The Transcriptional Activator Mirk/Dyrk1B Is Sequestered by p38α/β MAP Kinase*

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Mirk/Dyrk1B protein kinase was shown in an earlier study to function as a transcriptional activator of HNF1α, which Mirk phosphorylates at Ser249 within its CREB (cAMP-response element-binding protein)-binding protein (CBP) binding domain (1). The MAP kinase MKK3 was also shown to activate Mirk as a protein kinase, implicating Mirk in the biological response to certain stress agents. Another MKK3 substrate, p38MAPK, is now shown to inhibit the function of Mirk as a transcriptional activator in a kinase-independent manner. Co-immunoprecipitation experiments demonstrated that kinase-inactive p38AF, as well as wild-type p38, sequestered Mirk and prevented its association with MKK3. Only the p38α and p38β isoforms, but not the γ or δ isoforms, complexed with Mirk. p38αMAPK blocked Mirk activation of HNF1α in a dose-dependent manner, with high levels of kinase-inactive p38αAF completely suppressing the activity of Mirk. Size fractionation by fast protein liquid chromatography on Superdex 200 demonstrated that Mirk is not found as a monomer in vivo, but is found within 150–700 kDa subnuclear complexes, which co-migrate with the nuclear body scaffolding protein PML. Endogenous Mirk, p38, and MKK3 co-migrate within 500–700-kDa protein complexes, which accumulate when nuclear export is blocked by leptomycin B. Stable overexpression of Mirk increases the fraction of Mirk protein and p38 protein within these 500–700 kDa complexes, suggesting that the complexes act as nuclear depots for Mirk and p38. Sequestration of Mirk by p38 may occur within these subnuclear complexes. Synchronization experiments demonstrated that Mirk levels fluctuate about 10-fold within the cell cycle, while p38 levels do not, leading to the speculation that endogenous p38 could only block Mirk function when Mirk levels were low in S phase and not when Mirk levels were elevated in G1/G0. These data suggest a novel cell cycle-dependent function for p38, suppression of the function of Mirk as a transcriptional activator only when cells are proliferating, and thus limiting Mirk function to growth-arrested cells.

Mirk1 (minibrain-related kinase) is a member of the Dyrk/minibrain family of dual specificity tyrosine-regulated, argininedirected protein kinases (2–4) and is identical to Dyrk1B (5). Mirk is a serine/threonine protein kinase, which is expressed at elevated levels in normal skeletal muscle and certain carcinoma cell lines and is expressed at low levels in many normal tissues (6). Mutant minibrain/dyrk1A flies are characterized by a marked reduction in size of the optic lobes and central brain hemispheres (7), so it is reasonable to hypothesize that minibrain/dyrk1A plays a role in neuronal survival and/or proliferation. In cell culture systems dyrk1A mediates neuronal differentiation by phosphorylating CREB in vivo, leading to the stimulation of subsequent CRE-mediated transcription during neuronal differentiation in hippocampal progenitor cells (8).

Mirk/dyrk1B functions as a transactivator of a different transcription factor, HNF1α, to which it binds through the dimerization co-factor DcoH of HNF1α (1). The kinase activity of mirk is essential for its biological function. Mirk has constitutive protein kinase activity on the exogenous substrate myelin basic protein (6) and phosphorylates HNF1α at Ser249 within its CBP binding domain (1). However, mirk activation of HNF1α is enhanced by co-expressed MKK3 (1), a MAP kinase kinase that also can activate p38 MAP kinase (9–11). Therefore, the basal kinase activity of mirk is enhanced by a MAP kinase kinase, which mediates certain stress signals in vivo. Possibly mirk can transactivate other transcription factors in addition to HNF1α. Mirk bound to a novel protein in yeast two-hybrid analysis, which had several characteristics common to the PAX6 group of transcription factors.2 However, the identity of genes up-regulated by mirk is unknown. Such genes must have some role in cell cycle regulation or cell survival as stable overexpression of mirk allows cells to remain cycling in the absence of serum growth factors (6). In the current study we demonstrate that the activation pathway that controls the HNF1α transcriptional transactivator activities of mirk includes the MAP kinase p38 (9–11). Mirk activation is shown to be inhibited by p38 MAP kinase in a novel cell cycle-regulated, kinase-independent manner.

EXPERIMENTAL PROCEDURES

Materials—Antibodies to MKK3, p38, HNF1α, and residues 37–51 of human PML (PG-M3) were from Santa Cruz, and to the FLAG epitope from Sigma. Rabbit polyclonal antibody to a unique sequence at the C terminus of mirk was raised as described (6). Polyvinylidene difluoride transfer paper Immobilon-P was purchased from Millipore. PLUS reagent and LipoFectAMINE were from Invitrogen, all radioactive materials were purchased from PerkinElmer Life Sciences, and ECL reagents were from Amersham. All other reagents were from Sigma.

Cell Culture—NIH3T3 cells, HD3 colon carcinoma cells, and 293T cells were maintained in Dulbecco’s modified Eagle’s medium containing 7% fetal bovine serum, modified, and supplemented as described (12).

Plasmids—pHX9P (mirk) and pHX9P (kinase-inactive YF mirk) had loytic leukemia; HIPK2, homeodomain-interacting protein kinase-2; ERK, extracellular signal-regulated kinase.

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been previously generated (6), as were the mirk deletion mutants. The (β-28)–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 pBj5-DcoH, and the expression plasmid pBj5-HNF1α were the kind gifts of Dr. G. Crabtree, Stanford. M KK3b, M KK3b(E), p38, and p38AF, each in pcDNA3-FLAG, were the kind gifts of Dr. J. Han, Scripps Institute. 3C-HNF1 (1–283 amino acids) had been constructed in an earlier study (1).

In Vitro Synthesized mirk and Kinase Reaction—In vitro kinase reactions were performed with glutathione S-transferase-mirk as described in Ref. 1.

Transient Transfections—293T cells were transiently transfected by adding a complex of LipofectAMINE (2–4 μg of DNA) in serum-free medium for 24 h. For reporter gene assays, NIH3T3 cells were seeded the day before transfection at 0.9 × 10^6 cells/well in 24-well plates in complete medium containing 7% fetal bovine serum. Leptomycin B (10 nM) was added 1–2 h before transfection. The amount of total DNA used was kept constant by 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.

Co-immunoprecipitations: mirk, p38, and M KK3E—293T cells in multiple 60-mm dishes were co-transfected with 1 μg of DNA of either M KK3E, p38 wild-type, or p38AF, together with either expression plasmids for mirk, kinase-inactive YF-mirk, or pHisA vector (1 μg/well), allowed to express for 24 h, then each dish was lysed in 0.25 ml of EBC buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM diethiothreitol, and a tablet of protease inhibitor (Roche Molecular Biochemicals). An aliquot of total lysate of 300 μg was immunoprecipitated with 5 μl of anti-C2 mirk anti-peptide rabbit polyclonal antibody overnight at 4 °C, then the complexes were collected by addition of 20 μl of protein A-agarose and incubation for 2 h at 4 °C, then washed three times with EBC buffer and separated by SDS-PAGE.

Immunodetection—Following treatment as indicated and washing with cold phosphate-buffered saline, cells were lysed in EBC or RIPA buffer (1× phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors: 20 μg/ml leupeptin, 20 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 200 μg/ml sodium orthovanadate, and 20 mM sodium fluoride). Lysates were pelleted in a microcentrifuge for 15 min to remove insoluble material. Depending upon the experiment 10–50 μl of cell lysate were blotted onto polyvinylidene difluoride membranes after separation on SDS-PAGE. The blots were blocked in 5% milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature, 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 affinity-purified polyclonal antibody directed to the mirk auto-regulatory domain. Band density in autoradiograms was measured using a Lacie Silverscanner and silverscanner III software and analyzed by the IP LabGel program.

Size Fractionation by FFLC—HD3 colon carcinoma cells were synchronized by maintenance at confluent density for 2 days (14), serum-starved for 2 days, then re-seeded into multiple 150-mm dishes in complete medium containing 7% fetal bovine serum. Leptomycin B (10 μM final concentration) was added at the time of release. Cells in mid-G1, (6-h-released) and cells in mid-S phase (18-h-released) were lysed in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, and a tablet of protease inhibitors (Roche Molecular Biochemicals). The total lysate was adjusted to 4 mg/ml and 0.2 ml loaded onto a Superdex 200 column (Amersham Biosciences) and eluted in EBC buffer (20 mM HEPES, pH 7.5, 250 mM NaCl, 1 mM EDTA, 0.1% β-mercaptoethanol, and 0.01% Tween 20) at 0.4 ml/min with fractions of 0.4 ml dropped into tubes containing 10 μg of BSA per tube. Proteins were precipitated with acetone, 1 ml per tube, at −20 °C overnight, then pelleted and dried before resuspension in SDS sample buffer following SDS-PAGE and Western blotting. Samples, which were subjected to immunoprecipitation with anti-MKK3 or anti-p38, were dialyzed against lysis buffer overnight, then incubated with either 2 μg of antibody or 2 μg of normal rabbit serum for 24 h at 4 °C, then 20 μl of a slurry of protein A-agarose conjugates (Santa Cruz Biotechnology) was then added and incubated for an additional 6 h. The agarose beads were extensively washed four times, followed by SDS-PAGE and Western blotting.

RESULTS

Co-expressed p38 Blocks Mirk Transactivation of HNF1α—Recently mirk protein kinase has been shown to function as a transcriptional activator of HNF1α and to be stimulated as an activator by co-expression with the MAP kinase kinase M KK3 (1). Since M KK3 is an activator of p38 (9–11), M KK3 can activate either p38 or mirk. The choice of kinase substrate could depend on the identity of the upstream kinase activating M KK3 or the presence or absence of scaffold proteins such as I B2JIP2 (15). Mirk had also been shown to be a substrate of p38 in vitro (6), so in vivo p38 might directly activate or inactivate mirk by phosphorylation. We tested whether co-transfection of p38 would alter mirk transcriptional activation of HNF1 using the β-fibrinogen promoter reporter construct (β-28)–Luc in transient transfection experiments. Mirk increased the activity of HNF1 about 2.5-fold (Fig. 1A, compare lanes 1 and 2), while co-expression of p38 blocked the stimulation of HNF1 by Mirk. Inhibition of HNF1 activation was not due to the kinase activity of p38 as dominant negative p38AF, doubly mutated at its activation domain, also inhibited HNF1 activation by mirk. Co-expression of either p38AF or wild-type p38 with HNF1 in the absence of mirk decreased HNF1 transcriptional activity by 30–50%, which could be caused by either inhibition of endogenous mirk activity or direct inhibition of HNF1. Since either effect was mediated by kinase-inactive p38, this eliminated direct phosphorylation of HNF1 by p38 as a mechanism of action. Transcriptional activation by mirk was dependent on full-length HNF1. C-terminal-deleted HNF1 (ΔHNF1) containing only amino acids 1–283 could not substitute for full-length HNF1 in this assay. Only background level activation of the reporter gene could be detected when ΔHNF1 was co-transfected with or without mirk (Fig. 1B), although mirk binds to ΔHNF1 (1–283) and phosphorylates it (1). Neither kinase-inactive p38 (AF) or wild-type p38 affected ΔHNF1 (1–283).

Physical Interaction between mirk and p38—We then tested whether mirk and p38 directly interact in vivo by determining whether they could co-immunoprecipitate. FLAG epitope-tagged wild-type p38 or dominant negative p38AF were co-transfected into 293T cells with either wild-type mirk or mirk plus M KK3E (Fig. 2A). Mirk, p38 wild-type and p38AF were synthesized at comparable levels in each experimental mixture, as shown by Western blotting of the cell lysates (Fig. 2A, lower two lanes). In vivo interaction between p38 and mirk was demonstrated by their co-immunoprecipitation (Fig. 2A). Kinase-inactive p38AF bound mirk about twice as avidly as wild-type p38. Each of the four isoforms of p38, α, β, γ, and δ, were expressed in vivo alone or in the presence of mirk, and the ability of each isoform to interact with mirk in vivo was tested by immunoprecipitation of mirk. Only α and β were capable of complexing and co-immunoprecipitating with mirk (Fig. 2B).

The complexing of p38 to mirk blocked the kinase activity of mirk. Increasing amounts of purified recombinant non-activated p38 (0.1–3 μg) were mixed together with 1 μg of purified glutathione S-transferase-mirk, and an in vitro kinase assay was performed on myelin basic protein (MBP). Without activation p38 had little kinase activity on MBP, while mirk exhibited MBP kinase activity (Fig. 2C). Mirk kinase activity was inhibited by roughly equimolar concentrations of inactive p38, consistent with the model that p38 sequesters mirk. Since p38AF is a potent inhibitor of mirk activation of HNF1 (Fig. 1), it is likely that p38α and p38β sequester mirk and prevent its activation by M KK3E.

X. Deng, S. Lim, Z. Yan, S. Mercer, and E. Friedman, submitted for publication.
Mirk Levels Are at the Highest in G₀/G₁ Phase but Fall 10-fold when Cells Enter S Phase—If endogenous p38 sequesters endogenous mirk in vivo, the stoichiometry of the two kinases would be critical. We next investigated whether there were any cellular conditions in which sequestering of mirk might be favored or unfavored. Mirk protein levels had been shown to vary several-fold when cells were placed in serum-free conditions (6). Mirk protein levels had also been shown to be highest in G₀/G₁ and fall when cells entered S phase.³ We compared mirk levels and p38 levels in NIH3T3 cells that had been synchronized in G₀/G₁ by growth to high density, then released by culture at lower density. The level of mirk in G₀/G₁ at the release time was about 7-fold higher than the mirk level when NIH3T3 cells had entered S phase 18 h post-release, while in contrast the level of p38 varied no more than 8% (Fig. 3A). A similar imbalance between mirk and p38 levels was seen when HD3 colon carcinoma cells were placed in serum-free medium (Fig. 3B). Mirk levels increased 11-fold, while p38 levels remained constant in HD3 cells placed in serum-free medium for 22 h. Therefore, p38 might only be effective in sequestering mirk in proliferating cells when mirk levels were lowest.

p38 Displaces mirk in Binding to MKK3E—The ability of p38 to sequester mirk was tested by co-expressing mirk with MKK3E in the presence of increasing levels of FLAG-p38AF and then analyzing the molecules bound to either immunoprecipitated mirk (Fig. 4A) or immunoprecipitated MKK3E by Western blotting (Fig. 4B). In the absence of p38AF, mirk bound well to MKK3E. When the concentration of p38AF increased 10-fold, more FLAG-p38AF and less MKK3E was found associated with mirk in a dose-dependent manner (Fig. 4A, lower panels). At the highest concentration of p38AF, all of the MKK3E in the mirk immunoprecipitates had been replaced with FLAG-p38AF (Fig. 4A, last lanes). Similarly when MKK3E immunocomplexes were analyzed, increasing amounts of p38AF led to the displacement of mirk (Fig. 4B, lower panels). This competition experiment was repeated using increasing levels of wild-type p38. In the absence of wild-type p38, MKK3E bound effectively to immunoprecipitated mirk, but when wild-type p38 was co-expressed, the amount of MKK3E bound to mirk decreased in a dose-dependent manner (data not shown). These experiments demonstrated that p38 blocked the association of MKK3E and mirk in a kinase-independent manner.

p38 Blocks MKK3E Activation of mirk—In earlier studies we had shown that the constitutively active form of MKK3, MKK3E, enhanced the kinase activity of mirk on myelin basic protein (1). In addition (1), mirk and MKK3E each activated HNF1α’s transcriptional activity 3–5-fold, while wild-type and kinase-inactive p38AF complexes functioned synergistically to increase the activity of HNF1α 15-fold (1). The synergistic activation of HNF1 by mirk and MKK3E was strongly inhibited by p38 in a dose-dependent manner in promoter reporter assays (Fig. 5, lanes 1–7). Substitution of p38AF for wild-type p38 also strongly inhibited HNF1 activation by mirk and MKK3E. The dominant negative p38AF, which complexed more strongly to mirk than wild-type p38 (Fig. 2A and data not shown), appeared to be a more effective inhibitor of mirk activation of HNF1. The same range of concentrations of p38 and p38AF were co-transfected with mirk, yet the lowest concentration of p38AF was still inhibitory, while the lowest concentration of wild-type p38 was ineffective (Fig. 5). Both wild-type p38 and p38AF inhibited HNF1 activation mediated by ΔCmirk, as well (Fig. 5, last three lanes). Thus, when p38 or p38AF is co-transfected, they bind to mirk, sequester it, and prevent the activation of mirk as a kinase by MKK3E and the subsequent transactivation of HNF1 by mirk.

Mirk Does Not Inhibit p38 Activation—Mirk does not inhibit p38 activation in vivo. Mirk was expressed at two concentrations, 1× and 4×, together with wild-type p38α and the constitutively active p38 activator MKK3E. Neither wild-type mirk nor the deletion mutant ΔN-mirk prevented activation of p38 by MKK3E, as shown by immunoblotting with antibody specific for the dually phosphorylated activation domain of p38 (Fig. 6, top panel). Similar amounts of p38 were expressed in the absence of mirk or in its presence, as shown by Western blotting. Therefore, p38 binds to mirk and prevents the activation of mirk as a kinase by MKK3E, while mirk bound to p38 does not block the activation of p38 by MKK3E.

Co-localization of mirk and p38 in Subnuclear Complexes with PML—Biological and physical interaction between exogenous, overexpressed mirk and p38 had been readily detected,
but no association between endogenous Mirk and endogenous p38 could be detected by similar immunoprecipitation studies. However, mirk-enhanced green fluorescent protein localized within discrete bodies, some intranuclear, within NIH3T3 cells so we speculated that such bodies might have to be dissociated to detect interactions between endogenous mirk and p38. Accordingly, we tested this hypothesis by determining whether endogenous mirk migrated as a 70-kDa monomer, or whether endogenous Mirk and p38 would co-localize in these complexes. Lysates from a stable mirk transfectant of HD3 cells, T10, and a vector control NIH3T3 cells were grown to confluent density and maintained for 2 days in 0.2% serum (zero time) then replated at one-third density in replicate plates, which were examined 3, 6, 9, 12, or 18 h post-release by Western blotting for mirk, p38, or cyclin A. B: HD3 colon carcinoma cells in log phase growth were placed in serum-free medium for 0–22 h and replicate cultures examined by Western blotting for mirk or p38.

Variation in Mirk Abundance with Cell Cycle Progression

A. NIH3T3 Cells

| Hours Released from Density Arrest | WB: p38 |
|-----------------------------------|---------|
| 0                                 |         |
| 3                                 |         |
| 6                                 |         |
| 9                                 |         |
| 12                                |         |
| 18                                |         |

B. HD3 colon carcinoma cells

| Hours in Serum-free Medium | WB: p38 |
|----------------------------|---------|
| 0                          |         |
| 2                          |         |
| 4                          |         |
| 22                         |         |

Fig. 3. Endogenous mirk protein levels are highest in G0/G1. A, NIH3T3 cells were grown to confluent density and maintained for 2 days in 0.2% serum (zero time) then replated at one-third density in replicate plates, which were examined 3, 6, 9, 12, or 18 h post-release by Western blotting for mirk, p38, or cyclin A. B: HD3 colon carcinoma cells in log phase growth were placed in serum-free medium for 0–22 h and replicate cultures examined by Western blotting for mirk or p38. Over a broad size range, 150–700 kDa. Mirk was seen to migrate in a broad size range during gel filtration chromatography in each of six experiments with synchronized cells and in each of five experiments with non-synchronized cells. The relative abundance of the different mirk-containing complexes on gel filtration varied somewhat from experiment to experiment suggesting that our extraction procedure disrupted mirk-containing intranuclear structures, possibly ND10/PML nuclear bodies, which have been postulated to act as depots for proteins released upon cellular stress (18). There is some precedent for this interpretation. The serine/threonine kinase homeodomain-interacting protein kinase-2 (HIPK2), which is a member of the dyrk/mirk family, co-localizes and interacts with p53 and CBP within PML nuclear bodies (17, 18). To test this hypothesis, the FPLC fractions were examined for the presence of PML protein by Western blotting. PML protein was detected at its presumed monomer size of about 70 kDa and in two overlapping peaks at 660 kDa and at 150 kDa (Fig. 7A). These data indicate that mirk and PML co-migrate through Superdex 200 and may represent portions of disrupted nuclear bodies.

We next tested whether any cellular conditions could cause co-migration of p38 and mirk through Superdex 200. Leptomycin B is a specific inhibitor of nuclear export, which interferes with the binding of nuclear export signals to the export receptor CRM1. Treatment of cells with leptomycin B caused the accumulation of 500–700-kDa mirk-containing complexes in both G0 and S phase cells (Fig. 7, B and D, and data not shown) and also caused the accumulation of p38 and MKK3, the upstream activator of p38 and of mirk, within complexes of similar molecular weight (Fig. 7, B and D). Approximately 17–20% of the total mirk, MKK3, and p38 accumulated within these diffusely migrating subnuclear complexes (Fig. 7B).

We next tested whether increasing the concentration of mirk by stable transfection would increase the fraction of mirk in the high molecular mass 500–700-kDa complexes and whether p38 would co-localize in these complexes. Lysates from a stable mirk transfectant of HD3 cells, T10, and a vector control transfectant, RV1 (6), were subjected to size fractionation on Superdex 200. In the transfectant cells, more mirk proteins were found in the 500–700-kDa complexes compared with con-

Fig. 2. Physical interaction between mirk and p38 MAP kinase. A, 293T cells were co-transfected with either MKK3E, wild-type mirk, and either FLAG-p38 wild-type or kinase-inactive FLAG-p38AF, and following protein expression, mirk and its associated proteins were immunoprecipitated by antibody to mirk. The mirk immunoprecipitates were examined by Western blotting for the FLAG epitope on the expressed p38 and p38AF proteins. Similar amounts of p38 wild type and p38AF were synthesized in the lysates, and equal amounts of mirk were found in the appropriate lysates (Western blot of total lysates shown in lower two panels). One representative experiment of four repeat experiments with the same results is shown. B, Physical interaction between mirk and only the or or β isoforms of p38 MAP kinase. Equal amounts of the four p38 MAP kinase isoforms, α, β, γ, δ, were synthesized in 293T cells following transfection in the presence of co-expressed mirk or vector DNA (Western blots of lysates shown in lower two panels). Mirk and its associated proteins were immunoprecipitated by antibody to mirk. The mirk immunoprecipitates were examined by Western blotting for the FLAG epitope on the expressed p38 isoforms. C, in vitro kinase assay demonstrates that p38 MAP kinase inhibits mirk. Kinase mixtures in vitro contained either recombinant purified mirk or increasing concentrations of recombinant purified p38, as indicated. Kinases were added to MBP, and in vitro kinase assays were performed with [32P]ATP and analyzed by autoradiography after SDS-PAGE. One of three replicate experiments with similar results is shown.
trol cells (compare Fig. 7, C and A). In mirk transfectant T10 cells, a small peak of p38 protein, 5% of the total, migrated at 500–700 kDa, the same position as mirk and PML (compare Fig. 7, C and A), and the monomer peak of p38 developed a shoulder, indicating intermediate formation of larger complexes. Thus, when mirk levels increase in the 500–700-kDa nuclear complexes, p38 also accumulates in these complexes where it can potentially sequester mirk and block its capacity to function as a transcriptional co-activator.

We next attempted to determine whether mirk, p38, MKK3, and PML are associated within the same complex by immunoprecipitation experiments, using lysates of G1 synchronized HD3 cells treated with LMB to accumulate the large complexes (Fig. 7D). We attempted to immunoprecipitate MKK3 from the total lysates before fractionation on Superdex 200, from pooled fractions 20–25 (the 500–700 kDa complexes) and from pooled fractions 28–34 (proteins and protein complexes of 66–150 kDa). The putative immunoprecipitates were then analyzed by Western blotting following size fractionation by SDS-PAGE (Fig. 7E), with an aliquot of either total lysate, pooled fractions 20–25, or pooled fractions 28–34 as controls. MKK3 was

**Fig. 5.** The amount of co-expressed wild-type p38 or kinase-inactive p38AF determines the extent of suppression of mirk activity. 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 NIH3T3 cells, with expression plasmids for mirk (M), ΔCmirk (with amino acids 436–629 deleted), HNF1 (H), wild-type p38 (p38), and kinase-inactive p38AF (AF). The amounts of p38 expression plasmids co-expressed were 1, 0.25, 0.125, and 0.05 µg of DNA, as noted. Data shown are the mean ± S.E. of triplicate measurements, normalized for co-transfected β-galactosidase, and are representative of three separate experiments. The S.E. is only shown if >5%.

**Mirk Does Not Inhibit the Activation of Co-Expressed p38**

**Fig. 6.** Mirk does not inhibit the activation of co-expressed p38. 293T cells were co-transfected with either MKK3E, wild-type FLAG-p38, or two concentrations of wild-type mirk or ΔN kinase-inactive mirk, 1× and 4×, as noted. ΔNmirk consists of amino acids 110–629. Increasing amounts of either mirk construct were synthesized, while similar amounts of p38 were synthesized in the appropriate lysates (Western blot of total lysates shown in lower two panels). Upper panel, Western blot for the dually phosphorylated activation domain of p38 MAP kinase. The data are representative of two replicate experiments.
FIG. 7. Size fractionation of endogenous mirk by FPLC. A, HD3 colon carcinoma cells were synchronized by growth to confluent density (see “Experimental Procedures”) and lysed in mid G1 phase. Lysates were subjected to size fractionation on Superdex 200 and fractions analyzed for the presence of mirk, p38, and PML by Western blotting. A graph of Western blots is shown with the position of size markers of 660, 66, and 29 kDa indicated and is representative of two independent experiments. B, HD3 colon carcinoma cells were synchronized by growth to confluent density and released in the presence of 10 nM leptomycin B, then lysed in mid G1 phase in parallel to the data shown in A. Lysates were subjected to size fractionation on Superdex 200 and fractions analyzed for the presence of mirk, p38, and MKK3 by Western blotting. A graph of Western blots is shown with the position of a size marker of 660 kDa shown. The p38 data shown are representative of two separate experiments, and similar data were seen in a parallel experiment performed on S phase synchronized cells. C, lysates of unsynchronized mirk transfectant T10 of HD3 cells were subjected to size fractionation on Superdex 200 and fractions analyzed for the presence of mirk and p38 by Western blotting. A graph of Western blots with the position of a size marker of 660 kDa is shown. PML was seen co-migrating with mirk in these experiments (not shown) as in A. D, Western blots for mirk, MKK3, and p38 from Superdex 200 fractions of lysates from G1 synchronized HD3 cells treated with leptomycin B as indicated. E, immunoprecipitation experiments for MKK3 were performed with either total lysates of G1 synchronized HD3 cells, shown in D, or from pools of FPLC fractions of size-fractionated lysates, also shown in D. TL, total lysate; 20–25, fractions 20–25 comprised of protein and protein complexes of 200–700 kDa; and 28–34, fractions 28–34 comprised of proteins and protein complexes of 66–150 kDa. The immunoprecipitates were analyzed by Western blotting for MKK3, with control lanes of either total lysates or of pools of fractions 20–25 or pools of fractions 28–34 before immunoprecipitation. F, immunoprecipitation experiments for p38 were performed with either total lysates of G1 synchronized, leptomycin B-treated HD3 cells, shown in D, or from pools of FPLC fractions 19–24 of size fractionated lysates, also shown in D.
readily immunoprecipitated from total lysates (Fig. 7E, upper panel) and from the pool of the 66–150-kDa fractions, fractions 28–34 (Fig. 7E, lower panel). However, MKK3 could not be immunoprecipitated from the pool of 500–700-kDa protein complexes, although the MKK3 in this pool could readily be detected by Western blotting with the same antibody following boiling of the complexes in SDS sample buffer and electrophoresis. Since MKK3 could be immunoprecipitated from the smaller molecular weight complexes, but not the larger ones, the difference could not be due to the fractionation procedure or subsequent dialysis. We conclude that the antibody used for immunoprecipitation was not able to detect MKK3 in the complexes, because the immunogenic epitope was masked by some component in the complex, possibly the PML scaffolding protein. This experiment was repeated with antibody to p38. Again, p38 was readily immunoprecipitated from total lysates, but could not be immunoprecipitated from pooled high molecular mass complexes (Fig. 7F). These data, taken together, indicate that mirk, p38, and MKK3 can be found in large molecular weight subnuclear complexes, some of which contain the nuclear body scaffolding protein PML. However, it was not possible to demonstrate that the proteins were found within the same large molecular weight complex by co-immunoprecipitation experiments. Immunogenic epitopes on both p38 and MKK3 were blocked by some component of the subnuclear complex, which could only be dissociated by boiling in SDS sample buffer.

**DISCUSSION**

We have uncovered a novel function of the MAP kinase p38, direct inhibition of the transcriptional transactivator protein kinase mirk/dyrk1B by blocking access of this kinase to its upstream kinase activator, the MAP kinase kinase MKK3. The p38α and p38β isoforms bind to mirk, and in so doing displace mirk within in vivo complexes with MKK3. This activity of p38 is independent of its kinase activity as the kinase-inactive p38AF mutant was at least as potent as wild-type p38 in binding to mirk, in displacing mirk from association with MKK3, and in inhibiting the activity of mirk as a transcriptional activator. Although mirk and p38 bind together in vivo, the inhibition is unidirectional. While p38 inhibits the activation of mirk by MKK3, mirk does not inhibit the activation of p38. Elevated levels of mirk did not block activation of p38, which was detected by Western blotting for phosphorylation within the p38 activation domain.

A second novel observation of the current study is the localization of endogenous mirk in subnuclear complexes, some of which contain PML, p38, and/or MKK3. These complexes accumulate when nuclear export is blocked by leptomycin B and when mirk is overexpressed. We can identify mirk-containing complexes of roughly 500–700 kDa. The nuclear body scaffolding protein PML was found within these complexes, suggesting that they are portions of larger structures such as nuclear bodies, which are dissociated by our lysis buffer. There is precedent for this interpretation. The HIPK2, which is a member of the dyrk/mirk family, co-localizes and interacts with p35 and CBP within PML nuclear bodies (17, 18). HIPK2 binds to and activates p35 by directly phosphorylating it at Ser46. HIPK2 and p53 cooperate in the activation of p53-dependent transcription, which results in growth arrest and the enhancement of UV-induced apoptosis (17, 18). HIPK2 also co-localizes with the p53 family member p73 in nuclear bodies and modulates transcriptional regulation by p73 (19). Mirk, like HIPK2, phosphorylates a transcription factor; for mirk it is the transcription factor HNF1α within its CBP binding domain (1). Most HIPK2 was localized in subnuclear speckles, and a small amount was in the nucleoplasm. Transfected mirk-enhanced green fluorescent protein was seen within discrete bodies, some of which were intranuclear. It is unknown whether HIPK2 in the nuclear bodies functioned as a transcriptional modulator. Similarly, we do not know whether mirk associated with the 500–700-kDa intranuclear complexes functions as a transcriptional activator or whether this activity is limited to the mirk 150-kDa dimer. Some investigators have hypothesized that nuclear bodies are depots for unused proteins, which are released upon cell stress (16). However, subpopulations of nuclear bodies may have distinct functions. Nuclear DNA helicase II/RNA helicase A was found to be a component of a subset of PML nuclear bodies (20). After interferon treatment, however, NDHII co-localized with practically all nuclear bodies, particularly those with newly synthesized RNA and RNA polymerase II. Other investigators have identified another potential function for a subset of nuclear bodies. The hMre11-Rad50-NBS1 repair complex co-localized with PML nuclear bodies at sites of radiation induced DNA damage, suggesting that these nuclear bodies function in the recruitment of proteins required for both checkpoint and DNA repair responses (21).

Sequestration of mirk by p38 could occur in such nuclear bodies. Other investigators have found that sequestration of the Wnt-responsive transcription factor LEF1 into nuclear bodies mediates repression of LEF1 activity (22). PIASy, a nuclear matrix-associated SUMO E3 ligase, mediates sumoylation of LEF1 in vitro and in vivo. PIASy represses LEF1 transcriptional activity and targets LEF1 to nuclear bodies. Co-expression of LEF1 and PIASy redistributed LEF1 from whole nuclear staining into punctate structures that co-localized with PIASy.

The nucleus has recently been proposed as a site for ERK sequestration through nuclear anchoring proteins and for ERK signal termination, as well as a site for ERK action (23). Recently evidence was presented that activated p38α can sequester ERK1/2 and sterically block their phosphorylation and activation by MEK1 (24). The interaction of ERK1/2 with p38α and the interaction of mirk with p38α or p38β shown here differ. Phosphorylation and activation of p38 enhanced its interaction with ERK1/2, whereas kinase-inactive p38α appeared superior to wild-type p38α in sequestering mirk and blocking its activity. Activated p38 has also been reported to allosterically regulate the casein kinase CK2 (25). Cross-talk between signaling cascades has been seen in upstream activating kinases, but these reports indicate that direct interaction between the end point kinases themselves occurs.

p38 has been found to inhibit another transcriptional activation pathway in addition to MKK3/E/mirk/HNF1α, indicating that transcriptional down-regulation of certain genes may be a common response to stress signals. Stimulation of insulin gene promoter activity by insulinotropic hormone glucagon-like peptide 1 (GLP-1) was found to be inhibited by p38 (26). As a result treatment with SB203580, which blocks p38 activation, led to a pronounced increase in insulin promoter activity. The authors postulated that during periods of cellular stress in which p38 activity is stimulated, p38 may repress insulin gene expression. This would allow transcription levels of insulin to be precisely controlled. They further speculated that reduced insulin expression levels caused by chronic hyperglycemia or hyperlipidemia, which result in glucotoxicity or lipotoxicity, could be directly caused by p38 activation (26). We do not know the genes activated by MKK3/E/mirk/HNF1α signaling, but since stable mirk-overexpressing colon carcinoma cells show extended growth in serum-free media (6), we speculate that the genes may include those involved in cell survival.

In the current study MKK3 was shown to function at a cross-road in signaling, activating p38 in response to certain
stress signals, but being capable of activating mirk as a transcription factor transactivator when p38 levels are insufficient to complex and sequester mirk. The allosteric interaction between p38 and mirk is dependent on the relative abundances of both proteins within the same cellular compartment. While p38 protein levels were relatively stable throughout the cell cycle, mirk levels varied about 10-fold in two cell lines. The highest levels of mirk were found in serum-starved or density arrested cells in G0/G1, while one-tenth as much mirk was detected in cells in S phase. We speculate from these data that p38 is only able to sequester mirk and block the function of mirk when mirk levels are low in proliferating S phase cells, allowing mirk to operate as a transcriptional activator only in G0/G1. This is consistent with its likely mediation of neuronal maturation, leading to the stimulation of subsequent CRE-mediated transcription during neuronal differentiation in hippocampal progenitor cells (8), an activity consistent with its likely mediation of neuronal maturation in vivo. Thus the function of p38/mirk1B as an HNF1α kinase (1) may mediate some aspects of cellular differentiation, while dyrk1A mediates neuronal differentiation by functioning as a CREB kinase in response to the neurogenic factor, basic fibroblast growth factor (8).

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