Mirk/dyrk1B Kinase Destabilizes Cyclin D1 by Phosphorylation at Threonine 288*

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The phosphorylation of cyclin D1 at threonine 286 by glycogen synthase kinase 3β (GSK3β) has been shown to be required for the ubiquitination and nuclear export of cyclin D1 and its subsequent degradation in the proteasome. The mutation of the nearby residue, threonine 288, to nonphosphorylatable alanine has also been shown to reduce the ubiquitination of cyclin D1, suggesting that phosphorylation at threonine 288 may also lead to degradation of cyclin D1. We now demonstrate that the G0/G1-activated arginine-directed protein kinase Mirk/dyrk1B binds to cyclin D1 and phosphorylates cyclin D1 at threonine 288 in vitro and that the cyclin D1-T288A construct is more stable than wild-type cyclin D1. Transient overexpression of Mirk in nontransformed Mv1Lu lung epithelial cells blocked cells in G0/G1. Depletion of endogenous Mirk by RNA interference increased cyclin D1 protein levels but not mRNA levels, indicating that Mirk destabilizes cyclin D1 protein. Destabilization was confirmed by induction of a stable Mirk transfectant of Mv1Lu cells, which blocked cell migration (Zou, Y., Lim, S., Lee, K., Deng, X., and Friedman, E. (2003) J. Biol. Chem. 278, 49573–49581), and caused a decrease in the half-life of endogenous cyclin D1, concomitant with an increase in Mirk expression. In vitro cyclin D1 was phosphorylated in an additive fashion by Mirk and GSK3β. Mirk-phosphorylated cyclin D1 mutated at the GSK3β phosphorylation site and was capable of phosphorylating cyclin D1 in the presence of the GSK3β inhibitor LiCl. Mirk may function together with GSK3β to assist cell arrest in G0/G1 by destabilizing cyclin D1.

Cell cycle progression in eukaryotic cells is mediated by cyclin-dependent kinases (CDKs). The D-type cyclins, D1, D2, and D3, increase in nuclear abundance in G1 in response to mitogens, facilitate the import of CDK4 into the nucleus (1), and assemble combinatorially with CDK4 or CDK6 into complexes that phosphorylate the retinoblastoma protein, releasing factors needed for the progression into S phase. Cyclin D1 is translocated into the cytoplasm during S phase where it is destroyed by the proteasome following phosphorylation at threonine 286 by GSK3β (2, 3). Mutant cyclin D1-T286A, which cannot be phosphorylated by GSK3β, is stabilized in the nucleus and is capable of transforming murine fibroblasts, whereas overexpression of wild-type cyclin D1 cannot act alone to transform such cells (4). A cyclin D1 isoform derived by alternative splicing was shown to lack threonine 286, enabling this cyclin D1 isoform to remain nuclear throughout the cell cycle, remain highly expressed, and function to facilitate transformation of NIH3T3 cells (5). This cyclin D1 splice variant was also found in tumor-derived cells and primary human esophageal tumors (5). Overexpression of cyclin D1 occurs in several cancers including breast, pancreatic, and esophageal (6), suggesting that either increased transcription, transcription of stable splice variants, or dysregulation of cyclin D1 turnover may frequently occur in cancer.

In this study, we have studied the interaction of the ubiquitously expressed protein kinase Mirk/dyrk1B with cyclin D1. Mirk/dyrk1B is an arginine-directed serine/threonine kinase (7), which functions as a transcriptional co-activator and is activated through the stress-activated mitogen-activated protein kinase kinase MKK3 (8). We have shown recently that Mirk stabilizes the CDK inhibitor p27kip1 in the G0 phase of the cell cycle in NIH3T3 fibroblasts, whereas depletion of Mirk by RNA interference increases cell cycling as measured by increased PCNA expression (9). Mirk expression is decreased by mitogen activation of the MEK-ERK pathway during G1 (10), restricting Mirk function primarily to G0 and early G1. We now confirm that transient overexpression of Mirk in nontransformed Mv1Lu lung epithelial cells increases the length of G0/G1, by FACS analysis and that Mirk targets the G1 cell cycle regulator, cyclin D1, to maintain cells in growth arrest.

EXPERIMENTAL PROCEDURES

Materials—Affinity-purified rabbit polyclonal antibody to a unique sequence at the C terminus of Mirk and affinity-purified rabbit polyclonal antibody to a unique sequence at the N terminus of Mirk were raised as described previously (7). Antibodies to cyclin D1 and GSK3β were from Santa Cruz Biotechnology. Antibody to GSK3β phosphorylated at serine 9 was purchased from Cell Signaling Technology. Recombinant purified N-terminal histidine-tagged GSK3β was from Sigma. Polyvinylidene difluoride transfer membrane Immobilon-P was purchased from Millipore. PLUS reagent and LipofectAMINE were from Invitrogen. All of the radioactive materials were purchased from PerkinElmer Life Sciences, and ECL reagents were from Amersham Biosciences. All of the other reagents were from Sigma.

Plasmid Construction—The murine pFLEX-cyclin D1 construct and FLAG-tagged cyclin D1-T286A derivative were the kind gifts of Drs. Martine Roussel and Charles Sherr, and the wild-type GSK3β construct was the kind gift of Dr. Alan Diehl. Plasmids pcDNA3.1 (Mirk) and pcDNA3.1 (kinase-inactive YF Mirk) had been generated previously (7). Wild-type cyclin D1 was subcloned into pGEX-4T1 (Amersham Biosciences) to make GST-tagged mutants by site-directed mutagenesis by the GeneEditor system (Promega). Constructs were subcloned into

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‡ The abbreviations used are: CDK, cyclin-dependent kinase; GSK3β, glycogen synthase kinase 3β; MEK, mitogen-activated protein kinase kinase; PCNA, proliferating cell nuclear antigen; Mirk, mitogen-activated protein kinase/extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorter; GST, glutathione S-transferase; RNAi, small interfering RNA; GFP, green fluorescent protein; E3, ubiquitin-protein isopeptide ligase.
pCMV-tag2B to generate FLAG-tagged cyclin D1 constructs. All of the mutant cyclin D1 constructs were sequenced to confirm the mutated sequence.

Cell Culture—Mv1Lu mink lung epithelial cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, modified, and supplemented as described previously (11). The Mirk-inducible Mv1Lu C9 subline was isolated by stably transfecting cells with a pCMV-LacI-repressor construct, selecting the hygromycin-resistant clone, and then stably transfecting Mirk in a pOPR51/MCS vector using G418 (LacSwitch II System, Stratagene) (12).

Immunodetection—Following treatment as indicated and washing with cold phosphate-buffered saline, cells were lysed in radioimmune precipitation assay buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors 20 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 200 μM sodium orthovanadate, and 20 μM sodium fluoride). Lysates were pelleted in a microcentrifuge for 15 min to remove insoluble material. Depending upon the experiment, 10–50 μg of cell lysates were blotted onto polyvinylidene difluoride membranes after separation by 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 and incubated for 1 h at room temperature with primary antibody in TBST plus 4% bovine serum albumin, and proteins were detected subsequently by enhanced chemiluminescence. All of the Mirk blots used affinity-purified polyclonal antibody directed to the Mirk unique C terminus.

Transient Transfections—Mv1Lu cells were transfected transiently by adding a complex of PLUS reagent (3 μg DNA) and LipofectAMINE (2–4 μg μl DNA) in serum-free media for 3 h and then allowing the expression in growth medium for 24–36 h, or they were transfected using LipofectAMINE 2000 (3 μg μl DNA) in serum-containing medium for 24–36 h as noted. The amount of total DNA used was kept constant by the addition of empty vector DNA.

Co-immunoprecipitation of Mirk and Cyclin D1—Mirk and cyclin D1 expression plasmids were co-transfected into Mv1Lu cells and allowed to express for 48 h. After lysis in 50 mM Tris-HCl, pH 8.0, and 0.5% Nonidet P-40 with protease inhibitors, cyclin D1 was immunoprecipitated with antibodies to the FLAG epitope followed by rocking overnight at 4 °C. 20 μl of protein G-agarose conjugates (Santa Cruz Biotechnology) then were added and incubated for an additional 2 h at 4 °C. The agarose beads in each tube were washed three times followed by SDS-PAGE and Western blotting.

Co-immunoprecipitation of GSK3β and Cyclin D1—Endogenous GSK3β and associated proteins were immunoprecipitated following lysis in 50 mM Tris-HCl, pH 8.0, and 0.5% Nonidet P-40 with protease inhibitors by adding antibody to GSK3β (2 μg to 1 mg of total lysate) followed by rocking overnight at 4 °C. 20 μl of protein G-agarose conjugates were then added and incubated for an additional 2 h at 4 °C. The agarose beads in each tube were washed three times followed by SDS-PAGE and Western blotting. The immunoprecipitated samples containing GSK3β-associated cyclin D1 was performed by diluting the immunoprecipitates (in SDS sample buffer without dye) from 50 μl to 1 ml by adding 2% bovine serum albumin. The diluted sample was then incubated with anti-cyclin D1-conjugated protein G-agarose. The agarose beads were washed three times followed by SDS-PAGE and autoradiography.

In Vitro Kinase Reactions—GST-Mirk or its kinase-inactive YF mutant form was incubated together with GST-cyclin D1 as noted in kinase buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 10 mM MgCl2, and 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. In some experiments, 40 μl LCl was added to the reaction mixture to inhibit GSK3β. Reaction mixtures were then analyzed by SDS-PAGE and autoradiography.

Peptide Mapping—The cyclin D1 mutants were subcloned into pGEX-6P1 to take advantage of the cold cleavage conditions using the PreScission enzyme (Amersham Biosciences). Following an in vitro kinase reaction with phosphorylated cyclin D1, incubation at 4 °C, DBD was band cut from the SDS-polyacrylamide gel. The gel slice was washed five times with water for 20 min each and then soaked in a solution of urea/water/acetic acid (1 g/ml/1 ml) for 20 min and then in this solution containing 0.015 M N-chlorosuccinimide for 1 h. After washing with water, the slice was loaded onto a precast 20–10% gradient gel (Bio-Rad) and then subjected to electrophoresis and autoradiography. V5 cyclin D1 protein mapping was performed exactly as detailed previously (14).

RNA Interference (RNAi)—The RNAi to Mirk (si1) was synthesized and used as described previously (10). Positively transfected cells were sorted by co-transfected GFP using a BD FACSvantage SE cell sorter.

Band Density in Autoradiograms and Western Blots—Band density was measured using a Lacie Silverscanner and Silverscanner III software and analyzed using the IP LabGel program.

Metabolic Labeling—Mv1Lu cells were incubated for 5 h in serum-free low phosphate medium (9:1 ratio phosphate-free medium/normal medium) with 150 μCi of [3H]thymidine/2 ml of medium.

Northern Analysis—3–5 μg of total RNA from each cell line was electrophoresed on a 1.0% agarose-formaldehyde gel, transferred to nylon membranes by downward capillary transfer, and cross-linked by baking. The membranes were hybridized to a cDNA probe containing the cyclin D1 coding sequence in pCMV5, which had been labeled with 32P by random priming. The blot was hybridized overnight at 68 °C with at least 105 cpm of the labeled probe, washed at room temperature twice for 15 min with 1× SSC, 0.1% SDS, and then washed for 15 min at 65 °C in 0.2× SSC, 0.1% SDS and autoradiographed.

RESULTS

Cyclin D1 Turnover Enhanced by the G9/G7 Kinase Mirk—Mv1Lu cells were chosen to study the interaction between Mirk and cyclin D1, because Mv1Lu cells are nontransformed epithelial cells that exhibit some normal cell cycle regulation. Mv1Lu cells growth arrest in G1/G0 in response to transforming growth factor-β1 or to culture in serum-free conditions, whereas treatment with a cellular mitogen releases this arrest (15). In addition, we recently have made a stable Mirk-inducible subline from Mv1Lu cells and have shown that the induction of Mirk inhibited the migration of these cells in wound experiments and inhibited their invasion through polycarbonate Transwell filters (12). Therefore, we know that Mirk exhibits biological activity in these epithelial cells. We transiently transfected Mirk into subconfluent Mv1Lu cells, allowed the expression of Mirk for 24 h in serum-free medium, and arrested cells in G0 by continued culture in serum-free medium for an additional 48 h. In both Mirk-transfected cultures and vector control transfectedants, 78–79% of cells were in G0/G1 phase (Fig. 1A). An examination of cultures by fluorescence microscopy for co-transfected enhanced GFP showed that ~60–70% of cells were transfected. The rate of entry into S phase was determined by examining cultures by flow cytometry 7 and 12 h after the culture medium was changed to serum-containing growth medium. Both Mirk transfectedants and control transfectedants remained in G0 at 7 h after release (data not shown). However, 66% of vector control transfectedants entered S phase 12 h after the addition of serum, whereas only half of that many, 33% of Mirk transfectedants, entered the S phase at this time (Fig. 1A). Thus, transient overexpression of Mirk in epithelial cells blocked cells in G0/G1. In earlier studies, we had made a stable Mirk-inducible subline from nontransformed Mv1Lu lung epithelial cells (12). The induction of Mirk caused a 30–40% inhibition of Mv1Lu cell entry into S phase as measured by decreased uptake of [3H]thymidine (Fig. 1B), confirming the results of our transient transfection experiments (Fig. 1A). Complete cessation of growth did not occur in the presence of serum mitogens, but increased levels of Mirk inhibited cell cycling.

Transient overexpression of Mirk in Mv1Lu cells decreased cyclin D1 protein levels as shown by Western blotting (Fig. 2A). Similarly, the induction of Mirk protein in the stable Mirk-inducible Mv1Lu subline C9 decreased cyclin D1 levels by half, whereas treatment of cells with the proteasome inhibitor MG115 blocked the Mirk-induced reduction in cyclin D1 abundance (Fig. 2B). Translation arrest experiments with cycloheximide confirmed that the induction of Mirk decreased the half-life of cyclin D1 from ~40 to 20 min in Mv1Lu mink lung epithelial cells cultured in serum-free conditions (Fig. 2, C and D). The faster migrating cyclin D1 form (Fig. 2C, long arrow) is lost rapidly upon Mirk induction. Thus, Mirk enhanced the normal proteasomal turnover of cyclin D1 in Mv1Lu cells. Mirk is a kinase most active in G1/G0 in NIH3T3 cells when it is predominately localized in the nucleus (9). The major kinase that controls cyclin D1 levels in mammalian cells is GSK3β (2,
which is localized in the cytoplasm and perinuclear region during G1 in NIH3T3 cells and enters the nucleus during S phase. Possibly, Mirk maintains cells in G0 by limiting the abundance of nuclear cyclin D1 in G0 or, alternatively, functions in G1 to enhance the later occurring phosphorylation of cyclin D1 by GSK3/β.

Mirk Binds to Cyclin D1—We further explored the interaction between Mirk and cyclin D1 by determining whether they interact in vivo. Nontransformed Mv1Lu mink lung epithelial cells were co-transfected for 24 h with Mirk, FLAG-cyclin D1, or an equal amount of vector control DNA, cultured for 48 h in serum-free medium, and then immunoprecipitated with affinity-purified polyclonal antibody directed to a unique sequence at the N terminus of Mirk (Fig. 3A), affinity-purified polyclonal antibody directed to a unique sequence at the C terminus of Mirk (Fig. 3B), or antibody to the FLAG epitope on the cyclin D1 expression plasmid. The immunoprecipitates were separated by SDS-PAGE, and the abundance of Mirk and of FLAG-cyclin D were determined by Western blotting using the C-terminal directed antibody for Mirk and antibody to the FLAG epitope for cyclin D1. Five percent of the lysates were analyzed by Western blotting (Input panel in A). Antibody directed to the N terminus of Mirk was able to co-immunoprecipitate cyclin D1 (Fig. 3A), whereas antibody directed to the C terminus of Mirk could only immunoprecipitate Mirk and not cyclin D1 (Fig. 3B). These results suggest that Mirk interacts with cyclin D1 through Mirk-unique C terminus. Antibody to the FLAG epitope could also co-immunoprecipitate cyclin D1 and Mirk (Fig. 3B, last lane). Thus, Mirk and cyclin D1 interact in vivo within Mv1Lu cells as shown by their co-immunoprecipitation with antibodies directed either to Mirk or to cyclin D1.

Depletion of Mirk by RNAi in Postmitotic C2C12 Myoblasts Increases Cyclin D1 Abundance—We wished to determine the effect of depleting endogenous Mirk on cyclin D1 stability.
However, RNA interference experiments could not be performed reliably in Mv1Lu cells because Mirk had not been sequenced in the mink cell. We chose murine C2C12 myoblasts because Mirk mediates their differentiation into postmitotic fused myotubes when cells are placed into differentiation medium (10). The relative abundance of Mirk and cyclin D1 during the initial hours of differentiation was determined first. Mirk is expressed at very low levels in proliferating myoblasts but is rapidly induced at least 10-fold when primary-cultured muscle satellite cells or myoblast cell lines are placed in differentiation medium (10). The induction of Mirk in C2C12 myoblasts was accompanied by a sharp decrease in cyclin D1 levels detected within 6 h (Fig. 4A).

Cyclin D1 mRNA and protein decline rapidly during myogen-
Co-Immunoprecipitation of Mirk and Cyclin D1

**Fig. 3. Co-immunoprecipitation of Mirk and cyclin D1.** Nontransformed Mv1Lu mink lung epithelial cells were co-transfected for 24 h with Mirk, FLAG-cyclin D1, or an equal amount of vector control DNA and then cultured for 48 h in serum-free medium and lysed in 0.5% Nonidet P-40-containing lysis buffer. Cells were immunoprecipitated (IP) with antibody to the FLAG epitope (panels A and B), affinity-purified polyclonal antibody directed to a unique sequence at the N terminus of Mirk (panel A), or affinity-purified polyclonal antibody directed to a unique sequence at the C terminus of Mirk (panel B). The immunoprecipitates were separated by SDS-PAGE, and the abundance of Mirk was determined by antibody directed to a unique sequence at the N terminus of Mirk or antibody to the FLAG epitope to detect cyclin D1. Five percent of the lysates were analyzed by Western blotting as above (Input panel in A).

Mirk/dyrk1B Kinase Destabilizes Cyclin D1

To determine whether Mirk phosphorylated cyclin D1 in vivo, Mv1Lu cells were co-transfected for 24 h with FLAG-cyclin D1 and either wild-type Mirk or vector control and then metabolically labeled for 5 h with [32P]orthophosphate. Cyclin D1 was immunoprecipitated from cell lysates with anti-FLAG antibody and then analyzed by autoradiography and Western blotting after SDS-PAGE. Co-expression of wild-type Mirk increased in vivo phosphorylation of cyclin D1 (Fig. 5B). Thus, Mirk directly phosphorylated cyclin D1 in vitro and co-expression of Mirk increased the phosphorylation of cyclin D1 in vitro.

The region within cyclin D1, which contained the Mirk phosphorylation sites, was then determined by peptide mapping after in vitro phosphorylation. The GST epitope tag was cleaved from GST-cyclin D1 by the PreScission protease before assay. After the in vitro phosphorylation by Mirk, cyclin D1 was cleaved by N-chlorosuccinimide. The resulting peptide fragments were separated by 10–20% gradient SDS-PAGE and visualized by autoradiography. The largest phosphopeptide was 26 kDa, which encompassed the C terminus of cyclin D1, and was the most strongly labeled of the peptides (Fig. 5C). The 17-kDa peptide contained the N-terminal half of the molecule and was less strongly labeled. The smallest N-terminal fragment was 8 kDa and was not phosphorylated in vitro. These results indicated that Mirk must phosphorylate cyclin D1 at a site or sites within the C terminus or the central region.

Mirk Destabilizes Cyclin D1 by Phosphorylation at Threonine 288—Potential Mirk phosphorylation sites at Ser90, Thr105, Thr128, Thr134, Ser197, Ser219, Ser237, and Thr264 were mutated to alanine. These sites were within the 26-kDa cyclin D1 fragment phosphorylated by Mirk and were conserved between mouse and human. The closely related kinase to Mirk, Dyrk1A, has been reported to phosphorylate serines or threonines +3 from an arginine residue (20). In addition, we have found that Mirk phosphorylates hepatocyte nuclear factor 1α at serine 247, which is +3 from an arginine, R244GVS247PS (asterisk indicates phosphorylation site) (8). However, Mirk has phosphorylated other substrates at serines or threonines +5 to −5 from an arginine (data not shown), so we mutated a series of serines and threonines within +7 to −9 of an arginine residue and, in one case, +3 to a lysine. Initial in vitro experiments suggested that Mirk phosphorylated cyclin D1 at Thr134, Thr264, or both residues (data not shown). However, the activity of kinases can be very different in vitro and in vivo (21), so these experiments were taken only as guides for subsequent studies.

The in vitro phosphorylation studies were confirmed with in vivo expression studies. Co-expression of Mirk and FLAG

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**Co-Immunoprecipitation of Mirk and Cyclin D1**

**A**

**IP:** anti-N-term Mirk

**Flag-Cyclin D1**

**WB:** Mirk

**WB:** Flag

**Input**

**B**

**IP:** C-term Mirk

**Flag-Cyclin D1**

**IP:** Flag

**WB:** Mirk

**WB:** Flag

**Input**

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To determine whether Mirk phosphorylated cyclin D1 in vivo, Mv1Lu cells were co-transfected for 24 h with FLAG-cyclin D1 and either wild-type Mirk or vector control and then metabolically labeled for 5 h with [32P]orthophosphate. Cyclin D1 was immunoprecipitated from cell lysates with anti-FLAG antibody and then analyzed by autoradiography and Western blotting after SDS-PAGE. Co-expression of wild-type Mirk increased in vivo phosphorylation of cyclin D1 (Fig. 5B). Thus, Mirk directly phosphorylated cyclin D1 in vitro and co-expression of Mirk increased the phosphorylation of cyclin D1 in vivo.

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Phosphorylation. Threonine 288 was −3 from an arginine residue within the sequence LACTPTSVDRDYDVI (asterisk indicates the phosphorylation site).

Cyclin D1 migrates in two forms on SDS-PAGE, both of which are more highly phosphorylated in the presence of co-expressed Mirk (Fig. 6A, compare lanes 1 and 2). However, when cyclin D1 is mutated at T288A, only the upper band is phosphorylated (Fig. 6A). Thus, Mirk phosphorylates cyclin D1 in vivo at Thr288, yielding the faster migrating cyclin D1 species. It is of interest to recall that the faster-migrating cyclin D1 band in the half-life experiments was the form of cyclin D1 most rapidly decreased when Mirk was induced (Fig. 2C, long arrow). These data are consistent with a model in which Mirk phosphorylates Thr288 to produce the faster migrating cyclin D1 species.

The cyclin D1 double band was detected by Western blotting in several experiments in both Mv1Lu and C2C12 cells (Figs. 2, A and C, and 4A) and by immunoprecipitation after phosphorylation in vivo in Mv1Lu cells (Fig. 6A). We wanted to confirm that both bands were forms of cyclin D1, just posttranslationally modified in different ways. The phosphorylated double band in wild-type cyclin D1 and in cyclin D1 mutated at T184A was compared with the phosphorylated single band of cyclin D1 mutated at T288A by V8 protease peptide mapping (Fig. 6B). Digestion of all three cyclin D1 constructs yielded a similar phospho-peptide pattern following V8 cleavage, demonstrating that both the upper and lower bands were cyclin D1.

Translation arrest experiments with cycloheximide (Fig. 6C) demonstrated that a cyclin D1-T288A mutant construct was more stable than a wild-type construct. The turnover of Mirk was measured in both cultures as an internal control and showed similar stability when co-expressed with either wild-type cyclin D1 or the mutant T288A form. Thus, phosphorylation of cyclin D1 on Thr288 by Mirk destabilizes cyclin D1 in vivo.

Mirk Phosphorylates Cyclin D1 Bound to GSK3β and Can Phosphorylate Cyclin D1 When GSK3β Is Inhibited by LiCl—The major kinase that controls cyclin D1 levels in mammalian cells is GSK3β. Cyclin D1 is known to be rapidly degraded through the ubiquitin-proteasome pathway following phosphorylation by GSK3β at threonine 286, which facilitates the export of cyclin D1 to the cytoplasm where it is proteolyzed (2, 3). Mirk is most abundant and most active as a kinase in G0/G1 (9), so we hypothesized that Mirk functions in G0 to enhance the later occurring phosphorylation of cyclin D1 by GSK3β. Mirk and GSK3β phosphorylated cyclin D1 in an additive fashion when tested in vitro kinase assays. The activity of recombinant Mirk and recombinant GSK3β on GST-cyclin D1 was measured with and without treatment with the GSK3β inhibitor LiCl. When added together, GSK3β and Mirk phosphorylated cyclin D1 twice as much as either Mirk or GSK3β alone (Fig. 7A). In addition to the expected inhibition of GSK3β (25-fold inhibition), LiCl also blocked Mirk phosphorylation of cyclin D1 (3-fold inhibition). Mirk and GSK3β exhibit some homology, so this inhibition is not surprising. In the presence of LiCl, only the phosphorylation of cyclin D1 by Mirk was seen (note similar activities in Fig. 7A, lanes 4 and 6). Therefore, Mirk can phosphorylate cyclin D1 when GSK3β is inhibited.

To compare the relative effects of Mirk and GSK3β in vivo, Mv1Lu cells were co-transfected for 24 h with wild-type Mirk, kinase-inactive YF-Mirk, or vector control together with wild-type FLAG-cyclin D1, mutant FLAG-cyclin D1-T184A used as a control, or mutant FLAG-cyclin D1-T288A. GSK3β activity was inhibited in some cultures by LiCl, and cells were then metabolically labeled with [35]S]orthophosphate. Cyclin D1 was immunoprecipitated from cell lysates with anti-FLAG antibody and then analyzed by autoradiography and Western blotting.

epitope-tagged cyclin D1-T288A, T184A, or wild-type cyclin D1 in Mv1Lu cells was followed by in vivo labeling with [35]S]orthophosphate, and an analysis of the cyclin D1 immunoprecipitates by autoradiography and Western blotting after SDS-PAGE. In cyclin D1, the most effective mutation in blocking in vivo phosphorylation in the presence of co-expressed Mirk was T288A (Fig. 6A), whereas the T184A mutation caused no diminution of phospho-

Fig. 4. Increased Mirk expression in differentiating C2C12 myoblasts is associated with reduced cyclin D1 levels, whereas depletion of Mirk by RNA interference increases cyclin D1 protein levels and decreases phosphorylation of cyclin D1. A, C2C12 cells were cultured in growth medium containing 20% fetal bovine serum overnight and then switched to serum-free Dulbecco’s modified Eagle’s medium for 1–6 h to begin differentiation to postmitotic myotubes. Cell lysates were examined by Western blotting (WB) for Mirk, cyclin D1, GSK3β, and phosphorylated GSK3β. B, RNAi to Mirk increases cyclin D1 abundance by a posttranscriptional mechanism. C2C12 cells were co-transfected with an expression plasmid for Mirk RNAi and enhanced GFP, or vector DNA and enhanced GFP selected by cell sorting for enhanced GFP were placed in growth medium for 1 day and then switched to differentiation medium for 2 days. Vc, vector. Left panel, cell lysates were examined by Northern blotting for cyclin D1. 28S and 18S rRNA staining by ethidium bromide is shown. The ratio of the abundance of cyclin D1 mRNA to the abundance of the 28 S rRNA is given below the appropriate lanes. Right panel, cell lysates were examined by Western blotting for Mirk and cyclin D1. The abundance of a cross-reactive protein was similar in both lanes and served as an internal control (not shown). The ratio of Mirk protein and cyclin D1 protein in control and Mirk-directed RNAi-treated cultures is given to the right of the appropriate lanes. C, C2C12 cells were transfected for 24 h with either pSilencer plasmid encoding si1 to Mirk or control sequences and then switched to differentiation medium for 5 h containing [32]P]orthophosphate before cell lysis. CT, control-untreated cells. Cyclin D1 immunoprecipitates were analyzed by autoradiography and then Western blotting for cyclin D1. The ratio of labeled cyclin D1 to total immunoprecipitated (IP) cyclin D1 is listed under the appropriate lanes.
immunoprecipitation. The sizes of cyclin D1 (36 kDa) and its derivative phosphopeptides of 26, 17, 16, and 10 kDa are indicated.

Fig. 5. Mirk phosphorylates cyclin D1 in vitro and in vivo. A, GST-cyclin D1 was phosphorylated by Mirk in vitro. Kinase-inactive YF-Mirk served as the control. The in vitro kinase reaction mixtures were analyzed by SDS-PAGE and autoradiography, and the phosphorylated cyclin D1 band is shown (autorad). The total amount of purified cyclin D1 on the gel is shown by Ponceau S staining (Cyc D1). B, Mv1Lu cells were co-transfected for 24 h with FLAG-cyclin D1 and either wild-type (WT) Mirk or vector control and then labeled for 5 h with \(^{32}P\)orthophosphate in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. After lysis in buffer containing 0.5% Nonidet P-40, cyclin D1 was immunoprecipitated with anti-FLAG antibody and then analyzed by autoradiography and Western blotting (WB) for the FLAG epitope after SDS-PAGE. C, the phosphorylation sites in cyclin D1 were peptide-mapped after in vitro phosphorylation. The GST epitope tag was cleaved from GST-cyclin D1 by the PreScission protease before assay. After the in vitro kinase assay, the phosphorylated cyclin D1 was cleaved by N-chlorosuccinimide at Trp 63 and Trp 150 and the resulting peptide fragments were separated by 16%–25% gradient SDS-PAGE and visualized by autoradiography. The sizes of cyclin D1 (36 kDa) and its derivative phosphopeptides of 26, 17, 16, and 10 kDa are indicated. IP, immunoprecipitation.

After lysis in buffer containing 0.5% Nonidet P-40, cyclin D1 was immunoprecipitated with anti-FLAG antibody and then analyzed by autoradiography and Western blotting (WB) for the FLAG epitope after SDS-PAGE. Mirk phosphorylates cyclin D1 at Thr 288. A loss of the lower cyclin D1 phosphorylated band was seen in the T288A mutant (long arrow in Fig. 7B, similar to the results in Fig. 6A). LiCl treatment decreased the total amount of \(^{32}P\)orthophosphate incorporated into cyclin D1, but both bands remained phosphorylated, indicating that Mirk could phosphorylate cyclin D1 when GSK3\(\beta\) was inhibited (Fig. 7B, lanes 6 and 7). Moreover, only the mutation of cyclin D1 to Ala288, not LiCl treatment, caused a loss of phosphorylation of the lower band. The phosphorylation of cyclin D1 was decreased to a background level 20–30% of control values by mutation to T288A both in the absence and in the presence of LiCl (Fig. 7B, compare lanes 5 and 8). We conclude that, similar to their in vitro interaction, Mirk was capable of phosphorylating cyclin D1 at Thr\(^{288}\) in vivo, even when GSK3\(\beta\) was inhibited. These data suggested that Mirk might phosphorylate cyclin D1 before GSK3\(\beta\) and that blocking Mirk action on cyclin D1 by mutation at Thr\(^{288}\) could dramatically inhibit in vivo phosphorylation of cyclin D1 at other positions, possibly including the GSK3\(\beta\) site of Thr\(^{296}\) (compare lane 5 with 3).

We further explored the relationship between GSK3\(\beta\) and Mirk by measuring the kinase capacity of Mirk on immunoprecipitated GSK3\(\beta\) and its associated proteins (Fig. 7C). Mirk had little or no detectable kinase activity on immunoprecipitated GSK3\(\beta\) (Fig. 7C). However, a highly phosphorylated lower molecular weight band was observed in the GSK3\(\beta\) immunoprecipitates (Fig. 7C, upper blot), which co-migrated with cyclin D1 immunoprecipitated from the same lysates and run in parallel. To confirm the identity of this band, a portion of the immunoprecipitate, which had not been analyzed by SDS-PAGE, was re-precipitated this time with anti-cyclin D1 conjugated to agarose beads. Phosphorylated cyclin D1 was detected after SDS-PAGE and autoradiography (Fig. 7C, lower blot). Therefore, Mirk can phosphorylate cyclin D1 bound to GSK3\(\beta\).

**Mirk Phosphorylates Cyclin D1, Which Is Mutant at the GSK3\(\beta\) Site**—We next tested whether Mirk could phosphorylate cyclin D1 independently of GSK3\(\beta\) by co-expressing Mirk with Flag-cyclin D1-T286A (mutated at the GSK3\(\beta\) site), Flag-cyclin D1-T288A (mutated at the Mirk site), or wild-type Flag-cyclin D1 in both Mv1Lu lung epithelial cells and in NIH3T3 fibroblasts (Fig. 8). Following metabolic labeling with \(^{32}P\)orthophosphate, the cyclin D1 constructs were immunoprecipitated by antibody directed to their Flag epitope tag and their abundance was detected by Western blotting. There...
Mutation of cyclin D1 to Ala288. The ratio of immunoprecipitated [32P]orthophosphate-labeled cyclin D1 to abundance of FLAG-cyclin D1 is given.

duplicate experiments with similar results is shown. B, Mv1Lu cells were co-transfected for 24 h with wild-type Mirk, kinase-inactive YF-Mirk, or vector control together with wild-type FLAG-cyclin D1, mutant FLAG-cyclin D1-T184A, or mutant FLAG-cyclin D1-T288A. Cells were treated with 30 mM LiCl for 5.5 h in Dulbecco’s modified Eagle’s medium beginning 30 min before the addition of [32P]orthophosphate. After lysis in buffer containing 0.5% Nonidet P-40, cyclin D1 was immunoprecipitated (IP) with anti-FLAG antibody and then analyzed by autoradiography and Western blotting after SDS-PAGE. The short arrow marks the cyclin D1 upper band, whereas the long arrow points to the cyclin D1 lower band whose phosphorylation is eliminated by mutation of cyclin D1 to Ala288. The ratio of immunoprecipitated [32P]orthophosphate-labeled cyclin D1 to abundance of FLAG-cyclin D1 is given below each lane. One of duplicate experiments with similar results is shown. C, in vitro kinase assay of recombinant wild-type (Wt) Mirk or kinase-inactive YP-Mirk on immunoprecipitated endogenous GSK3β or cyclin D1. One of two experiments with similar results is shown. Upper panel, one-third of the in vitro kinase reaction mixture was analyzed by autoradiography following SDS-PAGE. The positions of GSK3β and of cyclin D1 are indicated. Lower panel, two-thirds of the in vitro kinase reaction mixture was denatured with SDS-sample buffer and diluted to non-denaturing conditions, and then a second immunoprecipitation was performed with antibody to cyclin D1 conjugated to agarose and then analyzed by autoradiography following SDS-PAGE. The position of the phosphate-labeled cyclin D1 corresponds to the large phosphorylated band, which co-migrates with immunoprecipitated cyclin D1 in the upper panel.

was a 30% increase in the abundance of cyclin D1-T286A compared with wild-type cyclin D1, probably because of the greater stability of the mutant form. The enrichment was predominately in the slower migrating cyclin D1 form and most clearly seen in NIH3T3 cells (Fig. 2B, small arrow). Mirk phosphorylation of cyclin D1 was not blocked by the T286A mutation. In fact, cyclin D1-T286A was phosphorylated twice as much as wild-type cyclin D1 in both cell types. Phosphorylation of cyclin D1 by co-expressed Mirk was inhibited by the T288A mutation as seen before with the loss of phosphorylation of the faster migrating band (autoradiography panels in Fig. 8, long arrows). These data show that the phosphorylation of cyclin D1 by GSK3β is not a precondition for phosphorylation by Mirk. We speculate that after Mirk phosphorylates cyclin D1 at Thr286, there is another modification of this phosphorylated form, which confirms its faster mobility. Because the faster migrating form of cyclin D1 is rapidly lost in translation arrest experiments (see Fig. 2C) and mutation to T286A stabilizes the slower form (Fig. 8), we speculate that both Mirk and GSK3β phosphorylate the slower migrating form. In this model, cyclin D1 phosphorylated at both 286 and 288 might then be rapidly modified to the faster migrating form and then proteolyzed.

**DISCUSSION**

Mirk/dyrk1B is a member of the Minibrain/dyrk family of arginine-directed protein kinases. Mirk, similar to Dyrk1A, functions as a transcriptional activator (8, 22, 23). We have shown in the current study that Mirk also functions by mediating protein degradation. Mirk phosphorylates cyclin D1 at Thr288, which is close to the GSK3β phosphorylation site of Thr286 that is known to mediate cyclin D1 ubiquitination and degradation. Furthermore, we have shown that depletion of endogenous Mirk by RNA interference increases the abundance of cyclin D1 by a posttranscriptional mechanism and that up-regulation of Mirk levels leads to a faster turnover of cyclin D1 and decreases the rate of entry into the S phase. These effects were seen in two nontransformed cell types, Mv1Lu lung epithelial cells and C2C12 myoblasts. A related Dyrk family kinase, MBK-2, has been shown to coordinate the degradation of several maternal proteins, which is essential for Caenorhabditis elegans zygotes to complete cytokinesis (24). Depletion of MBK-2 by RNA interference arrested the development at the one-cell stage with multiple nuclei. The mechanism of MBK-2 action is not known, but MBK-2 was not a general activator of protein degradation by the proteasome. MBK-2 was postulated to target some unknown factor but not an E3 ubiquitin ligase common to the specific group of maternal proteins, which were degraded (24).

In an earlier study, we had observed that stable overexpression of Mirk in colon carcinoma cells enhanced the turnover of cyclin D1 and the CDK inhibitor p27kip1 (25). These results appeared contradictory and may have resulted from the
changes in cell physiology that occur in the presence of elevated expression of an active kinase with many potential cellular targets. However, the present study has confirmed the role of Mirk in inducing rapid turnover of cyclin D1 by three methods: RNA interference; transient overexpression of Mirk; and induction of a stable inducible Mirk construct. Moreover, we have identified threonine 288 in cyclin D1 as a specific substrate for Mirk kinase and have shown that the nonphosphorylatable mutant construct T288A is more stable in vivo than wild-type cyclin D1.

Cyclin D1 was phosphorylated almost exclusively on threonine in vivo in NIH3T3 cells or in Sf9 insect cells co-expressing FLAG-cyclin D1 and CDK4, whereas the mutation of cyclin D1 to T286A blocked the phosphorylation of a FLAG-cyclin D1 construct in both cell types (2). These data would appear to rule out any role for endogenous Mirk in the phosphorylation of cyclin D1 at Thr286. However, we have found that depletion of Mirk by RNA interference in C2C12 myoblasts stabilized cyclin D1 protein without affecting cyclin D1 mRNA (Fig. 4). Furthermore, induced overexpression of Mirk in Mv1Lu lung epithelial cells led to the phosphorylation of cyclin D1 at Thr286 (Fig. 6) and a more rapid turnover of cyclin D1 (Fig. 2), all of which argue that Mirk can modulate cyclin D1 stability, at least in lung epithelial cells and in myoblasts. In this study, CDK4 was not co-expressed with exogenous cyclin D1, leaving most of the overexpressed cyclin D1 as a free monomer, possibly presenting a different substrate to cellular kinases. GSK3β is a more efficient kinase for cyclin D1 when cyclin D1 is bound to CDK4 (3). Thus, in our in vivo studies, the ectopic cyclin D1 would largely be in free form not complexed to CDK4, so we may have amplified the kinase effect of Mirk while decreasing the relative effect of GSK3β.

Another possibility is that Mirk and GSK3β may function together, so elimination of the Mirk site may block the function of GSK3β. In support of this hypothesis, the Mirk related kinase Dyrk1A has been hypothesized to act as a "primer" kinase for GSK3. Dyrk1A phosphorylates eIF2B at Ser539, greatly increasing the ability of GSK3 to phosphorylate the nearby residue Ser279 (26). Purified recombinant Mirk and GSK3β phosphorylate cyclin D1 in an additive fashion in in vitro kinase assays in this study, and blocking GSK3β activity by a posttranscriptional mechanism (Fig. 4B), so we conclude, at least in the cell types we have tested, that phosphorylation by Mirk can increase the turnover of cyclin D1. In recent studies, we have shown that Mirk is a G1 kinase, which acts in maintaining a G0 arrest by stabilizing the CDK inhibitor p27 by phosphorylation at Ser10 (9). This study demonstrates that Mirk phosphorylates the cell cycle regulator, cyclin D1, at Thr286, which enhances its rapid turnover. Thus, Mirk has the novel function of both stabilizing a CDK inhibitor and destabilizing a G1 cyclin to assist cells in remaining arrested in G0.

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