]ATP for 10 min at 30 °C. Purified p38 was incubated together with GST-RanBPM for 30 min at 30 °C in kinase buffer as above and 10 μM cold ATP plus 2.5 μCi of [32P]ATP and 10 μg of purified recombinant p21 protein as substrate. Reaction mixtures were then analyzed by SDS-PAGE and autoradiography.

RESULTS

Identification of RanBPM as a Mirk-interacting Protein—Full-length wild-type Mirk was used as bait in yeast two-hybrid screening. Seventeen candidate genes were identified after screening 3.5 × 10⁶ clones of a human skeletal muscle cDNA library. DNA sequencing revealed that one of the Mirk-binding proteins encoded a partial sequence of RanBPM. The RanBPM clone isolated in the yeast two-hybrid assay contained a 2.7-kb fragment that coded for an N-terminal deleted RanBPM90 (amino acids 118–729) and additional 3′-untranslated sequences. Thus the initial 1–117 amino acids of RanBPM are not essential for binding to Mirk.

Deletion analysis using Mirk mutants demonstrated that RanBPM interacts with the nonconserved C terminus of Mirk (Fig. 1, A and B). GST pull-down assays were used with [35S]-prelabeled Mirk constructs to map the site of Mirk-RanBPM interaction. Full-length wild-type Mirk, a kinase-inactive construct, YF-Mirk (active site double mutant Y271F/Y273F), and a quadruple mutant of the putative leucine zipper (L4), and Mirk deletion mutants ΔN (Δ1–110), ΔN2 (Δ1–233), ΔKC (Δ237–437), and ΔC (Δ436–629) were [35S]-prelabeled during coupled transcription/translation reactions. The prelabeled Mirk mutants were bound to GST-RanBPM (see “Experimental Procedures”), and the bound proteins were then analyzed by SDS-PAGE followed by autoradiography (bottom panel). Controls, which demonstrate no binding of lysate proteins to glutathione-Sepharose 4B beads alone, are not shown. The top panel shows the input prelabeled Mirk mutants as resolved by SDS-PAGE followed by autoradiography. One of two replicate experiments with similar results is shown. B, Mirk wild-type (WT) and Mirk deletion mutants ΔC7 (aa1–519), AP (Δaa440–480), and ΔC (Δ436–629) were [35S]-prelabeled during coupled transcription/translation reactions. The prelabeled Mirk mutants were bound to GST-RanBPM (see “Experimental Procedures”), and the bound proteins were then analyzed by SDS-PAGE and autoradiography. One of four replicate experiments with similar results is shown. C, co-immunoprecipitation of Mirk and RanBPM from total cell lysates. Mirk and FLAG epitope-tagged RanBPM were co-transfected into 293T cells. RanBPM was immunoprecipitated with antibody to its FLAG tag, and then the immunoprecipitates were analyzed by Western blotting for associated Mirk protein as well as anti-FLAG antibody for RanBPM. Both RanBPM and Mirk migrated at their expected molecular weights on the SDS-polyacrylamide gel. The four lanes at the left show Western blotting of 10% of the input proteins. One of three replicate experiments with similar results is shown. Similar data were obtained with Dyrk1A (not shown). D, Mv1Lu cells were co-transfected with FLAG-RanBPM and Mirk, and the following day the cells were treated for 30 min at 37 °C with the permeable cross-linking agent dimethyl-3,3-dithiobispropionimidate. Mirk was immunoprecipitated, and the immunoprecipitates were analyzed by Western blotting for co-immunoprecipitated RanBPM and Mirk. A sample of the lysate was run in the first lane. WB, Western blot; IP, immunoprecipitation.
kinase homeodomain-interacting protein kinase 2 (HIPK2), which has been reported to bind to RanBPM using sequences within its nonconserved C terminus and N terminus (24), and with the closest relative of Mirk/Dyrk1B in the Dyrk/minibrain family, Dyrk1A. A 14-amino acid-long sequence (DT/CEXX-SPQ(V/A) within the binding region for RanBPM in Mirk was homologous to sequences within the C-terminal nonconserved regions of Dyrk1A and HIPK2 (Fig. 2B). There is also a repeat of the SPQ sequence within HIPK2. These data demonstrate that a 40-amino acid-long segment of the nonconserved C terminus of Mirk mediates binding to RanBPM.

To demonstrate that RanBPM and Mirk associate in vivo, RanBPM was immunoprecipitated from 293T cells following transient co-expression of FLAG epitope-tagged RanBPM and Mirk. The RanBPM immunoprecipitates contained co-precipitated Mirk as shown by Western blotting of the immunoprecipitates after size fractionation by SDS-PAGE (Fig. 1C). Thus ectopically expressed Mirk and RanBPM complexed in vivo. The relatively low amount of co-immunoprecipitation between FLAG-RanBPM and Mirk shown here might reflect the mislocalization of much of the ectopic FLAG-RanBPM (discussed later, see Fig. 6A). To confirm in vivo association, Mv1Lu cells were co-transfected with FLAG-RanBPM and Mirk, and the following day the cells were treated for 30 min at 37 °C with the permeable cross-linking agent dimethyl-3-3′-dithiobispropionimidate. Mirk was then immunoprecipitated from the lysates that were examined by Western blotting for co-immunoprecipitated RanBPM, which was detected (Fig. 1D). Repeated experiments failed to detect Met protein within the immunoprecipitates (data not shown). However, Mirk and RanBPM associate in vivo.

RanBPM Blocks the Kinase Activity of Mirk—We next explored the functional significance of the binding of RanBPM to Mirk. In vitro kinase reactions were performed with Mirk and increasing amounts of RanBPM using MBP as substrate. The highest concentration of RanBPM tested was approximately equimolar to Mirk, and this concentration of RanBPM inhibited Mirk activity 4-fold (Fig. 3A). Lower concentrations of RanBPM were less active with a 1:5 ratio of RanBPM to Mirk producing no detectable inhibition. Mirk phosphorylates the transcription factor HNF1α on at least one unique position within its CREB-binding protein binding domain, and this kinase activity is necessary for the coactivation by Mirk of the transcriptional activity of HNF1α (7). Phosphorylation of HNF1α in vitro by Mirk was also inhibited by RanBPM with the highest concentration tested inhibiting Mirk activity by 70% (Fig. 3B). Phosphorylation of RanBPM by Mirk was not detected in these in vitro kinase experiments (data not shown).

Mirk also did not phosphorylate DCoH, another binding protein identified by the yeast two-hybrid assay, but phosphorylated HNF1 to which DCoH bound (7). In analogous fashion, we hypothesize that Mirk may phosphorylate a protein to which RanBPM binds, such as Met.

Dyrk1A is a kinase related to Mirk/Dyrk1B. Despite the odd nomenclature, they are not splice variants. Mirk and Dyrk1A exhibit 54% overall identity with 90% identity or close homology within their conserved kinase domains. RanBPM co-immunoprecipitated with co-expressed Dyrk1A (data not shown) similar to its co-immunoprecipitation with Mirk (Fig. 1C). RanBPM also inhibited the activity of Dyrk1A (Fig. 3C). HA epitope-tagged Dyrk1A was expressed in 293T cells, immunoprecipitated, and then tested in an in vitro kinase assay using MBP as an exogenous substrate. RanBPM inhibited Dyrk1A kinase activity in a dose-dependent manner with the highest concentration tested producing a 7-fold inhibition. Kinase-inactive Mirk (YP double mutation in the activation domain) and kinase-inactive Dyrk1A (KR mutation in its ATP binding site) served as the negative controls in these experiments. Since Mirk and Dyrk1A are serine/threonine kinases activated by tyrosine phosphorylation, we considered that RanBPM might inhibit the activity of other such kinases, for example members of the MAP kinase family such as p38MAPK. The cyclin-dependent kinase inhibitor p21 is phosphorylated at two (S/T)P sequences by p38MAPK (23). A 4-fold ratio of RanBPM to p38MAPK caused no detectable inhibition of p38 kinase activity on p21 in in vitro kinase reactions (Fig. 3D). Therefore, the blocking activity of RanBPM against Mirk kinase and Dyrk1A is selective and does not occur against other serine/threonine kinases such as p38 activated by tyrosine phosphorylation.

RanBPM Modulates the Transcriptional Transactivator Activity of Mirk—RanBPM also regulates transcriptional activity of the androgen receptor and glucocorticoid receptor (14). We next tested the ability of RanBPM to modulate the transactivator activity of Mirk on a co-transfected HNF1α-responsive reporter gene construct in NIH3T3 cells. No Met expression occurs in NIH3T3 cells unless they spontaneously form spheroids (25), so we could test RanBPM-Mirk interactions in the absence of this signaling system. Mirk stimulated HNF1α reporter activity about 50% in the absence of RanBPM but only activated the reporter gene by 34 and 38% following transfection of 0.5 and 1 μg of RanBPM, respectively, with the differences statistically significant by Student’s t test (p < 0.001) (Fig. 4A). Thus RanBPM inhibited the transcriptional transactivator activity of Mirk in NIH3T3 cells in the absence of Met.

In addition, the highest concentration of RanBPM inhibited the activity of the HNF1α reporter gene about 50% both in the absence and in the presence of Mirk (Fig. 4A). The inhibition of HNF1 in the absence of exogenous Mirk may indicate that RanBPM blocks the activity of endogenous Mirk, which is expressed in NIH3T3 cells (data not shown).

RanBPM has been reported to induce expression of an SRE-luciferase reporter gene construct when transfected into HeLa cells (15). We detected no activation of a SRE-reporter con-
Fig. 3. RanBPM specifically blocks Mirk/Dyrk1B and Dyrk1A kinase activity in vitro. A representative experiment of three replicate experiments is shown for each panel. A, kinase mixtures contained 200 ng of either recombinant purified wild-type GST-Mirk or kinase-inactive (YF) GST-Mirk with recombinant purified RanBPM (0, 20, 40, 100, and 200 ng). In vitro kinase assays were performed with [32P]ATP and MBP as substrate and analyzed by autoradiography after SDS-PAGE. B, kinase mixtures contained 200 ng of recombinant purified wild-type GST-Mirk with 0, 50, 100, 200, or 300 ng of recombinant RanBPM. In vitro kinase assays were performed with [32P]ATP and recombinant purified ΔHNF1α-(1–283) as substrate and analyzed by autoradiography after SDS-PAGE. C, kinase mixtures contained immunoprecipitated recombinant wild-type HA-Dyrk1A or kinase-inactive KR-Dyrk1A (KR) together with 0, 50, 150, or 300 ng of recombinant RanBPM. In vitro kinase assays were performed with [32P]ATP with MBP as substrate and analyzed by autoradiography after SDS-PAGE. D, kinase mixtures contained 200 ng of recombinant purified wild-type p38MAPK with 0, 80, or 400 ng of recombinant RanBPM. In vitro kinase assays were performed with [32P]ATP and recombinant purified GST-p21 as substrate and analyzed by autoradiography after SDS-PAGE. P*, phospho.

Fig. 4. RanBPM inhibits Mirk activation of HNF1α and SRE reporter activity. A, the transcriptional activity of HNF1 was inhibited by co-expression of increasing amounts of RanBPM both in the absence and in the presence of co-expressed Mirk. B, Mirk activation of SRE-luciferase reporter gene blocked by co-expressed RanBPM or by treatment with HGF. Mv1Lu cells were co-transfected with SRE-luciferase reporter plasmid together with RanBPM, Mirk (M), or both, and after 24 h of expression, one set of cells was treated in serum-free medium for 6 h with 10 ng/ml HGF. Mean values, normalized for expression of co-transfected β-galactosidase, of three experiments are shown. CT, control.
the Mirk-inducible C9-Mv1Lu subline (Fig. 5B), Mv1Lu cells bearing only the LacI repressor construct (lacI cells), or the parental line. A wound in the shape of a $-1 \times 1$-mm cross was made in the center of confluent monolayers in 6-well tissue culture plates. The wound was photographed at zero time and after 48 h later. Data are representative of four replicate experiments. C, quantitation of two duplicate experiments performed as in B with parental Mv1Lu cells, lacI stable transfectants, and the C9 Mirk-inducible transfectant. The wounding assays were performed as in B and then quantitated. Mean ± S.E. is shown (S.E. > 5%). D, migration of Mv1Lu cells through 8-μm polycarbonate Transwell filters was inhibited by induction of Mirk and stimulated by overexpression of exogenous RanBPM. Mean (±S.E. if over 5%) of four separate experiments, each performed in triplicate, is shown. In RanBPM experiments, control cells were transfected with vector DNA. HGF was added at 10 ng/ml as noted, and migration was assayed after 24 h. In this assay, HGF caused a slight but consistent decrease in migration with $p < 0.05$ by Student's two-tailed t test. E, inhibition of migration by Mirk is dependent on the kinase activity of Mirk. Mv1Lu cells were transiently transfected with expression plasmids for wild-type Mirk or kinase-inactive mutant YF-Mirk or with vector DNA. On the day following transfection, the cells were pulse-labeled with [3H]thymidine, trypsinized, and counted, and equal numbers were seeded into multiple Transwells. Migrated cells were counted 24 h later (see "Experimental Procedures"). The mean (±S.E.) of duplicate experiments, each performed in triplicate, is shown. HR, hours; I, IPTG; H, HGF; RBPM, RanBPM.

Fig. 5. Mirk inhibition of Mv1Lu migration. A, Western blot showing Mirk induction in the C9 Mirk-inducible transfectant Mv1Lu cell clone treated with 200 μM IPTG for the hours as indicated. CT is a cross-reactive protein on the same blot demonstrating equal loading. B, photomicrographs of wounding assay on the C9 Mirk-inducible transfectant Mv1Lu cell clone treated with 200 μM IPTG, 10 ng/ml HGF, both IPTG and HGF, or no additions. Photomicrographs were taken at time 0 just after changing the medium over the monolayers after wounding and 48 h later. Data are representative of four replicate experiments. C, quantitation of two duplicate experiments performed as in B with parental Mv1Lu cells, lacI stable transfectants, and the C9 Mirk-inducible transfectant. The wounding assays were performed as in B and then quantitated. Mean ± S.E. is shown (S.E. > 5%). D, migration of Mv1Lu cells through 8-μm polycarbonate Transwell filters was inhibited by induction of Mirk and stimulated by overexpression of exogenous RanBPM. Mean (±S.E. if over 5%) of four separate experiments, each performed in triplicate, is shown. In RanBPM experiments, control cells were transfected with vector DNA. HGF was added at 10 ng/ml as noted, and migration was assayed after 24 h. In this assay, HGF caused a slight but consistent decrease in migration with $p < 0.05$ by Student's two-tailed t test. E, inhibition of migration by Mirk is dependent on the kinase activity of Mirk. Mv1Lu cells were transiently transfected with expression plasmids for wild-type Mirk or kinase-inactive mutant YF-Mirk or with vector DNA. On the day following transfection, the cells were pulse-labeled with [3H]thymidine, trypsinized, and counted, and equal numbers were seeded into multiple Transwells. Migrated cells were counted 24 h later (see "Experimental Procedures"). The mean (±S.E.) of duplicate experiments, each performed in triplicate, is shown. HR, hours; I, IPTG; H, HGF; RBPM, RanBPM.

the Mirk-inducible C9-Mv1Lu subline (Fig. 5B), Mv1Lu cells bearing only the LacI repressor construct (lacI cells), or the parental line. A wound in the shape of a $-1 \times 1$-mm cross was made in the center of confluent monolayers in 6-well tissue culture plates. The wound was photographed at zero time and after 48 h later. After wounding the medium was changed to one containing IPTG to induce Mirk expression, HGF to activate the Met receptor, both agents, or neither.

Induction of Mirk significantly inhibited C9 cell migration into the wounded area. The area of the monolayer in which cells have not migrated is outlined (Fig. 5B). The wounded area remaining free of cells was 7 times greater in the C9 cultures following Mirk induction compared with uninduced cells (Fig. 5B, +I, −H versus −I, −H; one of four replicate experiments shown). Additional controls were performed to evaluate the effect of IPTG (Fig. 5C). No inhibition of migration was seen when IPTG was added to either lacI transfectants or the parental cultures (Fig. 5C). Thus Mirk had a significant inhibitory effect on cell migration in the wounding assay.

HGF was added to activate the Met receptor in Mv1Lu cells as seen by others (27). HGF by itself had little detectable effect on Mv1Lu cells in wound healing (Fig. 5, B and C, −I, +H) and
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Fig. 6. While purified Mirk forms a dimer in vitro, Mirk associates with other proteins including RanBPM in higher molecular weight complexes in vivo. A, Mv1Lu cells were transfected with Mirk and FLAG-RanBPM. Following expression, lysates of log phase cells were subjected to size fractionation on Superdex 200, and fractions were analyzed for the presence of Mirk, FLAG-RanBPM, Ran, and Jab1 by Western blotting. Western blots are shown of FPLC fractions containing proteins of 20–670 kDa with the position of FPLC size markers of 660, 150, 66, and 29 kDa indicated over their respective fractions. Monomer forms were 70 kDa for Mirk, 90 kDa for RanBPM, 36 kDa for Jab1, and 24 kDa for Ran. The blots shown are representative of two independent experiments. Endogenous Mirk from HD3 colon carcinoma cells was also observed to migrate in fractions of 66–660 kDa. B, recombinant Mirk was purified after growth of its expression plasmid in SF21 insect cells and then either directly sized on Superdex 200 by FPLC or treated with tyrosine phosphatase-β (PTBβ) before sizing. Protein abundance by A280 is shown. WB, Western blot; CT, control.

proliferation (data not shown). This lack of effect was consistent with earlier reports showing that HGF exerted only a slight effect on Mv1Lu proliferation and migration (28). However, when Mirk was induced in cells treated with HGF, the effect of Mirk was nullified, and the cells filled in the wounded area as quickly as control cells (Fig. 5B, compare +I−H to +I+H). This ability of HGF to block Mirk function in Mv1Lu cells was seen in each of four experiments (Fig. 5C) and was consistent with the capacity of HGF to block the activation of the SRE reporter construct by Mirk in Mv1Lu cells (Fig. 4B). HGF activates the Met receptor and enhances the downstream signaling of Met, perhaps increasing the activity of Met-bound RanBPM. We also questioned how HGF could block Mirk when HGF itself did not detectably affect Mv1Lu cell growth. In earlier reports, HGF by itself exerted little effect on Mv1Lu cell growth, but in the same studies HGF was still able to block the potent ability of transforming growth factor-β1 to arrest Mv1Lu cell growth (27). Therefore, HGF does alter Mv1Lu cellular responses to other agents, and HGF inhibited the antimitogitory effects of Mirk.

Mirk Inhibition of Mv1Lu Cell Migration Is Blocked by RanBPM—Since exogenously expressed RanBPM has been reported to enhance HGF-induced A704 human kidney carcinoma cell invasion (15), we decided to determine whether Mirk, as a RanBPM-binding protein, could also modulate cell motility in a RanBPM-dependent manner. The effect of Mirk on C9 cell motility was assayed by measuring cell invasion through 8-μm Transwell polycarbonate filters. Mirk was induced in C9 cells with or without added HGF to activate the Met receptor (Fig. 5D). Induction of Mirk inhibited the invasion of C9-Mv1Lu cells by 50% (data from four experiments, each performed in triplicate). When Mirk was induced in the presence of HGF, Mirk still inhibited invasion but only by 15%. Thus HGF, by activating Met, blocked some of the inhibitory action of Mirk on Mv1Lu cell invasion similar to the blocking effects of HGF on Mirk in wounding assays (Fig. 5, B and C) and in activation of the SRE reporter construct (Fig. 4B). Possibly activation of Met by HGF enhanced RanBPM binding to Mirk, enabling RanBPM to inhibit the kinase activity of Mirk.

As a further test of this hypothesis, RanBPM was transfected into C9-Mv1Lu cells. Transiently overexpressed RanBPM stimulated the migration of cells to 150% of control values (Fig. 5D). Since not all cells were transfected, the effect of RanBPM on migration would be greater on a per cell basis. When Mirk was induced in cells overexpressing RanBPM, the exogenous RanBPM completely blocked the inhibitory effect of Mirk on C9-Mv1Lu cell invasion (Fig. 5D). Inhibition of migration by Mirk was next shown to be dependent on the kinase activity of Mirk. Mv1Lu cells were transiently transfected with expression plasmids for wild-type Mirk, kinase-inactive mutant YF-Mirk, or vector DNA, and after expression, the ability of the cells to migrate through Transwells was determined. Transfected Mirk inhibited migration about 30%, and since not all cells were transfected, the effect of Mirk on migration would be greater on a per cell basis (Fig. 5E). However, there was no effect on migration of expression of YF-Mirk. Thus Mirk requires its kinase activity to block migration. In summary, the ability of RanBPM to inhibit the kinase activity of Mirk (Fig. 3) appears to enable RanBPM to block the antimitogitory ability of Mirk in vivo. Mirk blocked epithelial cell migration after wounding (Fig. 5, B and C) and invasion through a filter (Fig. 5D), but Mirk was unable to block migration of cells in which Met signaling had been activated by treatment with HGF.

RanBPM and Mirk Are Part of High Molecular Weight Complexes in Vivo—Both endogenous Mirk (12) and endogenous RanBPM (13) have been found to migrate as 660–670-kDa complexes in vivo as shown by analysis of cell lysates by gel filtration. Ran has also been found to be associated with RanBPM in these high molecular weight complexes (13). We hypothesized that in vivo association of Mirk and RanBPM could take place within these large molecular weight complexes. RanBPM is 90 kDa, and Mirk is 70 kDa, so other constituents of these complexes may exist. We decided to test whether RanBPM and Mirk are found in the same large complexes in vivo by co-expressing FLAG-tagged RanBPM and Mirk. RanBPM is a ubiquitously expressed protein with a very low level in lung (14, 15), and Mirk is widely expressed, again with low expression in lung (1). Since we had already shown that Mirk and
RanBPM interact in vivo in Mirk-inducible C9-Mv1Lu cells (Figs. 4 and 5), we co-expressed Mirk and FLAG-RanBPM in the parental Mv1Lu lung epithelial cell line. Cell lysates were fractionated by gel filtration on Superdex 200 by FPLC, and then fractions were analyzed by Western blotting for Mirk, Ran, Jab1, and the FLAG epitope tag of RanBPM. A second polyclonal antibody raised to recombinant RanBPM was used to detect both endogenous RanBPM and FLAG-RanBPM. Mirk migrated as a monomer (70 kDa), a dimer (140 kDa), and a continuum of higher molecular mass species up to 660 kDa (Fig. 6A). The migration pattern of exogenous Mirk was similar to the migration pattern of endogenous Mirk in colon carcinoma cells as demonstrated in earlier studies (12). To our surprise, endogenous RanBPM did not comigrate with the FLAG-RanBPM species. Under our conditions of cell lysis and gel filtration, FLAG-RanBPM was found primarily within a 320–440-kDa complex that co-migrated with Ran and with Jab1, a component of the 440-kDa signalosome (29). In contrast, endogenous RanBPM migrated predominantly in 660–670-kDa complexes, comigrating with the highest molecular mass Mirk species. The low fraction of FLAG-RanBPM found co-immunoprecipitating with Mirk (Fig. 1C) might reflect the incorrect localization of much of the ectopic FLAG-RanBPM.

We have speculated in earlier studies that the high molecular weight Mirk species might form a complex with comigrating p38MAPK or PML (promyelocytic leukemia) nuclear bodies (12). Another possibility is that Mirk can multimerize to form the 670-kDa complexes. To determine the extent to which Mirk can multimerize, purified Mirk protein was subjected to size fractionation on a Superdex 200 column by FPLC. Recombinant Mirk migrated primarily as a 140-kDa dimer and a smaller 70-kDa monomer. Treatment with tyrosine phosphatase-β converted all of the Mirk dimer to a monomer (Fig. 6B). For kinase activity Mirk must be phosphorylated within its activation domain.\textsuperscript{271}YQ\textsuperscript{273} so a tyrosine phosphatase would be expected to block its activity (1). These data suggest that tyrosine phosphorylation may occur intermolecularly within the Mirk dimer to maintain the dimer structure. Phosphorylation and activation of the related kinase ERK2 leads to conformation changes enabling ERK2 to form a dimer and exposing the Tyr\textsuperscript{202} within the Thr\textsuperscript{183}/Tyr\textsuperscript{185} activation domain, which is inaccessible when ERK2 is unphosphorylated (30). Phosphorylation of ERK2 has been shown to induce its dimerization, which is needed for nuclear localization (31). Thus Mirk, as a related Ser/Thr kinase activated by tyrosine phosphorylation (1), also requires tyrosine phosphorylation for its dimerization.

These data demonstrate that Mirk itself cannot be the only component of the 660-kDa Mirk-containing complexes. Much of the Mirk protein is found in the nucleus, but some is cytoplasmic.\textsuperscript{2} Therefore, it is possible that endogenous Mirk and endogenous RanBPM form complexes in the cytoplasm at the Mirk receptor cytoplasmic tail.

**DISCUSSION**

This study has demonstrated an interaction between the serine/threonine kinase Mirk and the Met adaptor protein RanBPM and furthermore has shown that this interaction provides a novel control point for cell motility and invasion. The Met receptor tyrosine kinase affects many cellular processes through its control of motility, including growth, differentiation, and morphogenesis (26, 32). Met was originally identified as the transforming gene from osteogenic sarcoma cells using focus-forming assays with NIH3T3 cells (33). The Met ligand HGF is secreted predominately by stromal cells, although some tumor cells co-express both HGF and Met, enabling autocrine activation of Met in these cancers (34). HGF was shown to enhance the migration of carcinoma cells and to stimulate invasion of NIH3T3 cells expressing exogenous Met (35–37). Met is overexpressed or activated in many human carcinomas, leading to enhanced cell motility and to tumor invasion. For example, the number of mRNA copies of Met significantly correlated with the depth of tumor invasion in early stage primary colorectal cancers suggesting the level of Met overexpression may be an important marker for invasion and regional disease metastasis (38). Met can sometimes be activated in tumors by germ line or somatic mutation. In a recent large screening study, no mutations were found in the Met kinase domain in 153 human sporadic cancers or in 25 cancer cell lines, but somatic Met mutations were found in 12 of 60 metastases (17). The mutations that were discovered were found to constitutively activate the Met kinase and to confer an invasive phenotype on human epithelial cells (17). Thus Met has been linked to motility and to tumor invasion.

The molecular mechanisms underlying physiological regulation of Met signaling have been studied intensively. Most tumors have areas of hypoxia, and within these areas Met overexpression is transcriptionally induced (39). Hypoxia strongly synergizes with HGF to induce cell scattering, while hypoxia and HGF also synergize in inducing invasion (39).

The Met receptor has tyrosines within its cytoplasmic tail that, when phosphorylated, serve as docking sites for other signaling molecules such as Shc and phosphatidylinositol 3-kinase. However, Met utilizes other signaling systems to amplify its signal. Met, HGF, and certain splice variants of CD44 associate into multimeric complexes and use the CD44 cytoplasmic tail to transduce Met signals (20). Likewise the integrin α\textsubscript{v}β\textsubscript{3} functions as an amplifier of HGF/Met signaling. Met selectively and constitutively associates with α\textsubscript{v}β\textsubscript{3} integrin and induces tyrosine phosphorylation of the integrin, which activates downstream signaling through Shc (18). This association is necessary for HGF-dependent responses including invasive growth and amplifies Met signaling. Another example is provided by ligand-induced activation of the proto-oncogene Ron. This results in heterodimerization of Met and Ron followed by the transphosphorylation of Met and induction of its downstream signaling (40). The binding of RanBPM to Met offers the cell an additional way to amplify HGF/Met signaling. RanBPM was recently reported to be a Met adaptor protein, binding to the Met tyrosine kinase domain and up-regulating its signaling (15). RanBPM interacts in vivo with Met without HGF stimulation, but HGF treatment magnifies the association between Met and RanBPM as demonstrated by enhanced co-immunoprecipitation. These data suggest that phosphorylated Met is more effective in recruiting RanBPM than is the unphosphorylated form (15). Thus cells utilize several mechanisms to up-regulate Met signaling at the receptor level.

In the current study, we have identified a novel antimotility function for the serine/threonine kinase Mirk/Dyrk1B through its interaction with the Met adaptor protein RanBPM. Mirk was shown to be a binding protein for RanBPM in yeast two-hybrid assays and by co-immunoprecipitation studies. We have further shown that RanBPM blocks Mirk kinase activity, Mirk transcriptional transactivator activity on two promoter constructs, and Mirk inhibition of epithelial cell migration. Furthermore HGF, through activating the Met downstream signaling pathway and enhancing the association between Met and RanBPM (15), attenuates the inhibitory effects of Mirk on migration and the capacity of Mirk as a transcriptional activator. One model for our results is that Mirk acts as an endogenous modifier of the HGF receptor Met by its ability to bind to

\textsuperscript{2} X. Deng, manuscript in preparation.
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the Met adaptor protein RanBPM. Our studies were performed by overexpressing Mirk and/or RanBPM. However, endogenous Mirk levels vary 20-fold within the cell, falling when cells are treated with mitogens and increasing when cells are deficient in mitogens (1). We speculate that under physiological conditions when Mirk levels are in excess of RanBPM levels cell migration would be inhibited, but when RanBPM levels are in excess of Mirk levels, cells would migrate. Thus under mitogen-poor conditions, Mirk levels would rise, and Mirk localized near the Met receptor could be in excess of RanBPM and block cell migration.

RanBPM was originally cloned as a 55-kDa Ran-binding protein (41) that localized in the centrosome. Its location suggested that RanBPM played a role in microtubule assembly, while its association with Ran suggested a function in either nucleocytoplasmic transport or nuclear membrane formation. However, RanBPM has little sequence homology with RanBP1 or related Ran-binding proteins, mitigating against a direct role in nucleocytoplasmic dissociation of RanGTP-importin and RanGTP-exportin-cargo complexes. In addition, more recent studies have demonstrated that the original RanBPM isolate resulted from an N-terminal deletion, whereas full-length RanBPM was 90 kDa. Full-length RanBPM was located in the nucleus and in the cytoplasm but not within the centrosome (13).

RanBPM also interacts with another member of the Dyrk/minibrain family, the HIPK2, as shown by yeast two-hybrid analysis, although the significance of this interaction was not explored (24). HIPK2 phosphorylates the tumor suppressor protein p53 at Ser15 and mediates apoptosis (42, 43). While Mirk is most highly expressed in skeletal muscle in vivo and expressed less in brain and heart (1), the closest family member of Mirk, Dyrk1A/minibrain, is most highly expressed in brain (44) and is the presumptive Down syndrome gene. Mirk/Dyrk1B exhibits 90% identity or homology to Dyrk1A within the conserved kinase domain. Dyrk1A has been mapped to the Down syndrome critical region of chromosome 21 (45), overexpression of Dyrk1A has been found in Down syndrome fetal brain (46), and transgenic mice overexpressing Dyrk1A exhibit cognitive deficits and motor abnormalities characteristic of Down syndrome (47). Like Mirk, Dyrk1A is a transcriptional coactivator. Dyrk1A phosphorylates the CAMP-response element-binding protein CREB in vivo, stimulating CREB transcription during neuronal differentiation of hippocampal progenitor cells (48). The current study demonstrates that RanBPM binds to and inhibits the activities of Mirk and Dyrk1A. RanBPM also has been found to bind to the Dyrk-related kinase HIPK2 (24). RanBPM may function as an endogenous inhibitor of the family of Dyrk/Mirk/minibrain kinases.

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