MOLECULAR AND CELLULAR BIOLOGY, Mar. 2005, p. 2384–2394 Vol. 25, No. 6
0270-7306/05/$08.00+0 doi:10.1128/MCB.25.6.2384–2394.2005

The *NF2* Tumor Suppressor Gene Product, Merlin, Inhibits Cell Proliferation and Cell Cycle Progression by Repressing Cyclin D1 Expression

Guang-Hui Xiao,[1] Ryan Gallagher,[1] Justin Shetler,[1] Kristine Skele,[1] Deborah A. Altomare,[1] Richard G. Pestell,[2] Suresh Jhanwar,[3] and Joseph R. Testa[1]*

Human Genetics Program, Fox Chase Cancer Center, Philadelphia, Pennsylvania[1]; Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, D.C.[2]; and Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, New York[3]

Received 14 September 2004/Returned for modification 25 October 2004/Accepted 7 December 2004

Inactivation of the *NF2* tumor suppressor gene has been observed in certain benign and malignant tumors. Recent studies have demonstrated that merlin, the product of the *NF2* gene, is regulated by Rac/PAK signaling. However, the mechanism by which merlin acts as a tumor suppressor has remained obscure. In this report, we show that adenovirus-mediated expression of merlin in *NF2*-deficient tumor cells inhibits cell proliferation and arrests cells at G_1 phase, concomitant with decreased expression of cyclin D1, inhibition of CDK4 activity, and dephosphorylation of pRB. The effect of merlin on cell cycle progression was partially overridden by ectopic expression of cyclin D1. RNA interference experiments showed that silencing of the endogenous *NF2* gene results in upregulation of cyclin D1 and S-phase entry. Furthermore, PAK1-stimulated cyclin D1 promoter activity was repressed by cotransfection of *NF2*, and PAK activity was inhibited by expression of merlin. Interestingly, the S518A mutant form of merlin, which is refractory to phosphorylation by PAK, was more efficient than the wild-type protein in inhibiting cell cycle progression and in repressing cyclin D1 promoter activity. Collectively, our data indicate that merlin exerts its antiproliferative effect, at least in part, via repression of PAK-induced cyclin D1 expression, suggesting a unifying mechanism by which merlin inactivation might contribute to the overgrowth seen in both noninvasive and malignant tumors.

Neurofibromatosis type 2 (NF2) is an autosomal dominant disorder that affects 1 in 33,000 to 40,000 people, with about one-half of cases representing new mutations. Germ line mutations of the *NF2* gene predispose individuals to the development of multiple benign tumors of the nervous system, the most common of which is bilateral vestibular schwannoma. Other tumor types include schwannomas of other cranial, spinal, and cutaneous nerves and cranial and spinal meningiomas, as well as ependymomas and gliomas of the central nervous system (9). The *NF2* gene is also implicated in the development of sporadic schwannomas and meningiomas, as well as in tumor types seemingly unrelated to the NF2 disorder, particularly malignant mesotheliomas, which are mesodermally derived, primarily pleural tumors (5–7, 29). Mutations of the *NF2* gene are present in about 50% of malignant mesotheliomas, and loss of heterozygosity at the *NF2* locus resulting in biallelic inactivation was reported in all cases of mesothelioma with *NF2* mutations (6). Heterozygous *Nf2* knockout mice develop a variety of metastatic tumors, mostly osteosarcomas (24), whereas conditional homozygous *Nf2* knockout mice, targeting Schwann cells, develop Schwann cell hyperplasia and schwannomas (10). How *NF2* inactivation plays a role in both benign and malignant tumors has remained obscure.

Consistent with its role as a tumor suppressor, ectopic expression of merlin in a variety of cell types has demonstrated that merlin plays an important role in regulating cell proliferation. Experiments with NIH 3T3 fibroblasts revealed that merlin can reverse Ras-induced anchorage-independent growth (33) and inhibit cell proliferation (22). Reexpression of merlin in schwannoma cells suppressed cell growth, which was accompanied by cell cycle arrest at G_0/G_1 (25, 28, 31). In *Drosophila melanogaster* mosaic tissue, *Merlin* mutant cells proliferate more rapidly than their wild-type neighboring cells (19). Despite these observations, the mechanism by which merlin regulates cell proliferation is not well understood.

Several lines of evidence have implicated merlin in the regulation of Rac/PAK signaling. Rac plays an important role in the regulation of cytoskeletal organization and intracellular pathways involved in cellular proliferation, transformation, and transcriptional activation. Merlin localizes at membrane ruffles, which can be induced by Rac activity. Extensive membrane ruffling observed in schwannoma cells can be reversed by inhibiting Rac1 (27) or by TAT-mediated merlin protein transfer (4), suggesting that Rac activity is deregulated in schwannoma cells and that merlin plays a role in Rac signaling. We and others have demonstrated a link between merlin and Rho GTPase signaling (17, 30, 35). In response to active Rac or Cdc42, but not active Rho, merlin is phosphorylated on serine 518 (30), and this phosphorylation is mediated by the PAK family of serine/threonine kinases that are immediate downstream effectors of both Rac and Cdc42 (17, 35). Recently, it has been shown that merlin expression inhibits Rac/PAK activation, which may be attributed to merlin's tumor suppressor function (12, 18).

Here we report that adenovirus-mediated expression of merlin inhibits cell proliferation and blocks cell cycle progression

* Corresponding author. Mailing address: Fox Chase Cancer Center, 333 Cottman Ave., Philadelphia, PA 19111. Phone: (215) 728-2610. Fax: (215) 214-1623. E-mail: Joseph.Testa@fccc.edu.

at G_1 phase in *NF2*-deficient tumor cells. Infection with an adenovirus expressing merlin resulted in a decrease in both cyclin D1 expression and CDK4 kinase activity, concomitant with dephosphorylation of pRB. The effects of merlin on cell cycle progression were found to be partially overridden by ectopic expression of cyclin D1. RNA interference experiments revealed that silencing of the endogenous *NF2* gene results in upregulation of cyclin D1 protein and S-phase entry. Furthermore, PAK1-stimulated cyclin D1 promoter activity was repressed by cotransfection of *NF2*, and PAK1 activity was inhibited by expression of merlin. Collectively, our findings strongly suggest that the tumor suppressor function of merlin is, at least in part, a consequence of its ability to inhibit PAK-induced cyclin D1 expression.

MATERIALS AND METHODS

Cell lines, plasmids, and antibodies. Human malignant mesothelioma cell lines Meso 17 and Meso 35 are *NF2* deficient (6). The expression plasmids pcDNA3-HA-NF2 and pcDNA3-HA-NF2 A518 have been described previously (35). pCMV-Myc-PAK1 and pCMV-Myc-PAK1 T423E were provided by J. Chernoff (Fox Chase Cancer Center, Philadelphia, Pa.). pRC-cyclin D1 and the human cyclin D1 promoter-reporter (−1745D1-Luc) were reported elsewhere (1). Monoclonal antibody antihemagglutinin (anti-HA; HA.11) was from BabCO. Monoclonal antibodies against cyclin D1 (DCS-6), cyclin D2 (G132-43), and pRB (G3-245) were from BD PharMingen. Polyclonal antibodies against cyclin E (M-20), CDK4 (H-22), p21 (C-19), PAK1(C-19), and β-actin (I-19) and monoclonal anti-p27 (clone 57) antibody were from Santa Cruz Biotechnology. Polyclonal anti-ERK1 (C-16) and anti-phospho-p44/42 ERK were from Cell Signaling. Monoclonal antibromodeoxyuridine (anti-BrdU)-Alexa 594 was from Molecular Probes.

Construction of adenoviral vectors. The *NF2* adenoviral vectors were generated by homologous recombination in *Escherichia coli* with the pAdEasy system (11). Briefly, the HA epitope-tagged wild type and the A518 mutant of the human *NF2* gene coding sequences were first cloned into a shuttle vector, pAdTrack-CMV, which also contains a separate cytomegalovirus (CMV)-regulated green fluorescent protein (GFP) expression cassette. The resultant plasmids were linearized and cotransformed into *E. coli* BJ5183 cells with an adenoviral backbone plasmid, pAdEasy-1. The recombinant plasmids were then transfected into the adenoviral packaging cell line 293 to generate recombinant adenoviruses AdNF2 and AdNF2 A518. The control adenovirus, AdCtrl, which carries a GFP gene driven by the CMV promoter, was constructed using pAdTrack-CMV and pAdEasy-1.

Immunofluorescence analysis of AdNF2 expression. Subcellular localization of adenovirus-mediated expression of merlin was examined by staining with monoclonal antibody anti-HA (HA.11), followed by C3-conjugated goat anti-mouse immunoglobulin G (IgG).

cDNA microarray analysis. Meso 17 cells were grown to approximately 70% confluence and transduced with the recombinant adenoviruses, AdCtrl or AdNF2. Total RNA was extracted using TRIzol reagent (Gibco BRL). Ninety micrograms of total RNA from each sample was reverse transcribed into cDNA using SuperScript II (Life Technologies). cDNA from cells infected with AdNF2 or AdCtrl were labeled with Cy3 or Cy5 fluorophores, respectively. Hybridization was performed on a cDNA microarray consisting of nearly 40,000 cDNA clones, available through the Fox Chase Cancer Center Microarray Core Facility. Following hybridization, arrays were scanned with an Affymetrix GSM 428 laser scanner, and the two acquired images (for Cy3 and Cy5) were analyzed by using Imagene software.

Cell culture, DNA transfection, and luciferase assays. *NF2*-negative mesothelioma cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. HeLa and A2780 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. For the cyclin D1 luciferase reporter gene assay, HeLa cells were grown in 12-well plates and transfected with 0.25 μg of cyclin D1 luciferase reporter construct (−1745D1-Luc) and 0.25 μg of pCMV-Myc-PAK1 wild type or pCMV-Myc-PAK1 T423E, plus 0.25 μg of pcDNA3-HA-NF2 or pcDNA3-HA-NF2 A518. The total amount of transfected DNA was kept constant by supplementation with pcDNA3 empty plasmid. After transfection for 32 h, cells were starved overnight in DMEM. Luciferase assays were performed by using a luciferase assay system (Promega) and a Monolight 2010 luminometer (Analytic Luminescence). The arbitrary units of luciferase content were normalized with total protein concentration and plotted relative to the pcDNA3 control. Each transfection was performed in triplicate wells.

CDK4 kinase assay. The CDK4 kinase assay was carried out essentially as described by Matsushime et al. (23). Briefly, 24 h after initiating the adenovirus infection, cells were collected by trypsinization and washed in a buffer solution consisting of 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, and 1 mM dithiothreitol. Cells were then lysed by sonicating in the wash buffer supplemented with 0.1% Tween 20, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin/ml, 10 μg of aprotinin/ml, 10 mM β-glycerophosphate, and 1 mM sodium orthovanadate at 4°C. Lysates were clarified by centrifugation, and 250 μg of protein was incubated with 10 μl of anti-CDK4 antibody. Protein A and protein G (1:1) Sepharose beads were added, and the mixture was incubated for 2 to 3 h. The immunoprecipitate was washed twice with wash buffer and twice with kinase buffer. The kinase reaction was carried out at 30°C for 30 min in the presence of 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM NaF, 20 μM ATP, and 5 μCi of [γ-^{32}P]ATP by using 0.5 μg of glutathione *S*-transferase–Rb (Santa Cruz) as substrate.

Cell proliferation and DNA synthesis. Cell proliferation was measured by counting viable cells on days 1, 2, 3, 4, and 5 after infection using the trypan blue dye exclusion method. DNA synthesis was evaluated by BrdU incorporation. Cells plated on coverslips were infected with adenoviruses; after 18 h 20 μM BrdU was added, and the cells were incubated for another 6 h. Cells were fixed in 3.5% paraformaldehyde for 5 min, permeabilized in 0.2% Triton X-100 for 5 min, and then treated with 4 N HCl for 3 min. BrdU incorporation was visualized with anti-BrdU–Alexa 594 antibody, and nuclei were visualized with 4′,6′-diamidino-2-phenylindole (DAPI) staining.

Two-dimensional gel analysis of PAK activity. To examine the effect of merlin expression on PAK1 activity, a two-dimensional gel electrophoretic analysis was performed following the protocol described by Kissel et al. (18).

RNA interference assays. A pool of small interfering RNA (siRNA) for *NF2* and a pool of negative control siRNA were produced by Dharmacon Research. HeLa cells and A2780 ovarian cancer cells were grown in DMEM containing 10% fetal bovine serum to 30% confluence in six-well plates and transfected with siRNA by using Oligofectamine 2000 (Invitrogen). After 72 h (for HeLa cells) or 48 h (for A2780 cells), the cells were lysed for immunoblot analyses. For BrdU incorporation experiments, cells were grown on coverslips. BrdU (20 μM) was added during the last 6 h, followed by immunofluorescence staining as described above.

RESULTS

Merlin downregulation of cyclin D1 expression. To identify novel merlin targets, we used cDNA microarrays to analyze gene expression in Meso 17 cells, which do not express merlin due to a nonsense mutation in one allele and loss of the remaining *NF2* allele. Since human mesothelioma cell lines have very low transfection efficiency, we employed an adenoviral delivery system to express merlin in *NF2*-deficient mesothelioma cells. Meso 17 cells were infected with AdCtrl or AdNF2 at a multiplicity of infection of 50, and 100% transduction efficiency was confirmed by examining GFP-positive cells under a fluorescence microscope (data not shown). Expression of AdNF2 was localized to the same subcellular compartment observed for endogenous merlin, i.e., predominantly at the cytoplasmic membrane (Fig. 1A). Total RNA was isolated, reverse transcribed, labeled, and hybridized to microarrays consisting of nearly 40,000 human cDNAs. Among the differences in gene expression profiles, we observed a 3.8-fold decrease in cyclin D1 expression in cells infected with AdNF2 compared to those infected with AdCtrl (Fig. 1B). Because of the prominent role of cyclin D1 in cell cycle progression and neoplastic transformation, we examined the effect of *NF2* on cyclin D1 expression.

We first confirmed our microarray finding by real-time PCR analysis. Meso 17 cells were treated in the same manner as in the microarray experiment. Total RNA was isolated and sub-

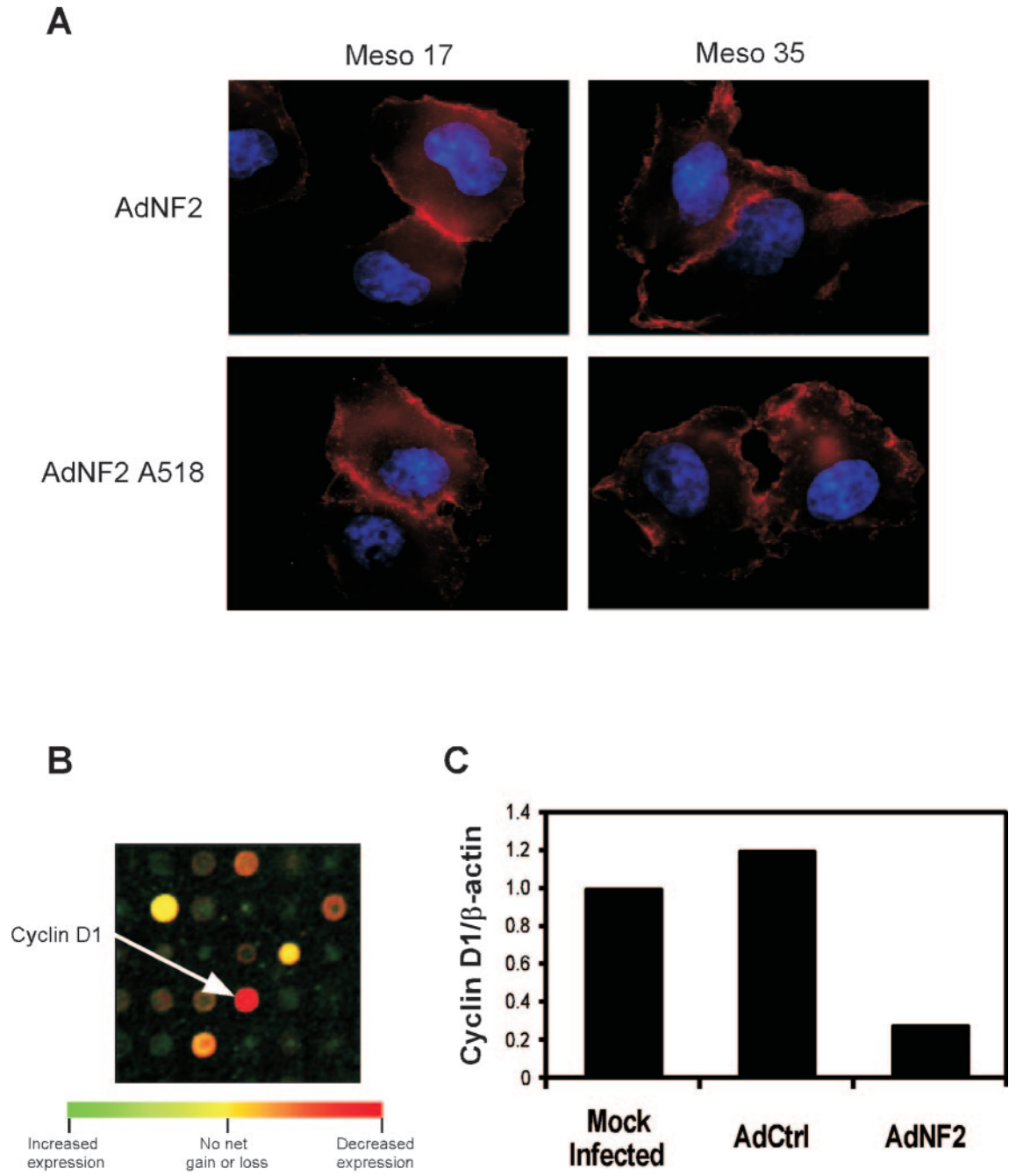

FIG. 1. Downregulation of cyclin D1 expression by merlin. (A) Immunofluorescence demonstrating that adenovirus-mediated expression of merlin in *NF2*-deficient Meso 17 and Meso 35 cells is localized predominantly at the cytoplasmic membrane. (B) Meso 17 cells were infected with either AdCtrl or Ad-NF2. After 24 h, total RNA was isolated, reverse transcribed, labeled, and hybridized to microarrays consisting of nearly 40,000 cDNAs. Among the differences in the gene expression patterns, a 3.8-fold decrease of cyclin D1 expression was observed in cells infected with AdNF2 compared to those infected with AdCtrl. A section of the microarray slide that includes the cyclin D1 spot is shown. The color scale shows the magnitude of the difference from the mean, with red and green indicating transcript levels below and above the median, respectively. (C) Total RNA isolated from Meso 17 cells mock infected or infected with AdCtrl or AdNF2 was subjected to real-time PCR analysis with cyclin D1- and β-actin-specific primers. The histogram depicts the range of cyclin D1 expression. The data are normalized to β-actin and presented as fold changes in cyclin D1 levels relative to those of the mock-infected sample.

jected to real-time PCR analysis using cyclin D1-specific primers. The results confirmed decreased cyclin D1 expression in Meso 17 cells after reexpression of merlin (Fig. 1C).

To determine if the decline in cyclin D1 mRNA level is accompanied by a decrease in cyclin D1 protein expression, immunoblotting experiments were performed. Exponentially growing Meso 17 cells were infected with the recombinant adenoviruses AdCtrl, AdNF2, or AdNF2S518A. Another *NF2*-

deficient mesothelioma cell line, Meso 35, was infected at a multiplicity of infection of 10 and, as with Meso 17, 100% transduction efficiency was achieved. Cells were lysed after 24 h of infection. Immunoblot analyses showed strong expression of HA-NF2 in the two cell lines. The AdNF2 protein migrated as a major faster-mobility band and a minor slower-mobility band, whereas the AdNF2 A518 mutant protein, which is refractory to phosphorylation by Rac/PAK signaling (17, 35), migrated as a single faster-mobility form (Fig. 2A). This same migration pattern on sodium dodecyl sulfate-polyacrylamide gel electrophoresis was previously observed in lysates from cells transfected with plasmids encoding wild-type *NF2* or the A518 mutant (35).

Infection with AdCtrl had no effect on cyclin D1 protein expression compared with the mock-infected cells. However, infection with either AdNF2 or AdNF2 A518 resulted in a marked decrease in cyclin D1 protein (Fig. 2A). Based on the intensity of the protein bands on the blots, wild-type *NF2* and the A518 mutant had a comparable effect on cyclin D1 expression. To test the specificity of cyclin D1 downregulation by merlin, we also examined the expression of other cell cycle regulators by immunoblotting. While the protein levels of cyclin D2, cyclin E, CDK4, and p21 were unaltered, accumulation of p27 protein was associated with repression of cyclin D1 expression (Fig. 2A).

Merlin inhibits CDK4 activity and induces pRB dephosphorylation. Cyclin D1 is a regulatory subunit of the CDK holoenzyme, which phosphorylates and inactivates the tumor suppressor pRB. This is a critical event in G_1 phase which allows cells to pass through a cell cycle checkpoint and enter S phase. Thus, we next examined whether downregulation of cyclin D1 by merlin is accompanied by inactivation of CDK4 and dephosphorylation of pRB. CDK4 catalytic activity was measured using an in vitro immunocomplex kinase assay. Infection with either AdNF2 or AdNF2 A518, but not AdCtrl, resulted in a decrease in CDK4 activity (Fig. 2B). pRB phosphorylation was assessed by immunoblotting. In mock-infected or AdCtrl-infected cells, pRB migrated as a doublet consisting of a faster-migrating, de- or hypophosphorylated form and a slower-migrating, hyperphosphorylated form of pRB protein (Fig. 2A). Expression of either wild-type or mutant forms of merlin dramatically decreased the hyperphosphorylated form of pRB, consistent with the observed decline in cyclin D1 protein level and CDK4 activity. In addition, merlin expression also resulted in a decrease in the total amount of RB protein.

Reexpression of merlin induces G_1 cell cycle arrest and inhibition of cell growth. We next assessed the effect of *NF2* on cell cycle progression in Meso 17 and Meso 35 cells by fluorescence-activated cell sorting (FACS) analysis. Infection with AdCtrl resulted in little change in cell cycle distribution, whereas infection with either AdNF2 or AdNF2 A518 impeded cell cycle progression, as revealed by an accumulation of cells in G_1 and a decrease in the proportion of cells in S phase (Fig. 3A).

To verify the effect of merlin on cell cycle progression, DNA synthesis was measured by assessing BrdU incorporation. Consistent with the finding of G_1 cell cycle arrest revealed by FACS, expression of NF2 or NF2 A518 significantly reduced DNA synthesis (Fig. 3B).

The effect of merlin on cell proliferation was examined using

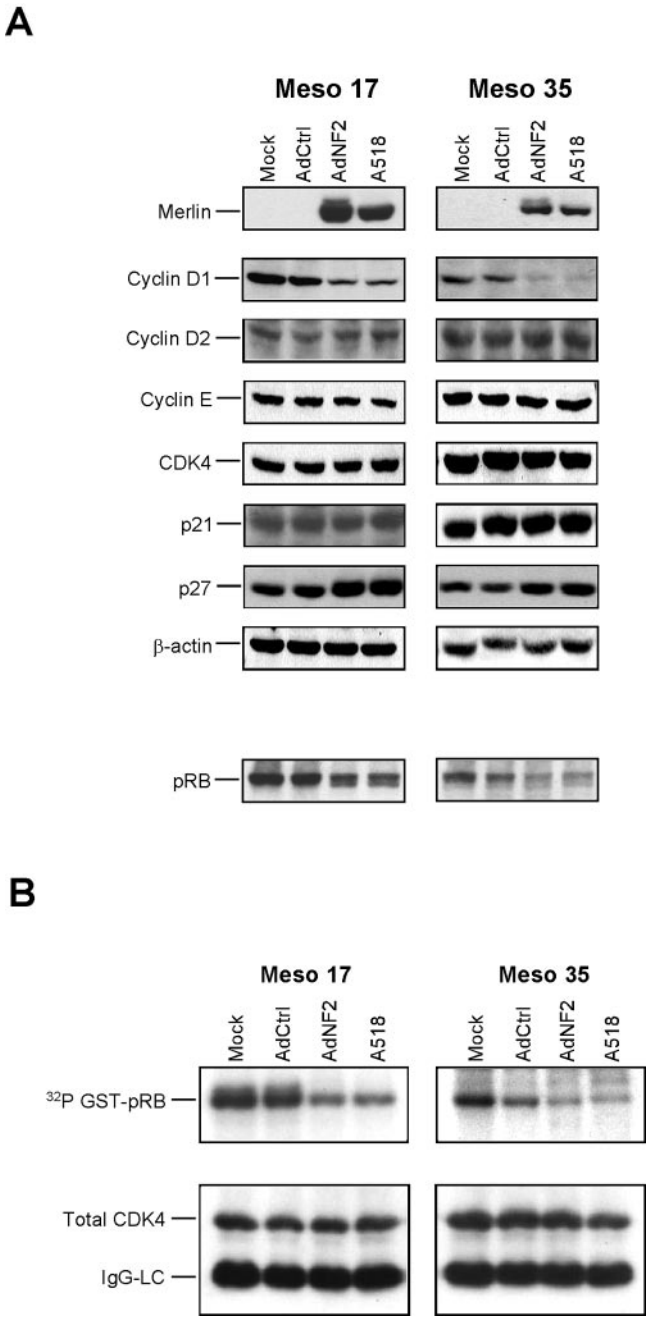

FIG. 2. Analysis of cell cycle regulators in Meso 17 and Meso 35 cells after adenovirus-mediated expression of merlin. (A) Cell lysates were electrophoresed and immunoblotted with the indicated antibodies. Infection with AdNF2 or AdNF2 A518 decreased cyclin D1 and increased p27 expression levels, which were accompanied by dephosphorylation of pRB. (B) Total cellular protein was immunoprecipitated with CDK4 antibody, and kinase assays were performed using glutathione *S*-transferase–Rb as a substrate, as described in Materials and Methods. Reexpression of AdNF2 or AdNF2 A518 inhibits CDK4 kinase activity (upper panel). Immunoblot analyses of immunoprecipitates with anti-CDK4 antibody demonstrate equivalent loading in each lane (lower panel). IgG-LC, Ig light chain.

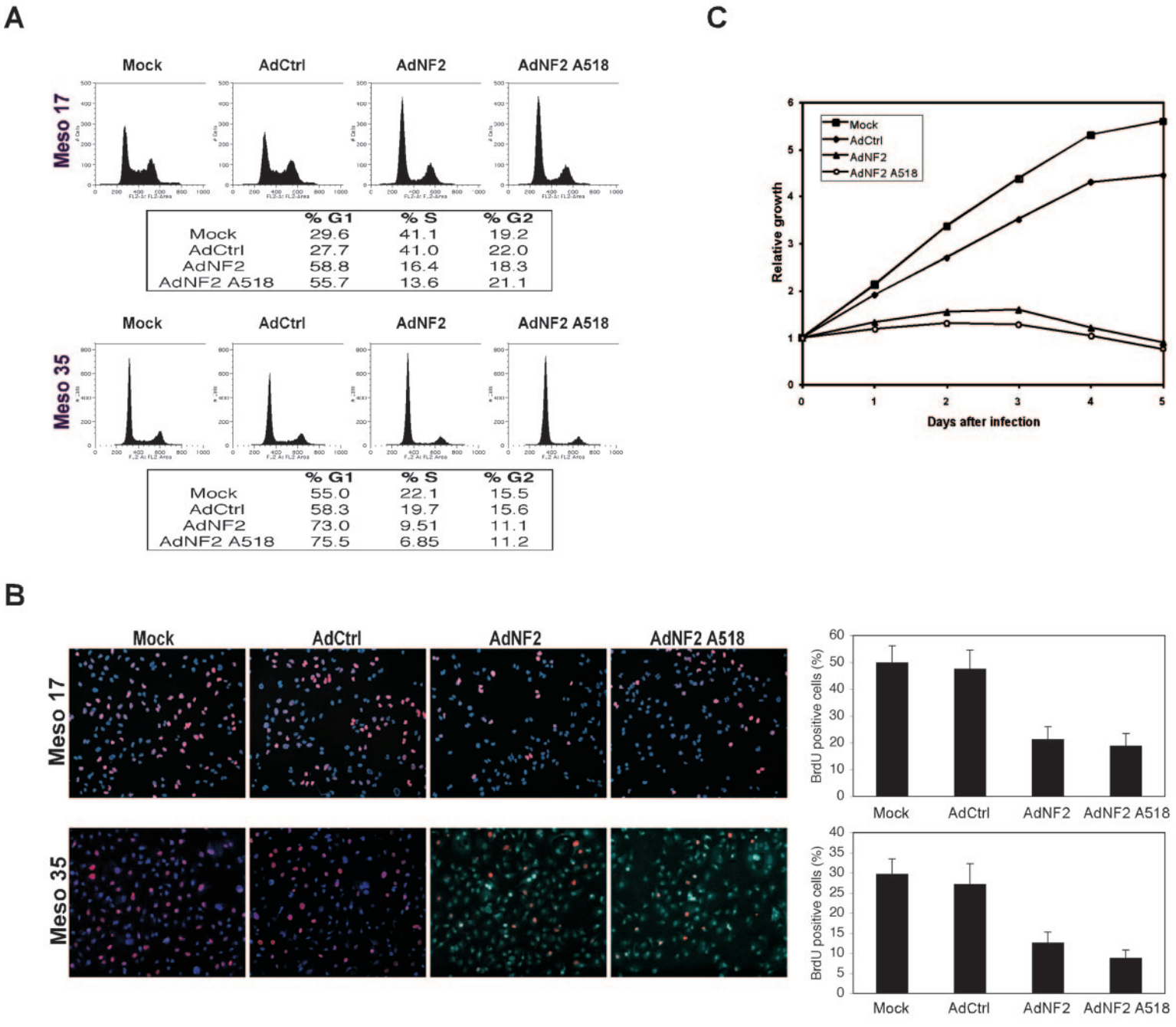

FIG. 3. Induction G_1 cell cycle arrest and inhibition of cell proliferation by reexpression of merlin. (A) After adenoviral infection for 24 h, Meso 17 and Meso 35 cells were stained with propidium iodide and subjected to FACS analysis to demonstrate cell cycle distribution based on DNA content. The experiments were conducted three times with similar results. (B) Expression of merlin inhibits S-phase entry. Meso 17 and Meso 35 cells were mock infected or infected with AdCtrl, Ad-NF2, or Ad-NF2 A518 for 18 h and incubated for an additional 6 h in the presence of 20 μM BrdU. Red staining depicts BrdU-labeled nuclei. DAPI staining (blue) was used as the counterstain. Histograms indicate percentages of BrdU-positive cells, which were calculated based on counts of 800 to 900 cells per treatment. Bars are means ± standard deviations of three independent experiments. (C) Equal numbers of Meso 17 cells were seeded in individual culture dishes. The cells were then mock infected or infected with AdCtrl, AdNF2, or AdNF2 A518. Viable cell counts (as determined by exclusion of trypan blue stain) were performed for 5 days at 24-h intervals. This experiment is representative of three independent experiments.

trypan blue staining. Adenovirus-mediated expression of merlin in Meso 17 severely inhibited cell growth (Fig. 3C). It is noteworthy that in all three experiments shown in Fig. 3, AdNF2 A518 was more efficient as a negative regulator of cell cycle progression and cell growth than was AdNF2.

Overexpression of cyclin D1 attenuates merlin's effect on cell cycle progression. To investigate whether cyclin D1 is an essential mediator of merlin's effect on cell cycle progression, we next performed a complementation experiment. Meso 17 cells were transfected with pcDNA3 empty vector or with a cyclin D1 expression plasmid (pRC-D1) and selected with G418. Infection of pcDNA3 control transfectants with AdNF2 or AdNF2 A518 resulted in pRB dephosphorylation and p27 upregulation, as was seen with the parental cells. Infection of pRC-D1 transfectants with AdNF2 or AdNF2 A518, however, did not have any effect on p27 expression, and merlin's regulatory effect on pRB dephosphorylation was inhibited (Fig. 4A). Furthermore, FACS analyses showed that infection of pcDNA3 transfectants with AdNF2 or AdNF2 A518 resulted in an accumulation of cells in G_1 and decrease in S and G_2 phases, a cell cycle distribution similar to that observed with the parental cells. Conversely, in pRC-D1 transfectants, the inhibitory effect of merlin on S-phase entry was severely attenuated (Fig. 4B). These results suggest that inhibition of cell cycle progression by merlin is mainly attributed to the fact that merlin functions upstream of cyclin D1 in regulating cell cycle progression.

NF2 inhibits cyclin D1 promoter activity. A recent study has shown that PAK1 stimulates cyclin D1 transcription in mammary epithelial and cancer cells (2), and merlin has been implicated as a negative regulator of PAK signaling (18). Thus, we asked whether PAK1-mediated transcription of cyclin D1 can be regulated by merlin. HeLa cells were cotransfected with an empty vector, wild-type PAK1, or PAK1 T423E, a constitutively active form of PAK1, together with the cyclin D1 reporter plasmid −1745 CD1-Luc. As reported previously (2), both wild-type PAK1 and PAK1 T423E expression induced transcription from the cyclin D1 promoter (Fig. 5). To determine the effect of merlin on PAK1-induced cyclin D1 promoter activity, cells were cotransfected with wild-type PAK1 or PAK1 T423E and HA-NF2 or HA-NF2 A518 plus the cyclin D1 reporter. Wild-type PAK1- or PAK1 T423E-induced transcription was repressed by coexpression of either HA-NF2 or HA-NF2 A518. Furthermore, PAK1-mediated induction of the cyclin D1 promoter was more effectively inhibited by cotransfection with the A518 mutant form of *NF2* than with wild-type *NF2* (Fig. 5).

Merlin blocks PAK activity. We have shown that PAK1-induced cyclin D1 promoter activity is attenuated by merlin, which is consistent with the notion that merlin functions as a negative regulator of PAK signaling in certain rodent systems (12, 18). To test if merlin can inhibit PAK activity in human mesothelioma cells, we next performed a two-dimensional gel electrophoretic analysis of PAK1 activity in Meso 17 cells transduced with AdNF2 WT or AdNF2 A518. In this assay, the phosphorylation status of PAK1 serves as a direct indication of the activation status of the kinase. The results showed that PAK1 activity was decreased by expression of either NF2 or NF2 A518 (Fig. 6A).

Since ERK activity is important in governing cyclin D1 ac-

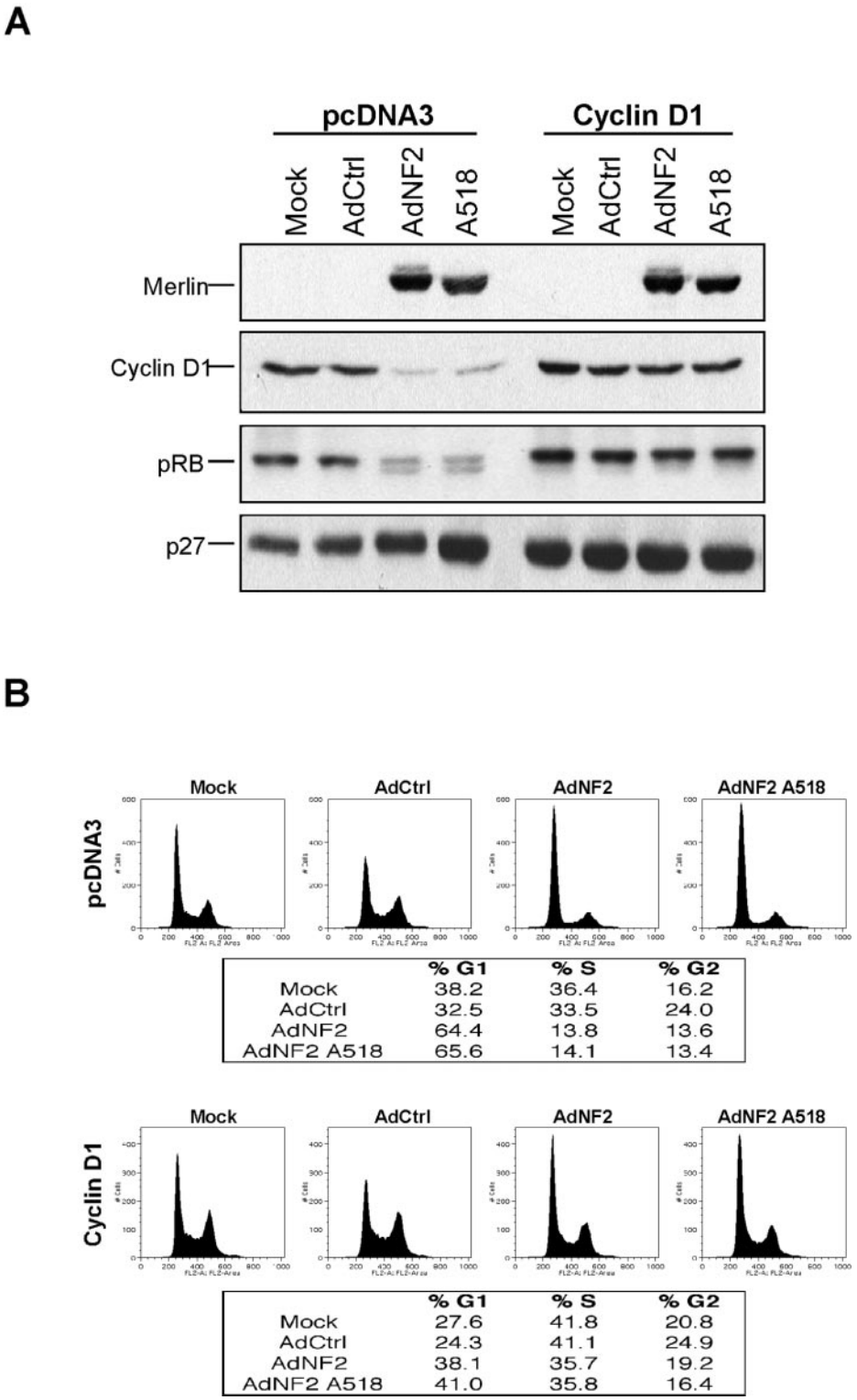

FIG. 4. Overexpression of cyclin D1 attenuates merlin's effect on cell cycle progression. (A) Meso 17 cells were transfected with pcDNA3 empty plasmid or cyclin D1 expression plasmid pRC-D1 and selected with G418. The pooled pcDNA3 or pRC-D1 transfectants were mock infected or infected with AdCtrl, AdNF2, or AdNF2 A518. After 24 h, cells were lysed and total cellular protein was electrophoresed and immunoblotted with the indicated antibodies. The pooled pRC-D1 transfectants exhibited a moderate increase in cyclin D1 protein level compared to pcDNA3 control transfectants (compare the lane of mock-infected pcDNA3 transfectants with that of mock-infected pRC-D1 transfectants). Infection of pcDNA3 transfectants with AdNF2 or AdNF2 A518 resulted in pRB dephosphorylation and p27 upregulation, as was seen with the parental cells (Fig. 2A). These regulatory effects of merlin, however, were markedly impeded in pRC-D1 transfectants. (B) Cells were treated as above and subjected to FACS analyses. Infection of pcDNA3 transfectants with AdNF2 or AdNF2 A518 resulted in an accumulation of G_1 cells and a decrease in the population in S phase, whereas infection of pRC-D1 transfectants with either AdNF2 or AdNF2 A518 attenuated the change in cell cycle distribution.

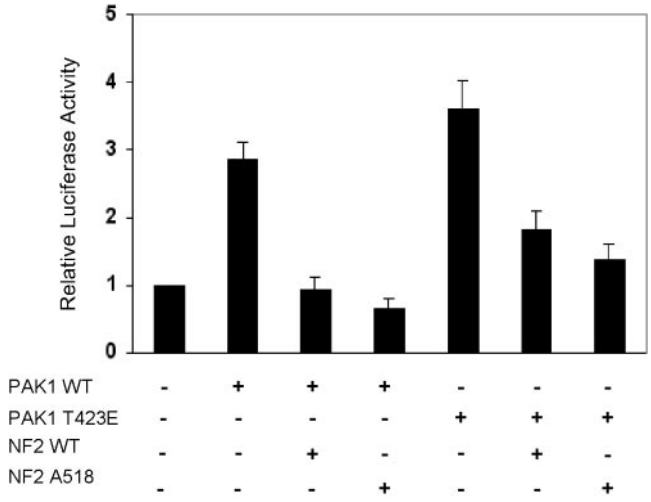

FIG. 5. Repression of PAK1-stimulated cyclinD1 promoter by merlin. HeLa cells were transiently cotransfected with the cyclin D1 luciferase reporter plasmid (−1745D1-Luc) and PAK1 wild type (WT) or the constitutively active form of PAK1 (PAK1 T423E), in the presence or absence of pcDNA3-HA-NF2 or pcDNA3-HA-NF2 A518. The arbitrary units of luciferase content were normalized with total protein concentration and plotted relative to the pcDNA3 control.

cumulation, we further examined if merlin could alter ERK signaling. Immunoblot analyses showed that reexpression of merlin in Meso 17 cells had no obvious effect on either phosphorylation or overall expression of ERK protein (Fig. 6B).

Inhibition of endogenous merlin augments cyclin D1 accumulation. The above experiments demonstrate that overexpression of merlin in *NF2*-deficient cells induces cell cycle arrest through downregulation of cyclin D1 expression. We next investigated the role of endogenous merlin in governing cell cycle progression. For this purpose, we used a siRNA pool targeting *NF2* to silence merlin expression in HeLa cells, which are highly amenable to siRNA treatment. As seen in Fig. 7A, cells transfected with *NF2* siRNA showed a dramatic reduction of endogenous merlin expression compared to cells transfected with a control siRNA. Consistent with our proposed role for merlin as a negative regulator of cyclin D1 expression, transfection with *NF2*-specific siRNA increased cyclin D1 accumulation. Similar results were obtained with the ovarian cancer cell line A2780 (Fig. 7A). Furthermore, silencing of endogenous *NF2* expression in HeLa cells accelerated S-phase entry, as indicated by elevated BrdU incorporation (Fig. 7B and C). These knockdown experiments complemented the preceding overexpression studies and strongly suggest that cyclin D1 expression is physiologically modulated by merlin.

DISCUSSION

In this report, we show that adenovirus-mediated expression of wild-type merlin inhibits cell proliferation and blocks cell cycle progression at G_1 phase in *NF2*-deficient mesothelioma cell lines. These results are in agreement with previous studies implicating merlin as a negative growth regulator in other cell types, including NIH 3T3 fibroblasts (22, 33), schwannoma cells (25, 28, 31), and meningioma cells (14).

We further demonstrated that merlin regulates cell proliferation by modulating cyclin D1 transcription. Furthermore, our results with human mesothelioma cells parallel the aberrant upregulation of cyclin D1 reported in mouse embryonic fibroblasts from *Nf2* null mice (20). Cyclin D1 (also known as bcl-1 or PRAD1) is a proto-oncogene that encodes a regulatory subunit of the cyclin-dependent kinase holoenzymes. Activation of the holoenzymes leads to phosphorylation and inactivation of the RB tumor suppressor and thereby promotes entry into S phase (32). Our results demonstrate that expression of merlin induces a decrease in both cyclin D1 RNA and protein levels and CDK4 kinase activity, concomitant with dephosphorylation of pRB and reduced DNA synthesis. We also found that cell cycle arrest at G_1 phase caused by AdNF2 or AdNF2S518A can be partially overridden by ectopic expression of a cyclin D1 plasmid, indicating that cyclin D1 is an essential mediator of merlin's observed effect on cell cycle progression. Moreover, our RNA interference experiments point to a physiological role for merlin in the regulation of cyclin D1 expression. Interestingly, Hulit et al. found that even a 50% reduction in cyclin D1 expression is sufficient to reduce tumor incidence and progression in *Min* mice harboring a mutant *Apc* gene (13). This is comparable to the downregulation observed in our studies and emphasizes the importance of cyclin D1 regulation in modulating tumorigenesis.

PAK has been suggested to serve as the key effector for Rac1 activation of cyclin D1 (34). Recent studies show that PAK signaling mediates cyclin D1 expression in mammary epithelial and cancer cells (2), and PAK is essential for RAS-induced upregulation of cyclin D1 during the G_1-to-S transition (26). The data presented here demonstrate that restoration of merlin in *NF2*-deficient mesothelioma cells inhibits PAK1 activity and represses PAK1-induced cyclin D1 promoter activity. Our results with human cancer cells are consistent with previous studies, using rodent cells, demonstrating that merlin can inhibit PAK activity (12, 18). More importantly, our findings indicate that merlin can repress cyclin D1 expression through inhibition of PAK. As noted above, *NF2* inactivation has been reported not only in malignant mesotheliomas, but also in benign neurological tumors, particularly schwannomas, seen in patients with NF2 disorder. How a tumor suppressor gene contributes to both malignant mesotheliomas and benign schwannomas may seem somewhat paradoxical, given that Rac-PAK1 signaling can regulate cell motility and invasiveness. Thus, a mechanism other than tumor invasiveness must be invoked to attribute a role for *NF2* inactivation in both benign and malignant tumors. Germane to this, Westwick et al. (34) showed that the Rac interaction with PAK was required for cyclin D1 stimulation but was dispensable for other Rac functions, such as induction of lamellipodia and activation of JNK. Their results therefore support our contention that inhibition of cyclin D1 expression represents perhaps merlin's major function as a tumor suppressor.

It has been shown previously that PAK1 regulates cyclin D1 transcription by means of an NF-κB-dependent pathway (2), and merlin can block NF-κB activity (16). Taken together, these data suggest a model in which merlin regulates transcription of cyclin D1 through PAK–NF-κB signaling.

Regulation of cyclin D1 expression by merlin might also involve ERK signaling. It is known that ERK activity is critical in the transcriptional induction of cyclin D1 in several cell

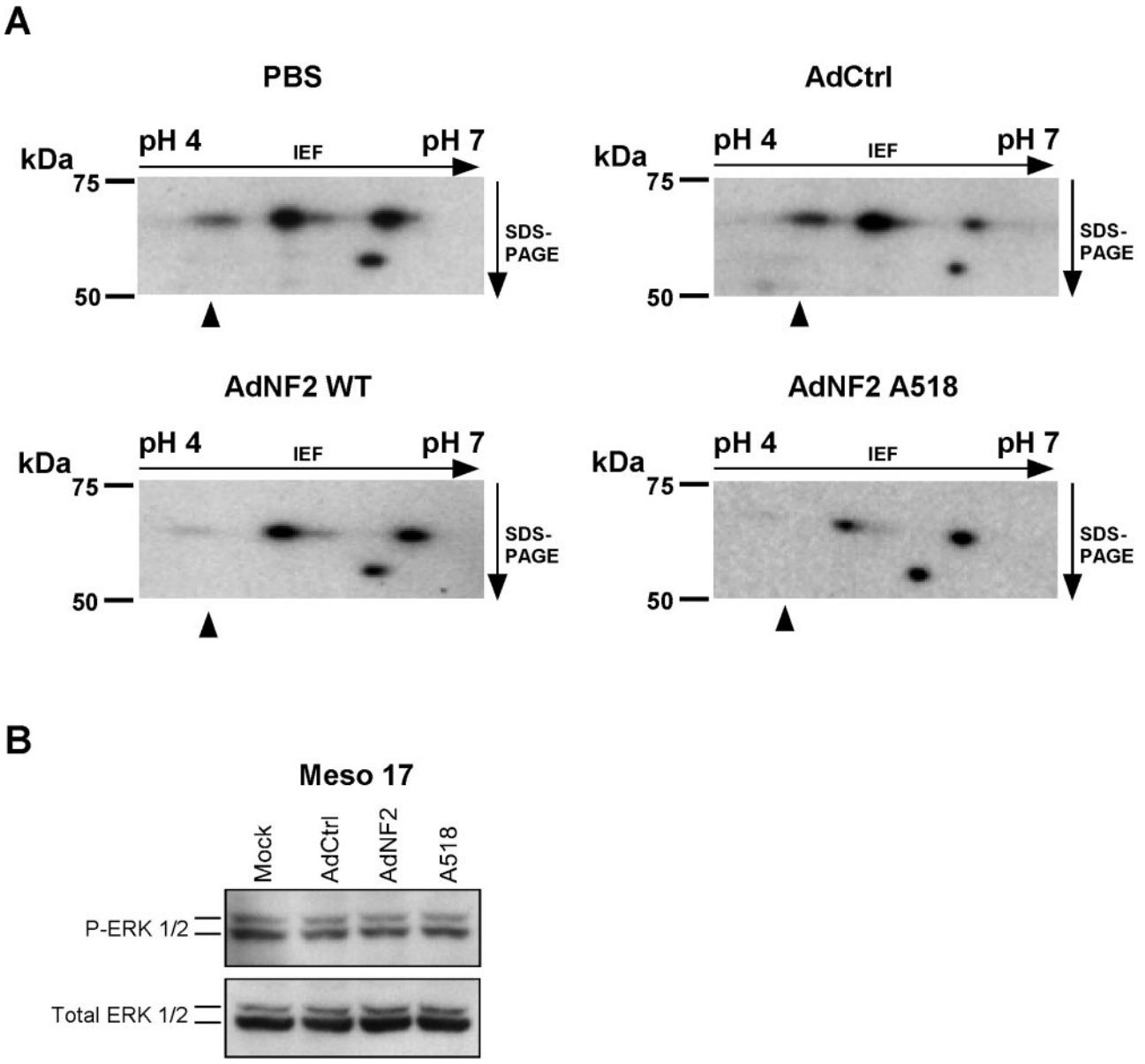

FIG. 6. Inhibition of PAK activity by merlin. (A) Two-dimensional gel analysis of PAK1 activity. Figures depict Western blot analyses of PAK1 in Meso 17 cells mock infected or infected with AdCtrl, AdNF2, or AdNF2 A518. The altered phosphorylated form of PAK1 is indicated by arrows. (B) Meso 17 cells were infected in the same way as for panel A, the cell lysates were immunoblotted with anti-phospho-ERK or global ERK antibody, and no obvious alteration was noted after reexpression of merlin.

types (reviewed in reference 3). ERK induction of the cyclin D1 promoter activity occurs through sequences targeted by ETS, which is distinct from the Rac/PAK pathway (15). Because PAK is thought to be required for efficient activation of Raf and MEK/ERK (8, 21), feedback inhibition of the Rac/PAK axis by merlin could play a role in downregulating ERK activity and therefore repressing cyclin D1 expression. However, neither ERK expression nor phosphorylation was changed in response to AdNF2 expression, indicating that this pathway is not involved in modulating cyclin D1 expression by merlin in our system.

Reexpression of merlin in Meso 17 and Meso 35 cells not only represses cyclin D1 expression but also results in the accumulation of the p27 protein. In addition to phosphorylation of the pRB protein, cyclin D-CDK4 complexes also function to sequester the cell cycle inhibitors p21 and p27, thereby inducing CDK2 activity and promoting cell cycle progression into S phase. Activated CDK2 then phosphorylates and triggers proteolysis of p27 (32). Thus, by repressing cyclin D-CDK4 activity, it is likely that merlin increases p27 accumulation by inhibiting sequestration and degradation of p27.

Previous work had demonstrated that the phosphorylation status of merlin specifies cell growth arrest or cell proliferation. Phosphorylation of merlin by Rac/PAK signaling inactivates merlin and potentiates Rac/PAK activities (17, 30, 35). The data presented here further support this feed-forward mecha-

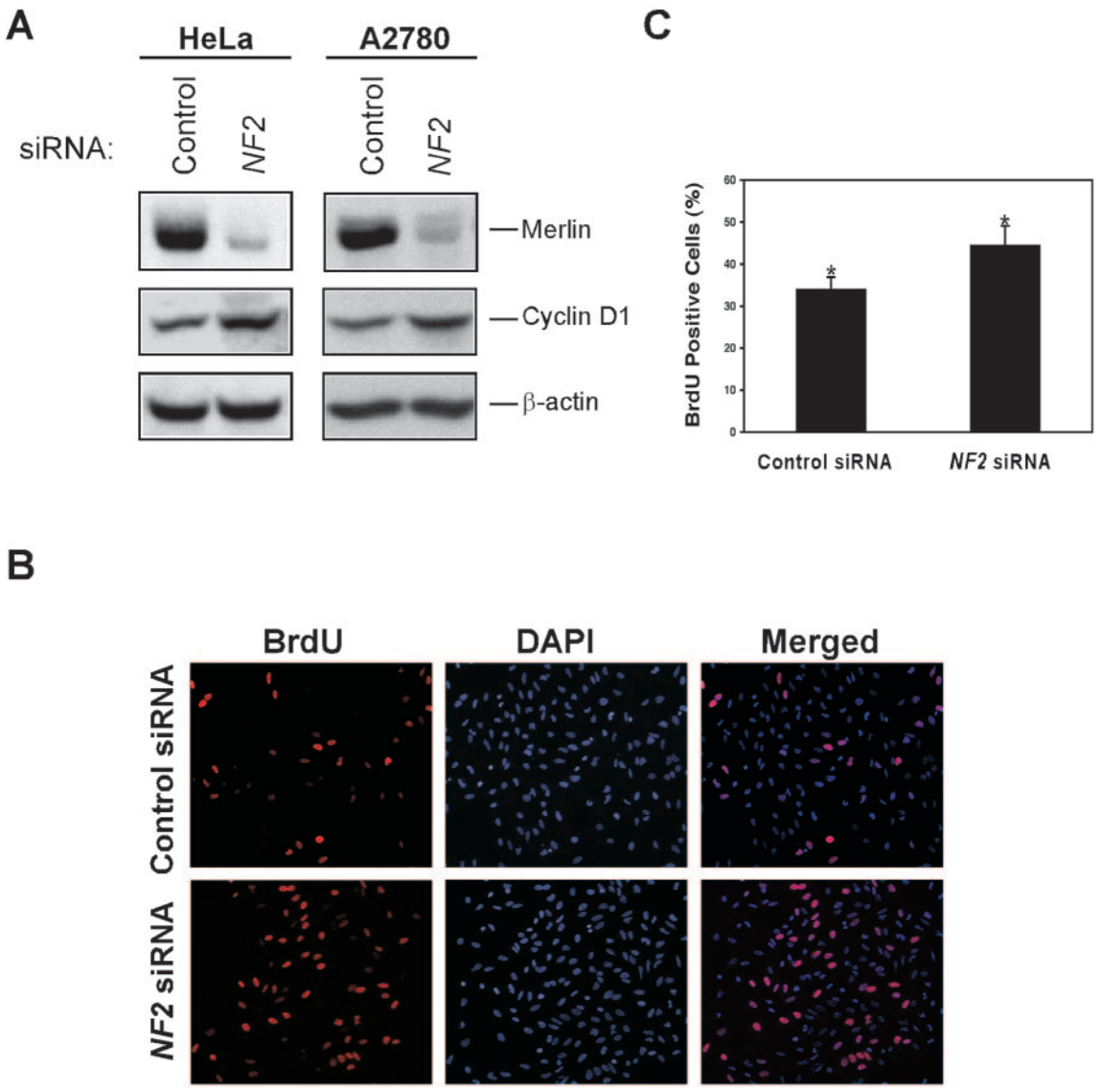

FIG. 7. Effects of silencing endogenous *NF2* expression. (A) HeLa and A2780 cells were transfected with control siRNA or siRNA specific for *NF2*. Cells were lysed, and total cellular protein was subjected to immunoblot analysis. In both HeLa and A2780 cells, exposure to *NF2* siRNA resulted in a marked reduction of merlin expression, concomitant with an increase in cyclin D1 accumulation. (B) Depletion of endogenous *NF2* accelerates S-phase entry. HeLa cells were grown on coverslips and transfected with control siRNA or *NF2* siRNA as described above. BrdU (20 μM) was added during the last 6 h of incubation, followed by immunofluorescence staining. BrdU incorporation was detected with an anti-BrdU–Alexa 594 antibody (red). Total nuclei were visualized with DAPI (blue). (C) Histogram depicting elevated BrdU incorporation resulting from silencing of endogenous *NF2* relative to control treatment. Bars are means ± standard deviations of three independent experiments. *, $P < 0.05$.

nism. In confluent cell cultures, hypophosphorylated merlin acts as an inhibitor of PAK and blocks Rac/PAK-stimulated cyclin D1 expression. This form of merlin is growth inhibitory and represents the functionally active tumor suppressor. At low cell density, however, merlin is phosphorylated in response to activation of Rac/PAK signaling. Phosphorylation of merlin attenuates its activity as a negative regulator of PAK and potentiates Rac/PAK functions. This form of merlin is growth permissive and therefore represents the functionally inactive tumor suppressor. Collectively, these findings indicate that there is a stimulatory-inhibitory loop between PAK and merlin and that a fine balance must be maintained between these two molecules to regulate normal cellular proliferation.

Merlin is phosphorylated at Ser518 by Rac/PAK signaling (17, 30, 35). As predicted, McClatchey and colleagues have shown that the A518 phosphorylation-defective form of merlin inhibits Rac-induced activation of AP-1 reporter activity as well as or better than the wild-type protein (30). In this study, we also examined whether the A518 mutant can act as a constitutively active form of the tumor suppressor in modulating PAK activity and cyclin D1 expression. We found that PAK1-mediated induction of the cyclin D1 promoter was more effectively inhibited by cotransfection with the A518 mutant form of *NF2* than with wild-type *NF2*. We also observed that AdNF2 A518 arrested more cells at G_1 phase than did AdNF2. In addition, Meso 17 cells transfected with a plasmid encoding the

A518 mutant showed significantly reduced colony-forming efficiency compared to cells transfected with wild-type *NF2* (unpublished observations). Based on these results and the findings by McClatchey and colleagues (30), we conclude that the phosphorylation-refractory A518 mutant represents an active form of the tumor suppressor.

Collectively, the data presented here suggest that the *NF2* tumor suppressor gene exerts its antiproliferative effect, at least in part, via repression of PAK-induced cyclin D1 expression. These observations have significant implications for elucidating tumorigenic mechanisms involved in neoplasms associated with merlin inactivation. Our experiments with AdNF2 achieved 100% transduction efficiency in mesothelioma cells, which demonstrates the technical feasibility of using adenovirus-mediated transfer of the *NF2* gene as a potential therapeutic strategy for human malignant mesothelioma.

ACKNOWLEDGMENTS

We thank Peter Adams for helpful suggestions regarding some of the experiments conducted in this study and Warren Kruger and Davide Ruggero for critical review of the manuscript. We also thank Bert Vogelstein for providing the pAdEasy system to generate the *NF2* adenoviral vectors.

This work was supported by National Institutes of Health grants CA-45745 (to J.R.T.), training grant T32-16850, ACS institutional grant 38102 (to G.H.X.), and CA-06927 (to Fox Chase Cancer Center), by an appropriation from the Commonwealth of Pennsylvania, and by a gift from the Local No. 14 Mesothelioma Fund of the International Association of Heat and Frost Insulators and Asbestos Workers in memory of Hank Vaughan and Alice Haas. R.G.P is supported by National Institutes of Health grants CA-70896, CA-75503, CA-86072, and CA-93596. The following Fox Chase Cancer Center shared facilities were used in the course of this work: Cell Culture Facility, DNA Sequencing Facility, DNA Synthesis Facility, DNA Microarray Facility, and Flow Cytometry and Cell Sorting Facility.

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