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PAK4 Is Required for Regulation of the Cell-Cycle Regulatory Protein p21, and for Control of Cell-Cycle Progression

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ABSTRACT

The serine/threonine kinase PAK4 regulates cytoskeletal architecture, and controls cell proliferation and survival. In most adult tissues PAK4 is expressed at low levels, but overexpression of PAK4 is associated with uncontrolled proliferation, inappropriate cell survival, and oncogenic transformation. Here we have studied for the first time, the role for PAK4 in the cell cycle. We found that PAK4 levels peak dramatically but transiently in the early part of G1 phase. Deletion of *Pak4* was also associated with an increase in p21 levels, and PAK4 was required for normal p21 degradation. In serum-starved cells, the absence of PAK4 led to a reduction in the amount of cells in G1, and an increase in the amount of cells in G2/M phase. We propose that the transient increase in PAK4 levels at early G1 reduces p21 levels, thereby abrogating the activity of CDK4/CDK6 kinases, and allowing cells to proceed with the cell cycle in a precisely coordinated way. J. Cell. Biochem. 112: 1795–1806, 2011.

KEY WORDS: PAK4; p21; PROTEIN KINASE; CELL CYCLE; G1

p21-activated kinase 4 (PAK4) is a member of a family of serine/threonine kinases, which consists of two groups, A and B [Jaffer and Chernoff, 2002]. The group A family includes mammalian PAK1, PAK2, and PAK3, whereas group B includes PAK4, PAK5, and PAK6 [Manser et al., 1994; Bagrodia et al., 1995; Brown et al., 1996; Abo et al., 1998; Yang et al., 2001; Dan et al., 2002; Pandey et al., 2002]. The PAKs were first identified as effector proteins for Cdc42 and Rac, members of the Rho GTPase family [Manser et al., 1994; Bagrodia et al., 1995; Brown et al., 1996; Abo et al., 1998; Dan et al., 2002]. More recently, they have also been found to have Rho GTPase-independent activators [Kaur et al., 2005; Ahmed, 2008]. PAK4, like other PAK family members, contains a highly conserved serine/threonine kinase domain, but it has both kinase-dependent and kinase-independent functions [Abo et al., 1998; Gnesutta and Minden, 2003; Barac et al., 2004].

PAK4 is highly expressed during development, and therefore it is not surprising that deletion of the *Pak4* gene results in embryonic lethality [Qu et al., 2003]. *Pak4* knockout embryos die in midgestation soon after E10.5, with defects in the heart and placenta, anomalies in vascular system, and abnormal migration of motor neurons [Qu et al., 2003; Tian et al., 2009]. In contrast to *Pak4*, deletion of *Pak5* or *Pak6* genes do not result in lethal phenotypes, although the mice demonstrated some behavioral deficits [Nekrasova et al., 2008]. This suggests that PAK4 has unique functions, which cannot be compensated by any other member of PAK family.

PAK4 is expressed at low levels in most adult tissues, but it is highly overexpressed in many different tumors and tumor cell lines [Callow et al., 2002; Liu, 2008]. A constitutively active PAK4 mutant promotes anchorage-independent growth when overexpressed in immortalized fibroblasts [Qu et al., 2001; Callow et al., 2002]. In fact, PAK4 transforms cells in culture as efficiently as oncogenic Ras, a strong oncogene [Qu et al., 2001]. In vivo overexpression of wild-type PAK4 leads to tumor formation in athymic mice, while deletion of PAK4 abrogates tumor formation [Liu, 2008]. The mechanism by which PAK4 leads to transformation is not well understood, but one possibility is that its role in cell survival contributes to its role in oncogenesis. PAK4 promotes cell survival by different mechanisms. Depending on the stimulus, PAK4 can promote cell survival by either kinase-dependent or kinase-independent mechanisms. The kinase-dependent mechanism includes phosphorylation of the pro-apoptotic protein Bad, while the kinase-independent mechanism includes inhibition of caspase-8 binding to death domain containing

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receptors [Gnesutta et al., 2001; Gnesutta and Minden, 2003; Li and Minden, 2005].

Improper regulation of the cell cycle can be another important factor in oncogenesis. An intriguing possibility, therefore, is that PAK4 plays a role in controlling the cell cycle, thus leading to oncogenesis when it is improperly expressed. The role for PAK4 in cell-cycle regulation has not previously been explored. In this article the role for PAK4 in cell-cycle regulation is investigated, and an inherent role for PAK4 in regulating the cell-cycle regulatory protein p21 (CDKN1A) was observed. p21 is a key regulator of cell-cycle progression, but also plays a role in cell differentiation and survival during development, as well as in senescence and apoptosis [reviewed in Abbas and Dutta, 2009]. p21 was originally identified as a cyclin-dependent kinase inhibitor, which functions to restrict cell-cycle progression. Later its function was found to be more complex, and it has been shown to have both negative as well as some positive roles in controlling the cell cycle. The role for p21 in positively regulating the cell cycle, at least early in G1, is supported by the fact that mitogens elevate p21 levels through the Ras-Raf-MEK-ERK signaling pathway [Olson et al., 1998; Bottazzi et al., 1999]. p21 also serves as a promoter for cyclin D1–Cdk assembly, nuclear retention, and stability during G1 phase [LaBaer et al., 1997; Cheng et al., 1999; Alt et al., 2002], thereby allowing cell-cycle progression. p21, however, also clearly has important roles in inhibiting cell-cycle progression. Negative regulation of the cell cycle by p21 is due in part to its ability to bind CDK2, and in turn inhibit its capacity to phosphorylate Rb [Mandal et al., 1998]. Another way that p21 negatively regulates the cell cycle is by binding to the proliferating cell nuclear antigen (PCNA), thereby inhibiting DNA replication [Waga et al., 1994].

As a result of extensive studies a complex picture of the regulation of p21 has emerged. Transcriptional induction of p21 is regulated by p53-dependent, as well as p53-independent mechanisms [el-Deiry et al., 1993; Li et al., 1994; Hiyama et al., 1998]. Transcriptional activators of p21 include growth factors (GFs), several nuclear receptors, numerous transcription factors, and some steroid hormones reviewed in Abbas and Dutta [2009]. Increased p21 levels can be also controlled at the level of mRNA stability [Giles et al., 2003].

p21 is a short-lived protein and its degradation is regulated by ubiquitin-mediated as well as ubiquitin-independent proteasomal degradation processes [Chen et al., 2004]. A number of different proteins engage in protein–protein interactions with p21 and may thereby influence p21 degradation [Bao et al., 2002; Scott et al., 2002; Densham et al., 2009]. p21 is a phosphoprotein and a target for several serine/threonine kinases, and phosphorylation of p21 may affect its stability [Li et al., 2002; Rossig et al., 2002; Scott et al., 2002]. The role for phosphorylation of p21 is complex, however, as phosphorylation can have different effects, ranging from stabilization to destabilization of the protein. The effects of p21 phosphorylation strongly depend on the cellular context [Li et al., 2002; Rossig et al., 2002; Scott et al., 2002]. Importantly, different signaling pathways can lead to either increased or decreased levels of p21 via multiple mechanisms. Ras, for example, can elevate p21 protein levels not only by activating p21 transcription, but also by increasing its stability. Increased stability in this case is mediated by induction of cyclin D1, which sequesters p21 from proteasome-dependent degradation [Coleman et al., 2003]. Rho-mediated repression of p21 is also regulated at both mRNA and protein levels [Coleman et al., 2006].

Here we have found that the absence of PAK4 in cells is associated with an increased level of p21 protein. Deletion of PAK4 increased both the level of p21 mRNA as well as stability of p21 protein. By analyzing PAK4 levels at different phases of the cell cycle, we found a strong but temporary increase in PAK4 in early G1 phase, which suggests that PAK4 plays a role in the initiation of the cell cycle. Our data support a model in which PAK4 downregulates p21 in the early, GF-dependent segment of the G1 phase, to prevent premature entrance of cells into the cell cycle.

MATERIALS AND METHODS

CELL CULTURE

NIH-3T3 and immortalized *Pak4*$^{+/+}$ and *Pak4*$^{-/-}$ fibroblasts were maintained in Dulbecco's modified Eagle's medium containing 10% FBS and 2 mg/ml L-glutamine at 37°C and 5% CO_2 (all reagents from Gibco). Cell lines were established from mouse embryonic fibroblasts isolated from *Pak4*$^{+/+}$ and *Pak4*$^{-/-}$ embryos at E10.5 and immortalized by 3T3 protocol. *Pak4* knockout mice were generated in our lab [Qu et al., 2003] and were maintained as heterozygotes. Early passage (30–50) immortalized cells were used in all experiments. For serum starvation cells were plated in DMEM supplemented with 0.5% FBS, 2 mg/ml L-glutamine and maintained for at least 48 h to arrest them in G0. *Pak4* knockout cells were transfected with Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. For apoptosis experiments cells were treated with 10 ng/ml TNF (BD Biosciences) in the presence of 10 μg/ml CHX (Sigma) overnight, or with 25 μM H_2O_2 (Sigma) overnight, or irradiated with UV at 50 J/cm^2.

ANTIBODIES AND WESTERN BLOTTING

Rabbit polyclonal anti-PAK4, anti-phosphoS474 PAK4, anti-p27, and anti-PARP antibody were from Cell Signaling. Rabbit polyclonal anti-laminB antibody were from Imgenex. Mouse monoclonal anti-α-tubulin antibody (clone B-512) were from Sigma. Mouse monoclonal antibodies against p21 (clone SX118) and against retinoblastoma protein (clone G3-245) were from BD Pharmingen. Anti-actin (clone AC-40) antibodies were from Sigma, anti-cyclinD (cloneSP4) from Thermo Scientific, anti-CDK4 (DCS-35) and CDK6 (K6.90) from Abcam. For Western blot analysis cells where homogenized in 0.25 ml of modified RIPA buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% triton X-100, 2.5 mM $Na_4P_2O_7$, 50 mM sodium fluoride, 2 mM sodium orthovanadate, and protease inhibitor cocktail (Set III, Calbiochem). Cytoplasmic and nuclear fractions were prepared using NE-PER nuclear and cytoplasmic extraction kit (ThermoScientific) according to manufacturer's protocol. Samples were centrifuged for 20 min at 13,000*g* at 4°C. Laemmli sample buffer was added to aliquots containing equal amounts of protein, and the samples were boiled at 95°C for 5 min. Samples were electrophoresed on 7–12% SDS–polyacrylamide gels and blotted electrophoretically to Immobilon membrane. Membranes were blocked in TBST (50 mM Tris–HCl,

pH 7.5, 150 mM NaCl, 0.1% Tween-20) with 5% nonfat milk. Blots were incubated with primary antibodies overnight followed by incubation with secondary antibody conjugated to horseradish peroxidase (1:10,000), and developed using the enhanced chemiluminescence method (GE Healthcare). Protein concentration was determined using the BioRad protein assay. Protein quantification was performed by densitometry of bands in low ECL exposures, using ImageJ software. All data were normalized by β-actin loading for the same samples. All experiments were repeated at least three times. Every repeat included examination of three wild-type and three *Pak4* knockout cell lines, and statistical analysis was performed on three cell lines for each genotype to see a genotype effect.

CYCLOHEXIMIDE CHASE

Cells were treated with 25 μg/ml cyclohexomide (Sigma) for 0, 20, 40, 60, and 80 min, rinsed with cold PBS, and lysed in RIPA buffer. β-Actin blots were used to verify equal loading. Linear regression analysis was performed using Prizm software.

FLOW CYTOMETRY

Cells were plated in 0.5% FBS in DMEM for 48 h and stimulated with 10% FBS for different times. After stimulation cells were detached by trypsinization, washed three times with PBS and fixed on ice in 70% cold ethanol for 30 min. For propidium iodide (PI) staining cells were washed with PBS and incubated in 10 μg/ml PI solution with 100 μg/ml RNAse A. The DNA content of cells stained with PI was determined by flow cytometry using Coulter Cytomics FC500 cell analyzer.

RT-PCR

RNA from cells was prepared using High Pure mRNA isolation Kit (Roche). After DNAse I treatment, 1 mg RNA was transcribed with AMV reverse transcriptase and fragments were amplified with Expand High Fidelity polymerases blend using Titan One tube RT-PCR System (Roche) according to the manufacturers protocol. The following primer sets were used: GAPDH primers: 5′-ACCCCTTCATTGACCTCAACTACA, 3′-AGTGATGGCATGGACTGTGGTCAT; p21 primers: 5′-AACATCTCAGGGCCGAAAACG, 3′-AGTTTGGAGACTGGGAGACA. Both primer sets were used in the same reaction tube and amplification was performed by 30 cycles with the annealing temperature of 58°C.

IMMUNOFLOURESCENCE

Cells grown on glass coverslips were fixed with 4% paraformaldehyde for 10 min, then blocked for 60 min in phosphate-buffered saline containing 5% bovine serum albumin (fraction V) and 0.25% Triton X-100 at room temperature. For immunofluorescence, fixed cells were incubated overnight with primary antibodies followed by fluorochrome-conjugated secondary antibodies for 1 h. Anti-mouse or anti-rabbit secondary antibodies, conjugated with DyLight 488 fluorochrome from Jackson Immunoresearch Laboratories were used at 1:200. The coverslips were counterstained with 4′6-diamidino-2-phenylindole (DAPI, 200 ng/ml), and then mounted using Prolong Antifade mounting media (Molecular Probes). Samples were examined, and pictures were acquired with a 60× oil immersion lens on a Leica TCS SP5 confocal system using the LASAF software (Leica). Recorded images were processed using Adobe Photoshop software using linear curve correction for adjusting contrast. Levels were adjusted equally for both images in a set.

RHO-GTP PULL-DOWN ASSAY

Measurement of GTP-bound Rho was performed using the Rho Activation Assay kit (Millipore), following the manufacturer's instructions. Briefly, the Rho-binding domain of Rhotekin expressed as a GST fusion protein was used to affinity precipitate GTP-bound Rho from cells lysed in 50 mm Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mm NaCl, 10 mm $MgCl_2$, supplemented with protease inhibitor mixture (Calbiochem). Precipitated Rho-GTP was then detected by immunoblot analysis, using a monoclonal anti-Rho(-A,-B,-C) antibody.

RESULTS

PAK4 LEVELS ARE INCREASED IN THE G1 PHASE OF THE CELL CYCLE

Asynchronous cultures of 3T3 cell lines growing in 10% serum or cells that were serum starved for 48 h were tested for expression of PAK4. Western blot analysis was used to examine the presence of endogenous PAK4 protein in both the cytoplasm and the nucleus. Serum starvation led to a strong increase in PAK4 protein levels (Fig. 1A). PAK4 was found primarily in the cytoplasm, but increased cellular amounts of PAK4 during serum starvation were associated with the appearance of small amounts of PAK4 protein in the nucleus as well (Fig. 1A). Interestingly, in cells that normally express higher amounts of PAK4, the protein is found in both the cytoplasm and the nucleus even under normal growth conditions (Supplemental Fig. S1).

Serum starvation can cause both growth arrest and apoptosis in different subsets of cells. We therefore tested whether the increase in PAK4 levels upon serum deprivation was associated with the synchronization of the cells with respect to the cell cycle, or if it was elevated in response to apoptosis. To analyze transition of the cells through the cycle after serum starvation, wild-type fibroblasts were serum starved for 48 h followed by stimulation with 10% serum, and analyzed by flow cytometry. The cells reached S phase after 17 h of stimulation and the mitotic peak was observed approximately 24 h after serum addition. When PAK4 levels were assessed under these conditions, PAK4 levels were found to be higher in the serum-starved cells relative to cells in S and G2/M phases of the cell cycle (Fig. 1B). Importantly, however, there was an increase in the level of PAK4 30 h after serum stimulation, when synchronized cells completed their first mitosis and entered the second G1 phase of the cell cycle. The mitotic peak (% of cells in G2/M) was maximal at 24 h poststimulation and was lower at 30 h, confirming that cells at this time point exited mitosis and were in the early G1 phase. There was no increase in PAK4 expression in other phases of the cell cycle. To test that PAK4 levels are elevated specifically in G1 and not earlier in mitosis, we assayed PAK4 levels in a pure population of mitotic cells and compared it to cells in G1, S, and G2/M phases of the cycle. We did not see any significant increase in PAK4 levels in mitotic cells compared with cells in the S and G2/M phases of the cycle (Fig. 1C).

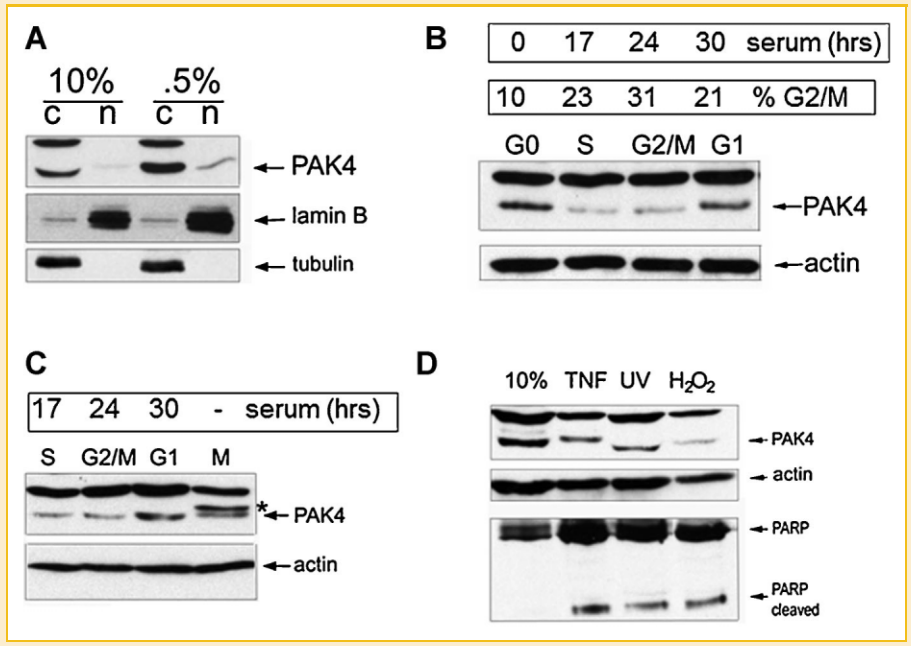

Fig. 1. PAK4 levels are increased in G1 phase. A: Wild-type fibroblasts were grown on 10% and 0.5% FBS for 48 h. The presence of PAK4 protein in cytosolic (c) and nuclear (n) fractions was analyzed by Western blotting. B: Wild-type cells were serum starved for 48 h and stimulated with 10% FBS for the indicated amounts of times. The corresponding cell-cycle phases are indicated. Western blots show PAK4 levels as well as actin levels, used for the loading control. C: Mitotic cells were shaken off the plates and whole cell lysates were prepared and tested by the Western blotting. Lysates of cells in S, G2/M, and G1 phases were loaded on the same gel. Asterisk indicates a nonspecific band that fluctuates during the cell cycle and can be seen in the *Pak4* null cells. D: Wild-type fibroblasts were treated overnight with TNF, hydrogen peroxide, or irradiated with UV. Western blots were used to assess PAK4 levels in treated cells compared to PAK4 levels in cycling cells. PARP cleavage resulting from apoptosis after treatments with different agents is also shown.

To test the possibility that the increase in PAK4 levels could result from apoptosis, we treated 3T3 cells with several apoptotic stimuli (TNF, UV, or H_2O_2) and assessed PAK4 levels 17 h later. None of the apoptotic stimuli influenced the levels of PAK4 protein (Fig. 1D). In fact, PAK4 levels were somewhat lower in the treated cells than in normal cycling cells. In accordance with this, serum starvation for 48 h increased the apoptotic fraction of cells by $<1\%$ ($2.38 \pm 0.6\%$ subG1 population in cells at 10% serum compared to $3.24 \pm 0.3\%$ at 0.5% serum). These results indicate that PAK4 protein levels are elevated specifically in the G1 phase of the cell cycle, and that the increase is not a response to increased levels of apoptosis that occur upon serum withdrawal.

PAK4 PROTEIN IS ELEVATED IN EARLY G1 BUT IT IS NOT HIGHLY PHOSPHORYLATED ON SER474

The G1 phase is the longest in the cell cycle and consists of GF-dependent and GF-independent segments. We were interested in determining which exact component of G1 is associated with the elevated amounts of PAK4 protein. We tested PAK4 levels in serum stimulated cells at different times after stimulation, when cells were synchronized and about 80% of them were in G1 phase. Western blot analysis of cell lysates revealed that the PAK4 protein levels were further increased after addition of serum to quiescent cells (Fig. 2A). Quantification of PAK4 expression revealed a major increase of PAK4 levels at 2 h poststimulation, after which PAK4 levels started to decline (Fig. 2B). In contrast, levels of PAK5, closely related to the PAK4 protein, remained at a constant level throughout the experiment (Fig. 2A,B).

Phosphorylation of serine/threonine kinases on a conserved serine or threonine residue in the kinase subdomain VIII is often associated with autophosphorylation and increased kinase activity [Taylor et al., 1992]. The corresponding serine on PAK4, serine 474, is therefore thought to be an indicator of its kinase activity [Abo et al., 1998]. We tested whether the increase in PAK4 levels in early G1 was accompanied by an increase in its phosphorylation at this site. Analysis of PAK4 phosphorylation on serine 474 after serum stimulation revealed its gradual increase during the first 6 h (Fig. 2A,C; pPAK4) without an equivalent increase in PAK4 protein levels (Fig. 2B). Analysis of serine 474 phosphorylation in other phases of the cell cycle revealed that despite the low expression of PAK4 in S and G2/M phases the protein was phosphorylated, but phosphorylation was somewhat lower in the next G1 phase (Fig. 2D,E). In contrast, phoshorylation of PAK5 on the corresponding serine, Ser602, did not change at any of the time points (Fig. 2A,C; pPAK5). Overall, PAK4 protein levels are strongly increased in early G1, but this is not accompanied by a corresponding increase in PAK4 phosphorylation on Ser474.

DELETION OF PAK4 LEADS TO AN INCREASE IN p21 LEVELS

Cyclin-dependent kinases and cell-cycle inhibitors are expressed in early G1 and are critical for regulation of the cell cycle. To gain a better understanding of the role for PAK4 in the onset of the cell cycle in G1, we used *Pak4*$^{-/-}$ fibroblast cell lines to test whether the

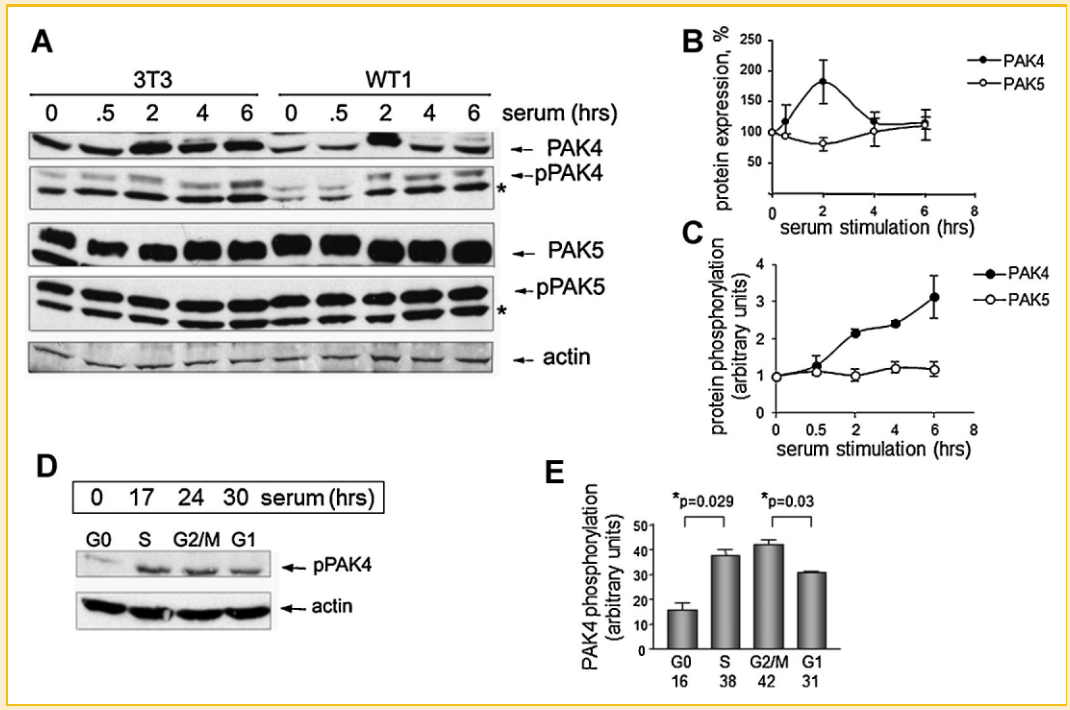

Fig. 2. PAK4 protein levels are elevated in early G1, without a corresponding increase in phospho-Ser474. A: Serum-starved NIH3T3 cells (3T3) or wild-type fibroblasts generated in our lab (WT1) were stimulated with 10% FBS. Western blot analysis was used to examine PAK4 and PAK5 protein levels as well as phospho-PAK4 and PAK5, at different times after serum stimulation. Asterisks indicate nonspecific bands. B: Quantification of PAK4 and PAK5 levels from panel A. Quantification was carried out as described in the Materials and Methods Section and plotted as arbitrary values against time. The average protein levels for two wild-type cell lines are shown. C: Quantification of phospho-PAK4 and phospho-PAK5 levels from panel A. The average phosphorylation levels of PAK proteins in independent two cell lines are shown. D: Phosphorylation of PAK4 on serine 474 assayed at different phases of the cell cycle was assessed by Western blotting in wild-type fibroblasts. E: Quantification of phospho-PAK4 levels from panel C. Average values for two cell lines are shown.

absence of PAK4 alters the balance of cell-cycle regulatory proteins. For these experiments we analyzed several 3T3 like cell lines made from *Pak4* null embryos and their wild-type littermates isolated at E10.5 (see the Materials and Methods Section). Strikingly, the absence of PAK4 caused a dramatic increase in the levels of p21 protein under low serum conditions, and serum stimulation further increased p21 levels in the *Pak4* null cells (Fig. 3A). Quantification of p21 levels revealed a statistically significant ($P < 0.05$) elevation of p21 protein in *Pak4* knockout cells compared with wild-type cells, under conditions of both serum starvation and serum stimulation (Fig. 3B). Expression of HA-tagged PAK4 in *Pak4* knockout cells led to a decrease in p21 levels (Fig. 3C). In contrast, the amounts of CDK4 (Fig. 3D,E) and CDK6 (not shown) were not changed in *Pak4* null cells growing on 10% or 0.5% serum. Amounts of cyclin D1 in serum-starved cells and at 9 h after serum stimulation, when cells have the highest amounts of cyclin D1, were not significantly different in *Pak4* null and wild-type cells, although there was a modest increase in cyclin D1 levels in serum-starved $Pak4^{-/-}$ cells (Fig. 3D,F). Expression of p27 in both serum-starved cells and 4 h after serum stimulation was slightly lower in *Pak4* null cell lines (Fig. 3D,G).

PAK4 REGULATES p21 AT BOTH THE mRNA AND PROTEIN LEVELS

p21 is a short-lived protein that can be regulated at the level of transcription as well as at the level of protein stabilization. To assess changes in p21 mRNA levels in $Pak4^{-/-}$ cells, we performed semi-quantitative RT-PCR analysis of the mRNA isolated from wild-type and *Pak4* null cells. RNA was isolated from serum-starved and serum-stimulated cells, and the amount of p21 mRNA was assessed. Serum stimulation increased p21 mRNA levels in both wild-type and *Pak4* knockout cells, although the levels of p21 mRNA were higher in $Pak4^{-/-}$ cells (Fig. 4A,B). Densitometric quantification indicated that the increase in p21 protein levels in *Pak4* null cells was about threefold (Fig. 3B,F), considerably higher than the 20–30% increase in the mRNA levels (Fig. 4B). These results indicate that PAK4 may regulate p21 at the protein level, as well as the mRNA level.

To determine whether PAK4 regulates p21 stability, we chased the presence of p21 protein in wild-type and $Pak4^{-/-}$ cells in the presence of cycloheximide, an inhibitor of protein translation. To obtain high amounts of p21 in the cells, all cells were serum starved for 48 h, then stimulated with serum for 4 h, followed by treatment with cycloheximide. In wild-type cells p21 degradation appeared normal and occurred as rapidly as expected. In contrast, in *Pak4* null cells the p21 protein levels decreased much more slowly (Fig. 4C). Quantification of the results showed that in wild-type cells, approximately 50% of p21 was degraded by 80 min during cycloheximide treatment, while only 10% of p21 was lost in the same time frame in *Pak4* null cells (Fig. 4D). Linear regression analysis of p21 degradation in *Pak4* null cells revealed insignificant ($P = 0.32$, $r^2 = 0.16$) changes in p21 compared to the highly

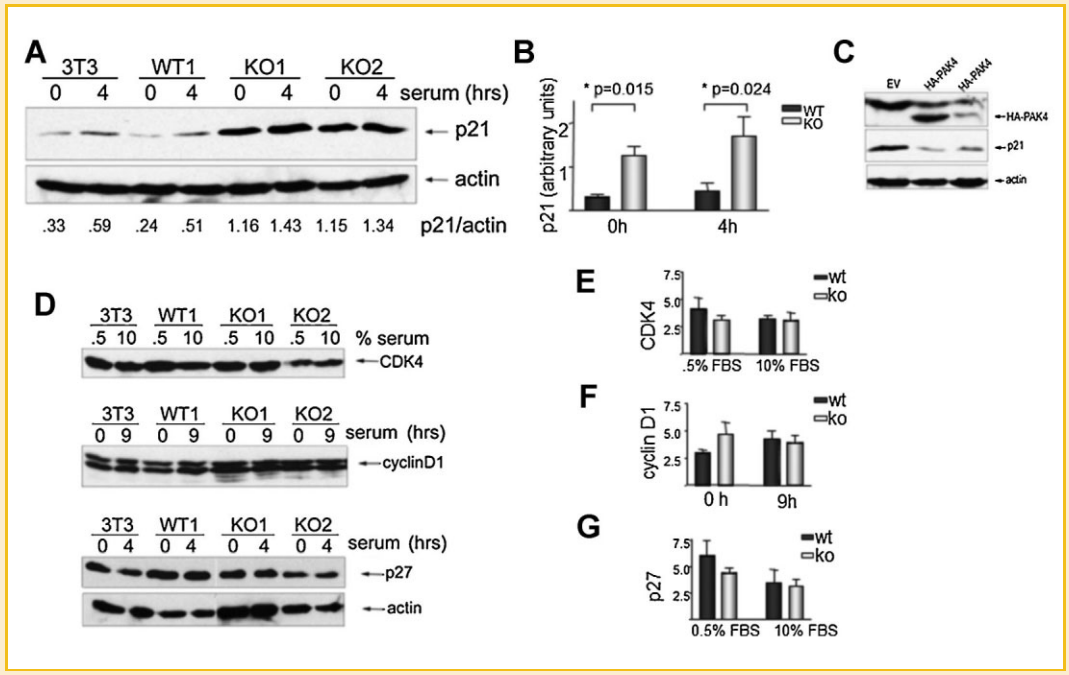

Fig. 3. Deletion of PAK4 leads to increased p21 levels. A: NIH3T3 cells (3T3) and wild-type cell line (WT1) and two *Pak4*$^{-/-}$ (KO1, KO2) cell lines were serum starved for 48 h followed by serum stimulation for 4 h. p21 levels were then assessed by Western blot analysis. The 3T3 cells were used as an external control. B: Quantification of p21 levels in *Pak4*$^{+/+}$ and *Pak4*$^{-/-}$ cell lines after serum starvation, followed by serum stimulation for 4 h. Protein levels are averaged for wild-type ($n = 3$) and *Pak4*$^{-/-}$ ($n = 3$) cell lines and presented in arbitrary units. C: *Pak4*$^{-/-}$ cells were transiently transfected with empty vector (EV) or HA-PAK4 vector (HA-PAK4) and p21 levels were assessed by Western blot analysis. Two different groups of HA-PAK4 expressing cells were examined, each having different levels of PAK4. D: NIH3T3 (3T3) and wild-type fibroblasts (WT1) and the two *Pak4*$^{-/-}$ cell lines were serum starved for 48 h followed by serum stimulation for the indicated amounts of time. Cyclin D1 and p27 levels were then assessed by Western blot analysis. Levels of CDK4 were tested in cells plated in 10% or 0.5% serum after 48 h in culture. E–G: Quantification of CDK4, cyclin D1, and p27 levels in wild-type and *Pak4*$^{-/-}$ cells. Graphs represent average expression of proteins in the cell lines as arbitrary values ($n = 3$ cell lines for each genotype).

significant and expected decay of p21 in wild-type cells ($P = 0.014$, $r^2 = 0.84$) (Fig. 4E). In sharp contrast, degradation of p27 in the presence of cycloheximide was comparable in both wild-type and *Pak4* knockout cells. In both cases, approximately 40% of p27 was degraded by 80 min (Fig. 4F,G). To explore the in vivo correlation between the PAK4 and p21 after serum stimulation we analyzed p21 protein at the same time points that PAK4 levels were analyzed. PAK4 levels peaked at 2 h after serum stimulation, and this correlated with a sharp decrease of p21, supporting the idea that PAK4 downregulates p21 levels in cells (Supplemental Fig. S2). These results suggest that PAK4 controls p21 at the level of mRNA, although the major mode of regulation of p21 by PAK4 occurs through controlling the degradation of the p21 protein.

ACTIVATION OF RHO IS NOT CHANGED IN *PAK4*$^{-/-}$ CELLS

The small GTPase Rho strongly suppresses p21 induction [Olson et al., 1998], and PAK4 is also involved in the regulation of Rho [Callow et al., 2002; Barac et al., 2004]. We therefore tested whether Rho activation is affected by the absence of PAK4. Serum-starved cells were stimulated with 15% FBS, and Rho loading with GTP was assessed. Cells of both genotypes showed similar activation profile of Rho after stimulation with serum (Fig. 5A,B), suggesting that Rho is not an important mediator in PAK4 regulation of p21.

PAK4 DELETION LEADS TO A DEFECT IN G1 CHECKPOINT CONTROL

Recent studies have demonstrated important but complex roles for p21 in the regulation of the cell cycle. Cellular localization of p21 has significant effect on cell behavior. While nuclear localization of p21 is associated with its function as a cell-cycle inhibitor, increased levels of p21 in the cytoplasm protect cells from apoptosis and can also affect the cytoskeleton [Li et al., 2002; Lee and Helfman, 2004]. To test whether increased levels of p21 in *Pak4* null cells are associated with its miss-localization to the cytoplasm, we performed immunostaining on wild-type and *Pak4*$^{-/-}$ cells. p21 was abundant in the nuclei in both types of cells, while only small amounts of p21 could be seen in the cytoplasms of both wild-type and *Pak4* null cells (Fig. 6A). Nuclear versus cytoplasmic location was unchanged regardless of the presence or absence of PAK4. We next tested whether the increased level of p21 protein induced by the absence of PAK4 was associated with changes in cell-cycle progression. In the cells growing in 10% serum, no notable differences between wild-type and *Pak4* knockout cells were observed. The percents of cells in G1, G2/M, and S phases of the cell cycle in the two populations were comparable (Fig. 6B–D, 10% point). However, since PAK4 levels are highly induced in the GF-dependent segment of G1 phase, we sought to determine whether in the absence of GFs, *Pak4* null cells would have a different type of cell-cycle profile. To test this, wild-type and *Pak4* null cell lines were serum starved for 48 h, followed by serum stimulation, to

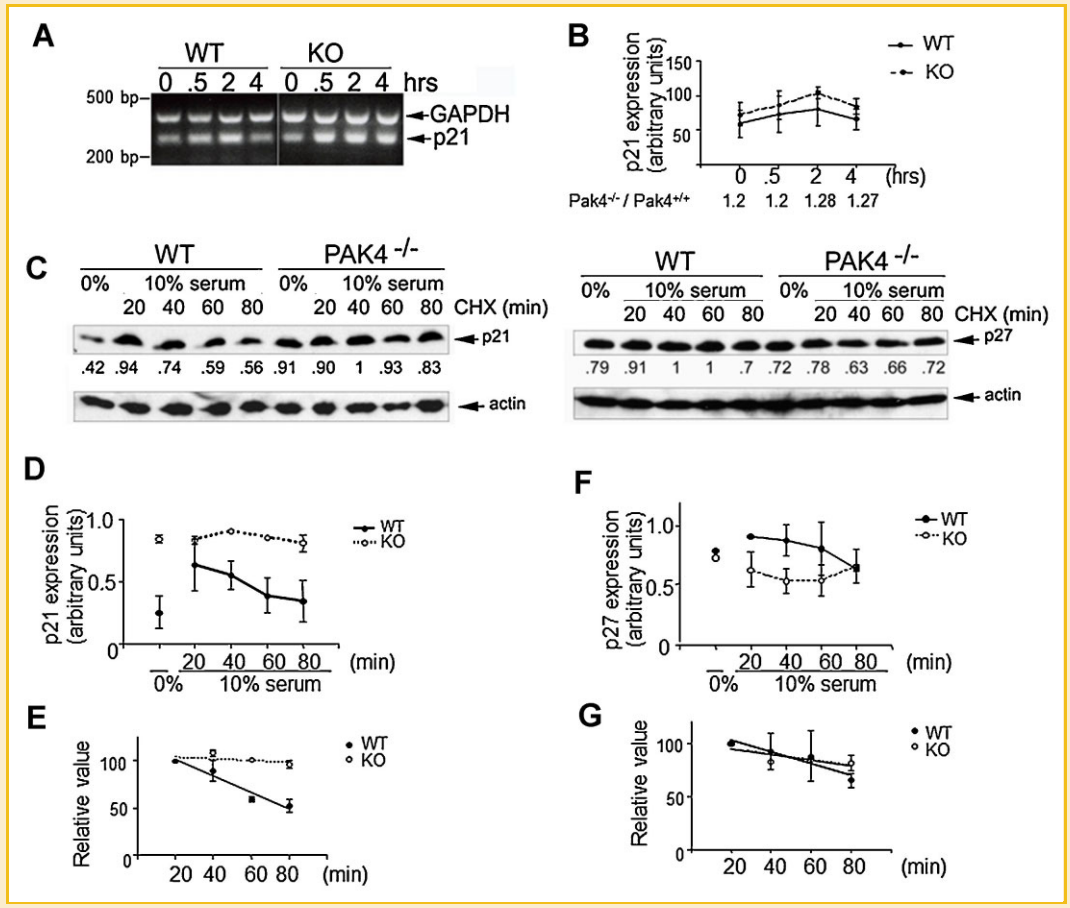

Fig. 4. PAK4 regulation of p21 mRNA and protein levels. A: RT-PCR was used to assess expression of mRNA encoding p21 in wild-type (WT) and *Pak4*$^{-/-}$ (KO) cell lines. Cells were serum starved, followed by serum stimulation. B: Quantification of p21 mRNA levels from panel A. mRNA levels are averaged for wild-type (n = 3) and *Pak4*$^{-/-}$ (n = 3) cell lines and presented in arbitrary units. C: Dynamics of p21 and p27 degradation in cycloheximide treated cells. Cycloheximide (25 μg/ml) was added to serum-starved cells after they were stimulated with serum for 4 h. Individual plates of cells were lysed every 20 min and processed for Western blot analysis. Actin was used as a loading control. D,F: Degradation of p21 or p27 in the presence of cycloheximide averaged for wild-type (n = 2) and Pak4 null (n = 2) cell lines expressed as arbitrary density units. E,G: Linear regression of p21 or p27 decay in wild-type (n = 2) and *Pak4*$^{-/-}$ (n = 2) cell lines.

induce re-entry into the cell cycle. Analysis of the cell population by flow cytometry at early times after serum addition (0–17 h) demonstrated that *Pak4* null cells had significantly higher percentage of cells in G2/M phase at both 0 h time point (serum-starved cells, Fig. 6D) ($P < 0.05$) and 17 h ($P < 0.05$) poststimulation (Fig. 6D). Quantification revealed an average increase from approximately 10% in wild-type cells to 25% in *Pak4* null cells. Accordingly, the percentage of cells in the G1 population was reduced from 75% in wild-type cells to 56% in *Pak4*$^{-/-}$ cells ($P < 0.001$) after serum starvation (Fig. 6B). Interestingly, the percentage of mitotic cells was not notably changed in *Pak4* null cells during the experiment and even 24 h poststimulation, when most of the wild-type cells were in mitosis, *Pak4* null cells did not show an increase in the mitotic population (Fig. 6D). In contrast, when cells were treated with cell-cycle inhibitors that block the cell cycle in late G1 or early S phases, *Pak4* null cells were indistinguishable from wild-type cells (Supplemental Fig. S3). Taken together, these results indicate that deletion of PAK4 results in changes in G1 as well as the G2/M phase of the cell cycle.

In order to gain a better understanding of the mechanisms underlying the cell-cycle-related abnormalities in *Pak4* null cells, we assessed proliferation of wild-type and *Pak4*$^{-/-}$ cells in the presence of 10% or 0.5% serum. Analysis of the growth curves did not reveal significant proliferation differences between wild-type and Pak4$^{-/-}$ cells under either condition (Fig. 6E). We next examined the cell-cycle profiles of wild-type and *Pak4*$^{-/-}$ cells in response to serum starvation, and compared them to cell-cycle profiles of proliferating cells of both genotypes. In wild-type cells serum starvation for 48 h led to a 30% increase in the amounts of cells in G1 phase of the cycle and a corresponding decrease in the amounts of cells in S and G2/M phases by 18% and 13%, respectively (Fig. 6F). In contrast, in *Pak4*$^{-/-}$ cells the increase in the G1 population was only 12%, and S and G2/M populations were reduced by 6% each (Fig. 6F). p21 is known to promote association of cyclin D1 with CDK4 to form functional kinase complexes [LaBaer et al., 1997]. To examine the contribution of increased p21 levels to the composition of CDK4 complexes we analyzed CDK4 complexes in wild-type and *Pak4*$^{-/-}$ cells. In cells proliferating in 10% serum, the amounts of p21 and cyclin D1 bound to CDK4 were comparable

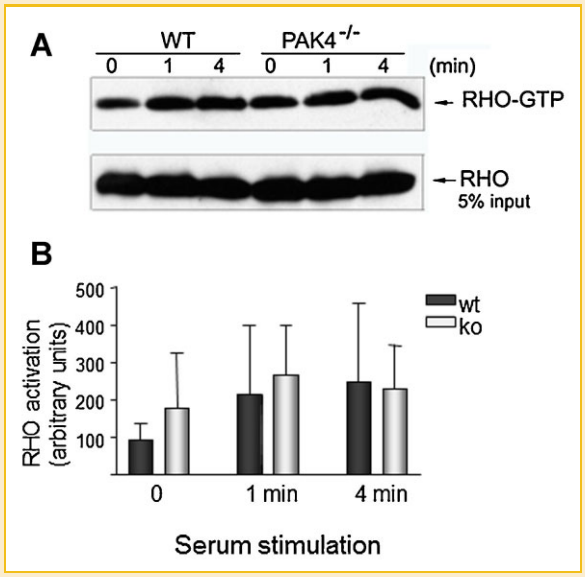

Fig. 5. Rho activation is not significantly affected by deletion of PAK4. A: Activation of Rho was assessed in wild-type (WT) and *Pak4* null cells by rhotekin binding assay as described in the Materials and Methods Section. Western blot analysis shows the amounts of GTP-bound (active Rho) and total amount of Rho protein in cells of both genotypes. B: Quantification of Rho activation in wild-type and *Pak4* null cells (n = 3 cell lines per genotype).

in wild-type and $Pak4^{-/-}$ cells (Fig. 6G). In serum-starved cells, however, there was a strong increase in both p21 and cyclin D1 in the CDK4 complexes (Fig. 6G). To understand the impact of this increase on the progression of the cell cycle we examined Rb phosphorylation in cells of both genotypes. As expected Rb protein was not phosphorylated in serum-starved wild-type cells, and only weak Rb phosphorylation could be detected at early time points of serum stimulation (Fig. 6H). In strong contrast Rb phosphorylation was visibly increased in serum-starved $Pak4^{-/-}$ cells and at 1 and 2 h after serum stimulation (Fig. 6H). Our results demonstrate that in the absence of PAK4 increased amounts of p21 in cells lead to increased amounts of cyclin D1 recruited to the CDK4 complex. These data are supported by the finding that $Pak4^{-/-}$ cells have elevated levels of Rb phosphorylation and directly demonstrate why $Pak4^{-/-}$ cells fail to arrest upon serum starvation.

DISCUSSION

We have identified a novel role for PAK4 in cell-cycle progression. Changes in PAK4 levels throughout the cell cycle have never been examined before, and it has often been assumed that PAK4 protein levels are stable during different phases of the cell cycle. Here we have found that PAK4 protein levels fluctuate throughout the cell cycle. PAK4 levels dramatically increase early in G1 phase and then decline within several hours, remaining low during the rest of the cycle. These results provide a new view on the role for PAK4 in proliferating cells. PAK4 has often been considered to be more important during embryonic development rather than in adults, since its expression is essential for development, while over-production of PAK4 in adults is associated with tumorigenesis [Qu et al., 2003; Liu et al., 2008]. Our data indicate that normal proliferating cells have a temporal increase in PAK4 expression, which is necessary to provide proper transition through the cell cycle. Mammalian PAK1, and PAK homologues in simple eukaryotes, are also implicated in controlling the cell cycle, specifically in the G2/M transition and mitosis [Zhao et al., 2005; Maroto et al., 2008]. PAK1 was found in the microtubule organizing centers and along parts of the spindles [Banerjee et al., 2002], where it regulates mitosis via phosphorylation of centrosomal adaptor GIT1 and centrosomal kinase Aurora A [Zhao et al., 2005], and controls multifunctional mitotic protein Plk1 [Maroto et al., 2008]. Phosphorylation and activation of PAK1 are increased in mitosis, but PAK1 protein levels were never shown to change during the cell cycle [Banerjee et al., 2002; Zhao et al., 2005]. Recently PAK4 was also found to have an important role in G2/M phase of the cell cycle via phosphorylation of Ran GTPase, a modulator of several biological functions including spindle assembly and chromosomal segregation [Bompard et al., 2010]. Members of the group A PAK family are also associated with upregulation of cyclin D1/D2 levels in the G1 to S transition phase of the cell cycle, although these results are controversial [Nheu et al., 2004; Thullberg et al., 2007]. Thus, our results indicate that PAK4 seems to have a new and unique role among the PAK proteins in regulating the initiation of the cell division. We find that PAK4 levels are highly upregulated in the early part of the G1 phase of the cell cycle when the cell makes a decision to divide, a decision that is controlled by a plethora of external signals. This work therefore provides evidence that PAK4 is the part of the cell-cycle regulatory machinery and is involved in the control of cell-cycle initiation.

We have made the important finding that PAK4 controls the level of p21 in the cell. We wished to determine whether PAK4 affects p21 levels through single or multiple mechanisms. We found that in the absence of PAK4, p21 mRNA levels are elevated, which suggests either control of p21 transcription, or destabilization of p21 mRNA by PAK4. The exact mechanism responsible for the increase in p21 mRNA still remains to be determined. It would be interesting to test whether PAK4 can affect the activity of HDAC1 and/or HDAC4, enzymes involved in the deacetylation of DNA, which affect transcription of p21 [Zupkovitz et al., 2006; Mottet et al., 2009]. The similarity between the phenotypes of *Hdac1* and *Pak4* null mice [Zupkovitz et al., 2006; Montgomery et al., 2007] makes this direction of study very intriguing.

Our data also revealed that PAK4 is important for rapid degradation of p21 protein, as deletion of PAK4 significantly extended the lifetime of p21. We are currently investigating the possibility that PAK4 and p21 are binding partners. Although this might not be a straightforward task as such binding could be transient and result in immediate degradation of p21. It remains plausible, however, that PAK4 binds to other components of the cell-cycle machinery, indirectly affecting p21 levels and stability.

Cell attachment to the extracellular matrix was shown to affect p21 stability [Bottazzi et al., 1999; Bao et al., 2002], and in ECV304/T24 human carcinoma cells down-regulation of p21 induced by adhesion was linked to cdc42 and Rac1 signaling [Bao et al., 2002]. Since PAK4 can serve as an effector for cdc42 and Rac1 there is a

possibility that PAK4 could act downstream of these GTPases to affect p21 levels. It is well documented that activated cdc42 and Rac1 can promote DNA synthesis in fibroblasts [Olson et al., 1995] and later studies on cdc42$^{-/-}$ cells confirmed that cdc42 plays a role in the cell cycle [Yang et al., 2006]. These studies indicate that cdc42 operates in late G1 and is important for G1/S transition [Yang et al., 2006]. Our results, however, demonstrated that PAK4 plays a role much earlier in the initiation phase of the cell cycle. Although the interdependence of these proteins within the early G1 phase of the cell cycle has not been thoroughly examined yet, this link would be interesting to explore in the future.

Another important question is whether PAK4 affects p21 levels in a kinase-dependent manner, and whether it phosphorylates p21. Like other PAKs, PAK4 has been shown to have both kinase-dependent and kinase-independent functions [Abo et al., 1998; Gnesutta et al., 2001; Qu et al., 2001; Gnesutta and Minden, 2003], and it will be interesting to determine whether PAK4 regulation of p21 and the cell cycle depends on its kinase activity. Multiple protein kinases phosphorylate p21 and in turn affect its stability [Li et al., 2002; Rossig et al., 2002; Scott et al., 2002]. Phosphorylation of p21 is not absolutely required for its degradation, however, as demonstrated by its regulation by the CDK2–cyclinE complex [Bornstein et al., 2003]. We found that an increase in the phosphorylation of Ser474 on PAK4 (which is thought to be associated with kinase activity) in G1 is gradual. It reaches its highest level after only 6 h of serum stimulation and does not correlate with increased PAK4 protein levels. This suggests that PAK4 kinase activity is not necessary for the reduction of p21 levels.

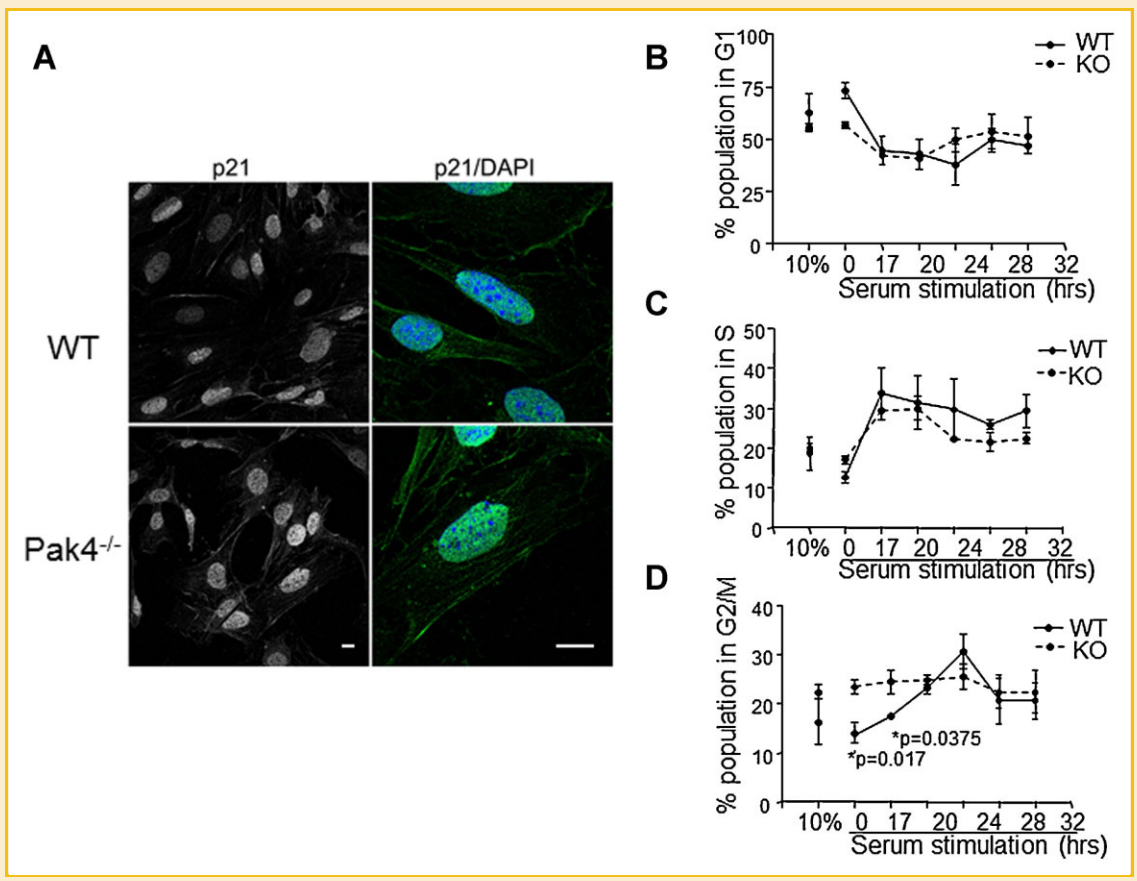

Fig. 6. Deletion of PAK4 does not affect localization of p21 but leads to the defect in G1 checkpoint control. A: Intracellular localization of p21 in wild-type (WT) and *Pak4*$^{-/-}$ cells. Cells were serum starved for 48 h, stimulated with serum for 4 h, and stained with antibodies to p21 and DAPI. Immunofluorescence was carried out and cells were examined by confocal microscopy. Images were acquired at 20× and 60× magnification. Bars, 5 μm. B–D: Cell-cycle progression in wild-type and *Pak4*$^{-/-}$ cells after serum stimulation. Serum-starved (48 h) wild-type and *Pak4* null cell lines (n = 3 per genotype) were stimulated with 10% FBS and examined by flow cytometry. The percentage of cells in each phase of the cell cycle was averaged for each genotype. B: Percent population in G1. C: Percent population in S phase. D: Percent population in G2/M phase. Average percent of wild-type and *Pak4*$^{-/-}$ cells in every phase of the cell cycle growing on 10% serum is also shown on the graphs. E: Growth analysis of wild-type and *Pak4*$^{-/-}$ cells. Cells (25×10^3) were plated in replicate culture dishes and counted every day. N = 3 cell lines per genotype. F: Cell cycle profiles of cycling and serum-starved wild-type and *Pak4*$^{-/-}$ cells. Cells were grown for 48 h in 10% or 0.5% FBS, harvested, and stained with propidium iodide. DNA content was determined by flow cytometry. Average percentages of the cells in G1, S, and G2/M phases of the cell cycle are shown for three wild-type and three *Pak4*$^{-/-}$ cell lines. G: Analysis of CDK4 binding complexes in wild-type and *Pak4*$^{-/-}$ cells grown in 10% and 0.5% serum. Lysates of wild-type and *Pak4*$^{-/-}$ cells were immunoprecipitated using anti-CDK4 antibodies and analyzed by Western blot. Total protein levels in wild-type and *Pak4*$^{-/-}$ cells were also determined by Western blot, as indicated. H: Wild-type and *Pak4*$^{-/-}$ cells were grown in 10% serum or serum starved for 48 h and stimulated with serum for the indicated amounts of times. pRb phosphorylation was assessed by Western blot analysis of cells of both genotypes. Actin expression is shown as a loading control.

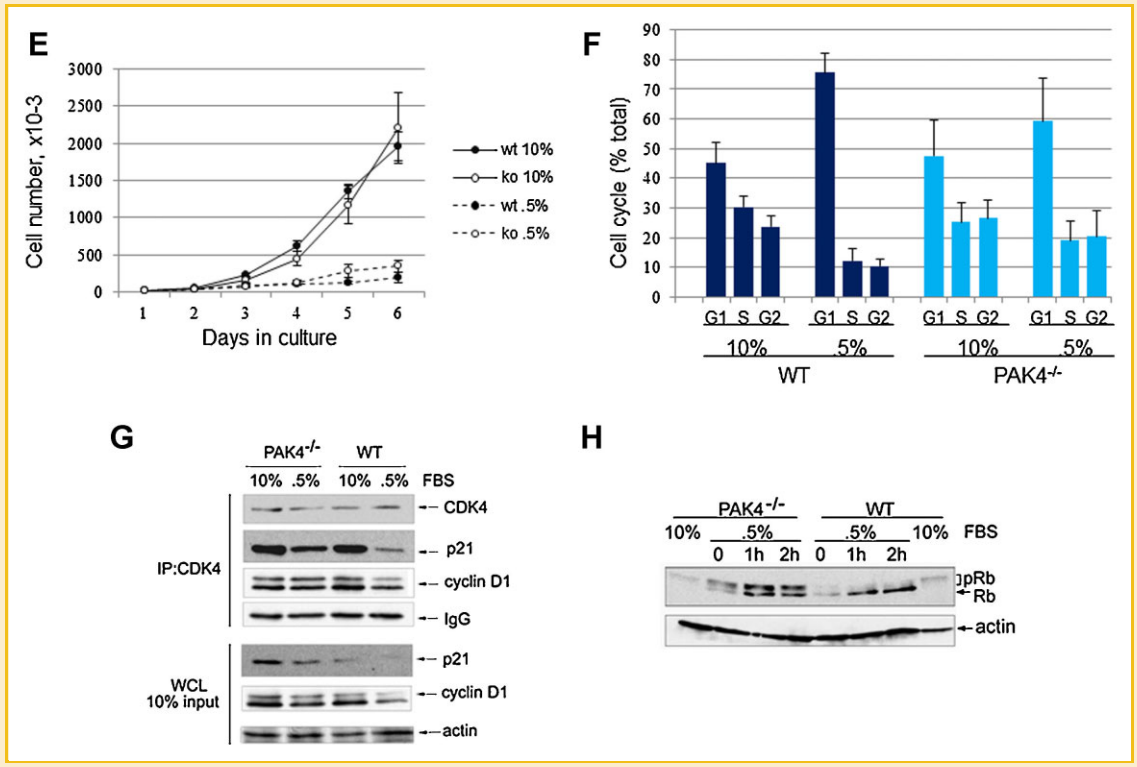

Fig. 6. (*Continued*)

Interestingly, studies by Murray et al. [2010] demonstrated that in HCT116 colon carcinoma cells, an inhibitor of PAK4 phosphorylation reduced both p21 and p53 levels induced by DNA-damaging agent. Thus, we cannot exclude the possibility that activated PAK4 might affect p21 levels by different mechanisms, possibly at the transcriptional level via p53, or by stimulating the ERK pathway via interaction with Raf1. This possibility is very intriguing and should be further explored in the future studies. The PAK4 inhibitor PF-3758309, however, has a similar strong potency against other members of PAK4 family (PAK1, PAK5, and PAK6) as well as several other protein kinases such as AKT, GSK3b, MEK1, and PKCγ and τ [Murray et al., 2010]. Some of these kinases can also affect p21 levels. Indirect regulation of p21 and p53 by PF-3758309 therefore remains a possibility.

We also found that deletion of *Pak4* leads to a slight increase in cyclin D1 levels. This is interesting because cyclin D1 was reported to stabilize p21, perhaps by blocking p21 interaction with the C8 proteasome subunit [Coleman et al., 2003]. It is possible that the increase in cyclin D1 levels could, at least partially, protect p21 from degradation in the *Pak4* null cells.

Our finding that the increased p21 levels in *Pak4* null cells are associated with a defect in G1 arrest upon serum starvation is in agreement with the positive role shown for p21 in the assembly of active CDK4/CDK6 complexes [LaBaer et al., 1997; Cheng et al., 1999; Alt et al., 2002]. We found that increased p21 levels in the absence of GFs promote premature assembly of CDK4 with cyclin D1, allowing improperly timed progression of the cell cycle. The increased PAK4 levels in early G1, leading to decreased p21 levels, may thus allow normal cells to avoid entering the cell cycle prematurely. In this way cell cycle initiation under PAK4 control might occur at a specific time within the GF-dependent segment of the G1. During this time a short and sharp increase of PAK4 may reduce p21 levels, abrogate the activity of CDK4/CDK6 kinases, and allow cells to properly sense the environment and enter the cell cycle at the right time.

PAK4 was first identified as a protein kinase that controls cell shape by regulating cytoskeletal organization [Abo et al., 1998; Dan et al., 2001; Qu et al., 2001]. It is very intriguing that PAK4 now also appears to have a role in regulating cell-cycle progression, by downregulating the activity of p21. The control of cell shape and control of the cell cycle need to work in a coordinated way, in order for cells to replicate properly. Our results suggest that PAK4 is involved in both of these processes. These results also have important implications in the findings that improper regulation of PAK4 is associated with uncontrolled cell proliferation and oncogenesis [Callow et al., 2002; Liu, 2008]. PAK4 is overexpressed in many types of tumors [Ahmed et al., 2008; Chen et al., 2008; Liu, 2008]. High amounts of PAK4 in tumors could result in an improper decrease in p21 levels, leading to unrestrained proliferation.

The finding that PAK4 controls p21 levels may help to explain the embryonic lethal phenotype of *Pak4* knockout mice. We propose that deletion of *Pak4* can lead to increased expression of p21 in the embryo, both prematurely and in inappropriate locations, and in turn affect proliferation of cells that are not yet differentiated. Developmental expression of p21 starts at midgestation at E11.5, and only a few p21 expressing regions in the mouse embryo can be

identified at this developmental stage [Vasey et al., 2010]. Normal proliferation of the cells in *Pak4 null* embryos should therefore be strongly affected by the upregulation of p21. Coordinated proliferation of cells in the embryo strongly relies on the specificity and availability of growth signals. The role for PAK4 in the development may thus be to coordinate proliferation with appropriate developmental cues. Premature cell-cycle progression can result in growth arrest, which is a likely cause of the smaller size of $Pak4^{-/-}$ embryos, seen at embryonic day 10.5 [Qu et al., 2003]. In addition, excessive amounts of p21 might act as cyclinE/CDK2 inhibitors [Mandal et al., 1998] leading to further disruption in the cell cycle in *Pak4* null embryos. The integrated roles for PAK4 and p21 in embryogenesis thus remain to be fully explored.

In summary, we have found that PAK4 levels are affected by the stage of the cell cycle, and that PAK4 has an important role in the regulation of p21 levels. This work provides evidence that PAK4 is necessary for efficient cell-cycle arrest upon serum starvation, and suggests that the normal function of PAK4 is to prevent premature activity of CDK4 in the early stages of G1 phase. These results have important implications for the role for PAK4 in cell-cycle regulation, as well as in normal development and in oncogenesis.

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