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# Changes in Motility, Gene Expression and Actin Dynamics: Cdk6-Induced Cytoskeletal Changes Associated With Differentiation in Mouse Astrocytes

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**Abstract** Cyclin dependent kinase (cdk) 4 and cdk6 have historically been understood to be D-cyclin kinases that phosphorylate pRb in the nucleus to regulate G1 phase of the cell cycle. In conflict with this understood redundancy are several studies that have demonstrated a novel role for cdk6 in differentiation. Cdk6 expression must be reduced to allow proper osteoblast and osteoclast differentiation, enforced cdk6 expression blocked differentiation of mouse embryo fibroblasts, and cdk6 expression in primary astrocytes favored the expression of progenitor cell markers (Ericson et al. [2003] Mol Cancer Res 1:654–664; Matushansky et al. [2003] Oncogene 22:4143–4149; Ogasawara et al. [2004a] J Bone Miner Res 19:1128–1136; Ogasawara et al. [2004b] Mol Cell Biol 24:6560–6568). Experiments shown here investigate novel cytoplasmic and nuclear functions of cdk6. These data demonstrate that cdk6 expression in mouse astrocytes results in changes in patterns of gene expression, changes in the actin cytoskeleton including loss of stress fibers, and enhanced motility. These changes in cdk6-infected cells are associated with the process of cellular differentiation. J. Cell. Biochem. 99: 635–646, 2006. 

**Key words:** Cdk6; astrocytes; differentiation; actin dynamics; cytoskeleton

In $G_1$ phase and early S-phase, positive and negative regulatory proteins control the onset of DNA replication. Cyclin dependent kinases (cdks) partner with cyclins to phosphorylate the retinoblastoma protein (pRb), causing it to release its binding partner E2F. E2F is a transcriptional activator that, once freed from pRb, is able to activate the transcription of genes necessary to replicate DNA. Two families of cell cycle inhibitor proteins regulate the mitogenic activity of the cdks; the INK family ($p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, $p19^{INK4d}$) and the CIP/KIP family ($p21^{Cip1/Waf1}$, $p27^{Kip1}$and $p57^{Kip2}$). Kinases cdk4 and cdk6 partner with D-type cyclins (cyclin D1, D2, D3) in early $G_1$ phase while cdk2 binds to both cyclin E and cyclin A in later $G_1$ and early S phase. (For review see [Ekholm and Reed, 2000]).

Cdk4 and cdk6 share 71% amino acid identity and both partner with D-type cyclins to phosphorylate pRb. Both cdk4 and cdk6 are expressed ubiquitously and historically have been understood to function redundantly in $G_1$ phase of the cell cycle [Meyerson et al., 1992; Meyerson and Harlow, 1994]. This understood redundancy is now being re-examined as accumulated data identify several differences in these two kinases. For instance, cdk4 and cdk6 have distinct functions in tumorigenesis, in halting cellular proliferation, and in T-cell activation [Lucas et al., 1995a,b, 2004; Wolfel et al., 1995; Zuo et al., 1996; Costello et al., 1997; Timmermann et al., 1997; Easton et al., 1998; Nagasawa et al., 2001; Piboonniyom et al., 2002]. These kinases also have distinct responses to p21 in human leukemia cells [Munoz-Alonso et al., 2005]. Cdk4 and cdk6 have demonstrated differences in in vitro substrate recognition—cdk4 preferentially phosphorylates the threonine residue at amino acid 826 on the retinoblastoma protein while cdk6 preferentially phosphorylates threonine 821 [Takaki et al., 2005]. Differences in localization

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have also been observed. In T-cells, breast epithelial cells, and astroctyes, cdk6 has been found to be more abundant in the cytoplasm than in the nucleus, where cdk4 is primarily localized [Kwon et al., 1995; Mahony et al., 1998; Ericson et al., 2003]. Cdk6 has even been found localized to the ruffling edge of human fibroblasts, suggesting a role in cell spreading [Fahraeus and Lane, 1999].

Recent evidence demonstrates a unique role for cdk6 in blocking differentiation. For instance, expression of cdk6, but not cdk4, in mouse astrocytes resulted in a drastic morphology change that correlated with changes in expression of markers of differentiation [Ericson et al., 2003]. Murine erythroid leukemia (MEL) cells expressing an inhibitor-resistant form of cdk6 (cdk6R31C), but not an inhibitor-resistant form of cdk4 (cdk4R24C), failed to differentiate [Matushansky et al., 2003]. Also, BMP-2-stimulated osteoblast differentiation was shown to be inhibited by overexpression of cdk6, but not cdk4, and cdk6 protein levels, but not cdk4 levels, were downregulated by osteoclast differentiation of murine monocytic RAW cells [Ogasawara et al., 2004a,b].

The role of cytoskeletal dynamics in the morphological changes accompanying differentiation has long been noted. The process of cellular differentiation requires cell division, cellular motility, and the formation of specialized structures; all dependent on regulated dynamics of the actin cytoskeleton. The architecture of the cytoskeleton and the movement of cells are controlled in part by the G-proteins Rho, Rac, and cdc42 [Ridley, 2001]. RhoA controls stress fiber formation through the well-characterized Rho/ROCK/LIMK pathway [Ridley, 2001]. RhoA activates Rho kinase (ROCK), which in turn phosphorylates and activates LIM-kinase (LIMK) to phosphorylate and inhibit the actin-depolymerizing protein cofilin, leading to the formation of stress fibers [Ridley, 2001].

We have previously shown that cdk6-infection of primary mouse astrocytes resulted in drastic morphology changes that correlated with less differentiated cells. Experiments shown here investigate these cdk6-induced changes. We show here that cdk6-infected cells display changes in the expression of proteins that regulate actin dynamics, lack stress fibers, demonstrate differences in the Rho/ROCK/LIMK/cofilin signaling pathway, and demonstrate enhanced motility. Data presented here suggest a role for cdk6 in the cytoplasm and further support a role for cdk6 in the process of differentiation.

## MATERIALS AND METHODS

### Infection and Culture of Primary Mouse Astrocytes

Virus-producing DF-1 cells [Holland et al., 1998b] were grown to 50% confluence in DMEM with 10% fetal bovine serum (FBS) and Penicillin/Streptomycin (Penn/Strep) (Cellgro, Hernondon, VA) and transfected with 10 μg RCAS plasmid (constructed as described in [Ericson et al., 2003]) and 10 μg carrier DNA by calcium phosphate precipitation [Chen and Okayama, 1987]. Immunoblots confirmed expression of AP, cdk4, or cdk6 protein in DF-1 cells. Virus-containing supernatants were harvested and passed through a 0.45 μm syringe filter onto $10^5$ primary mouse astrocytes isolated from newborn G-tva chimeric mice (a kind gift of Eric Holland). Cells were infected for 3 or 4 days with virus-containing supernatant that was changed twice and then carried in DMEM with 10% FBS and Penn/Strep. RCAS-AP, RCAS-cdk4, and RCAS-cdk6 cells were grown in DMEM (Media Tech, Herndon, VA) supplemented with 10% FBS (Media Tech, Herndon, VA) and Pen/Strep, and incubated at 37°C and 5% $CO_2$.

### Macroarray

Total RNA was isolated from RCAS-AP, RCAS-cdk4 and RCAS-cdk6 infected astrocyte cultures, DNAse-treated, and used to create a $^{32}P$ cDNA probe with the Atlas Pure Total RNA Labeling System (Clontech, Palo Alto, CA). Probes were hybridized to Atlas Mouse Cancer 1.2 nylon membrane arrays according to manufacturer's instructions (Clontech). Arrays with probes from AP and cdk4 cell lines were exposed 72 h, the cdk6 cell line array was exposed 161 h to normalize the results based on GAPDH, Phospholipase and GAB45 controls. The membranes were imaged with a Storm 820 Phosphoimager and data was processed using ImageQuant software, correcting for background using the local average. The volume data showing relative amounts of mRNA in the cells contained an inherent background error of plus or minus 2,000 U.

### Phalloidin Staining

Infected astrocytes were plated onto coverslips that had been pre-incubated in calf serum. Cells were washed twice in 37°C Dulbecco's PBS (JRH Biosciences, Lenexa, KS), fixed in 3.7% formaldehyde in PBS for 10 min, washed twice in PBS for 5 min, permeabilized in 0.1% Triton X-100 for 5 min and washed in PBS for 5 min. The cells were stained with Alexa 488 conjugated phalloidin (Molecular Probes, Eugene, OR) at ~5 U/ml (~0.16 μM) and Hoescht DNA Dye in 1% BSA in PBS 20 min, washed in PBS and mounted in Prolong (Molecular Probes). Results were examined using a Zeiss Axioskop microscope with digital imaging.

### Semiquantitative RT-PCR

Reverse transcriptase using the Advantage RT-for-PCR (Clontech) protocol was performed on 1 μg Dnase-treated Total RNA, extracted with the Atlas Pure Total RNA Labeling System (Clontech), as above. One tenth of that reaction was then used with TITANIUM Taq DNA Polymerase (Clontech) in a PCR reaction with primers designed for mouse genes as listed below. GAPDH Primers (Clontech) were used as a loading control. The products were resolved on a 1.8% agarose gel with Ethidium Bromide. Primers for each gene are as follows: **PN1:** CGGGCTGTCCTTGTTGGAAGGAACCAT and CACAGCATTGGCCACGGTCA CAATGTC **Tβ4**: AGCAAGCTGGCGAATCGTAATGAGGCG and CCCTCTCTGC TAGCCAGACCATCAGAT **crp2**: GCTGCTTCCTGTGCATGGTTTGCAGGA and TTTGTGCCAGGGCTTCCCAGCTCCAAT **TCP-1β**: TACTAAACTGGCCGTGGAAG CGGGTCT and TCCACACCTGCGAAATCCGCATGCTCA **cdc2**: GGAGTGCCCAG TACTGCAATTCGGGAA and CAAACGCTCTGGCAAGGCCGAAATCAG **D1:** GGTGAACAAGCTCAAGTGGAACCTGGC and GAAGGGCTTCAATCTGTT CCTGGCAGG.

### Immunoblots

Cells were rinsed and scraped in 4°C PBS on ice and resuspended in RIPA Lysis Buffer with DTT, phenylmethylsulfonyl fluoride, leupeptin, and aprotinin. Extracts were centrifuged at 16,000*g* for 20 min and pellets discarded. Protein concentrations were determined by protein assay (Bio-Rad, Hercules, CA) and equivalent amounts of protein were analyzed by SDS–PAGE and transferred to supported nitrocellulose (Schleicher & Schuell, Keene, NH). For direct lysis, cells were scraped in SDS/PAGE loading dye and loaded without determination of protein concentration. After blocking in 5% nonfat dry milk in PBS, blots were incubated with primary antibodies (RhoA: mouse monoclonal, Cytoskeleton, Denver, Co; ROCK-II: Mouse monoclonal, BD Transduction Laboratories, San Diego, CA; cofilin: mouse monoclonal, BD Transduction Laboratories; p-cofilin: rabbit polyclonal (ser 3), Santa Cruz Biotechnologies, Santa Cruz, CA). Secondary antibodies used were anti-rabbit or anti-mouse conjugated to horseradish peroxidase (Jackson Immunoresearch, West Grove, PA). Protein bands were visualized using enhanced chemiluminescence system (ECL) (Perkin-Elmer Life Sciences, Boston, MA).

### Wound Assays

RCAS-AP, RCAS-cdk4, and RCAS-cdk6 infected astrocytes were plated on Bio Coat collagen-treated 60-mm plates (Becton Dickinson, Bedford, MA) and grown to 70%–80% confluence. Cells were washed gently in phosphate-buffered saline (PBS) (JRH Sciences, Lenexa, KS). A sterile 1,000 μl pipet tip was used to scratch wounds through the cell monolayer. After scraping, cells were washed in PBS twice to remove loose cells, new media was added, and cells were incubated at 37°C for times indicated. Digital images were taken at 0, 6, 10, and 24 h after scraping. 10 μg/ml Mitomycin C (Sigma Chemical, St. Louis, MO) was added to the cells for 2 or 16 h prior to scraping, rinsed with PBS to remove mitomycin C, and carried out as described above. Images were superimposed with a 0.3 mm × 0.3 mm grid to quantify wound closure. Percent wound closure was calculated by comparing the number of grids in the defined wound area not occupied by cells after each time interval.

## RESULTS

### Changes in Expression of Genes Associated With Cdk6 Expression

Previous work from our lab has shown that cdk6 infection of primary mouse astroyctes immediately and reproducibly induced a morphological change from a typical star-shaped astrocytic morphology to a bipolar morphology that is typical of glial progenitor cells (see Fig. 2

for representative morphology). These primary mouse astrocytes were isolated from transgenic mice engineered to express the Avian Leukosis Virus (ALV) receptor (TVA) downstream of the GFAP promoter, allowing tissue-specific viral infection of cells in vivo and in vitro [Holland et al., 1998a,b]. These astrocytes, which lack detectable levels of endogenous cdk6, were infected with cdk6-expressing virus. Starting cultures displayed a typical star-shaped astrocytic morphology and enforced cdk6 expression resulted in morphological change to a bipolar morphology and favored the expression of glial progenitor cell markers [Ericson et al., 2003]. In contrast, primary astrocytes infected with cdk4 or with alkaline phosphotase (AP), as a control, retained both the morphology and the expression pattern of differentiation markers consistent with astrocytes.

To further investigate unique functions of cdk6 in these cells, array analysis was performed. A mouse cancer—based macroarray was analyzed to examine the mRNA levels of 1,200 labeled transcripts isolated from AP-, cdk4-, and cdk6-infected astrocytes. As many as 70 genes showed differences in expression between the three cell lines and those showing the most significant differences are presented in Table I. In the array analysis, all genes that showed significant up- or downregulation in the cdk6-infected cells, as compared with either the AP-infected or cdk4-infected cells, were chosen for further examination. Results of these analyses revealed that the vast majority of these differentially expressed genes encoded proteins that are involved actin dynamics. These genes included Protease Nexin 1 (PN1), Cysteine Rich Protein 2 (Crp2), Thymosin β4, three different TCP transcripts, γ-actin, tropomyosin 5, and transgelin. In addition to these actin-related genes, a cdc2 homolog and the cyclin D1 transcript were also upregulated in cdk6-expressing cells. A detailed discussion of these differentially expressed genes and the quantitative data from the array is presented below. Semiquantitative RT-PCR was used to confirm differences in gene expression identified on the array. Figure 1 shows RT-PCR analysis with increasing numbers of PCR cycles, as noted in the figure legend, using transcripts isolated from AP-, cdk4-, and cdk6-infected mouse astrocytes. These data support and extend the results of the quantitative analysis of the array and confirm the upregulation of these transcripts in cdk6-expressing cells.

The functions of proteins encoded by these genes were investigated. The largest differential regulation of gene expression was the upregulation of PN1 in the cdk6-infected cells. Array analysis indicated that cdk6 cells contained 10.5 times as much PN1 mRNA as compared to cdk4 cells and 6.7 times more than AP-infected cells. PN1 also showed clear upregulation by RT-PCR analysis. PN1 is a thrombin inhibitor and may regulate astrocyte shape by modulating the activity of thrombin, a process that is likely mediated by RhoA [Cavanaugh et al., 1990; Suidan et al., 1997]. Thrombin has also been shown to regulate the differentiation of primary glial cultures and may stimulate cell

**TABLE I. Macroarray Analysis Indicated Differential Expression of Genes**

| | Volumes | | | Ratios (of volumes) | |
|---|---|---|---|---|---|
| Name of gene | AP | Cdk4 | Cdk6 | Cdk6/cdk4 | Cdk6/AP |
| Protease nexin 1 | 3,600 | 2,300 | 24,200 | 10.5 | 6.7 |
| Cysteine-rich protein 2 | 21,600 | 9,500 | 58,200 | 6.1 | 2.7 |
| Thymosin β4 | 36,900 | 21,800 | 99,200 | 4.6 | 2.7 |
| TCP-1β | 9,900 | 9,600 | 27,700 | 2.9 | 2.8 |
| TCP-1δ | 1,400 | 3,700 | 7,200 | 2.0 | 5.3 |
| TCP-1η | 40,700 | 27,400 | 63,900 | 2.3 | 1.6 |
| γ-actin | 536,300 | 263,200 | 797,100 | 3.0 | 1.5 |
| Tropomyosin 5 | 18,100 | 17,100 | 35,600 | 2.1 | 2.0 |
| Transgelin | 24,800 | 3,800 | 12,300 | 3.3 | 0.5 |
| Receptor related to tyrosine kinase | 3,700 | 2,400 | 10,800 | 4.5 | 2.9 |
| Cell division cycle 2 homolog | 13,000 | 16,800 | 39,400 | 2.3 | 3.0 |
| Cyclin D1 | 16,000 | 37,800 | 58,800 | 1.6 | 3.7 |

Transcript volumes represent a relative value for the quantity of mRNA in the cell based on the radioactive emission from the array and contain a margin of error of ±2,000. A two- to five-fold increase in the volume indicates a difference of probable significance while an increase of more than five-fold indicates a significant increase (Clontech).

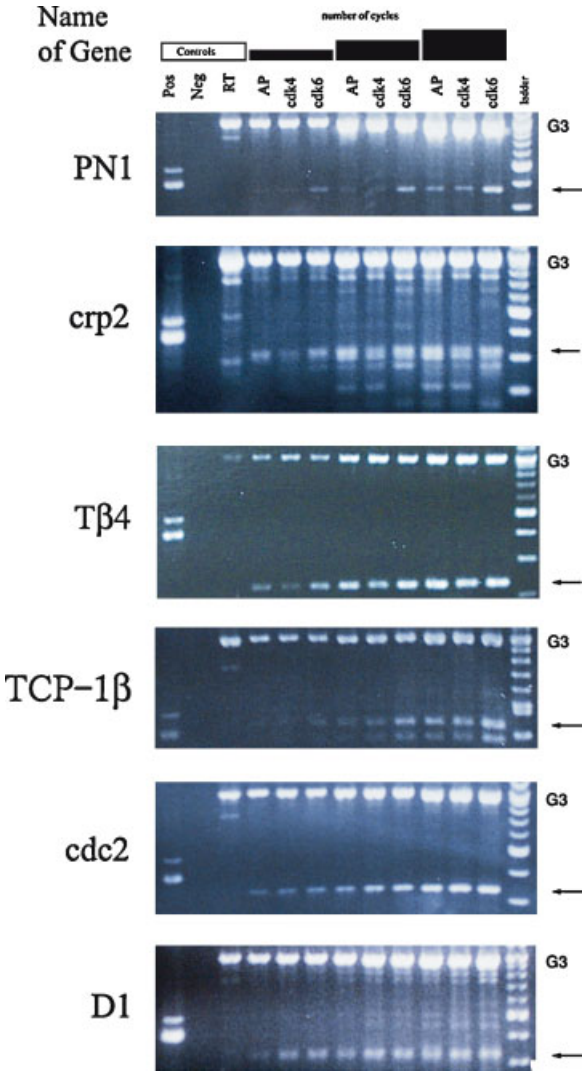


**Fig. 1.** Semiquantitative RT-PCR. Differentially expressed genes identified from a macroarray were subjected to reverse transcriptase PCR. A G3PDH transcript was included as a control. Varied numbers of PCR cycles were conducted for each transcript to distinguish differences in expression. From left to right: positive (POS) and negative (NEG) control for the PCR, positive control for the reverse transcriptase reaction (RT), increasing numbers of cycles for each of the three samples and 100 bp ladder. Arrows indicate expected band size based on mRNA sequence (GenBank) and G3 indicates G3PDH control. For each cDNA PCR cycles are as noted: PN1: 24, 26, 28; crp2: 27, 30, 33; Tβ4 and TCP-1β: 23, 25, 27; cdc2: 24, 26, 28; D1: 27, 30, 33. Panels are representative of at least three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

migration in some systems [Nelson and Siman, 1990; Suidan et al., 1997].

Cysteine-rich protein 2 was also upregulated in cdk6 cells. This protein is involved in differentiation and has roles in actin dynamics. RT-PCR analysis of the crp2 transcript confirmed the array data indicating upregulation in cdk6 cells as compared to cdk4-infected cells (six-fold) (compare on lowest number of cycles). Upregulation seen in cdk6 cells is slight compared to AP-infected cells, corresponding well with the quantitative array analysis (2.7-fold). The RT-PCR of crp2 highlighted size differences between cdk6, AP and cdk4 amplified crp2 bands. This pattern may represent a variable splicing of the amplified transcript. Crp2 displays dual subcellular localization and has distinct functions in each compartment. In the cytoplasm, crp2 binds to F-actin stress fibers and in the nucleus it is thought to act as a transcriptional cofactor in the process of differentiation [Grubinger and Gimona, 2004].

Other proteins analyzed from the genomic array include three of the eight subunits of TCP-1. The TCP-1 complex is a cytoplasmic chaperone that folds cytoskeletal proteins, including actin, to help regulate cytoskeletal function [Liang and MacRae, 1997]. Thymosin β4, also clearly upregulated in cdk6 cells (up 4.6-fold over cdk4), is an actin-sequestering protein. Thymosins bind monomeric actin in a 1:1 complex. Changes in expression of β-thymosins may be associated with differentiation of cells and increased expression of β-thymosins may increase cell motility [Huff et al., 2001]. The slight upregulation of Cyclin D1 and cdc2 homolog observed in the quantitative data (two to three fold) were apparent, but not striking when studied by RT-PCR. In addition to the well-known role these proteins play in cell division, it is also important to note that cdc2 has been shown to modulate cell migration [Manes et al., 2003]. Genes shown to be upregulated in the array but not chosen for further analysis by RT-PCR include RYK (a receptor tyrosine kinase), transgelin and tropomyosin. These transcripts, found to be upregulated in cdk6-expressing cells, all have impact on the organization of the actin cytoskeleton and especially influence cytoskeletal changes associated with the process of differentiation.

### Cdk6-Expressing Cells Lack Stress Fibers

To investigate possible changes in the actin cytoskeleton of infected astrocytes, cells were stained with fluorescent phalloidin. Consistent with observed changes in expression of actin-related transcripts and with the remarkable

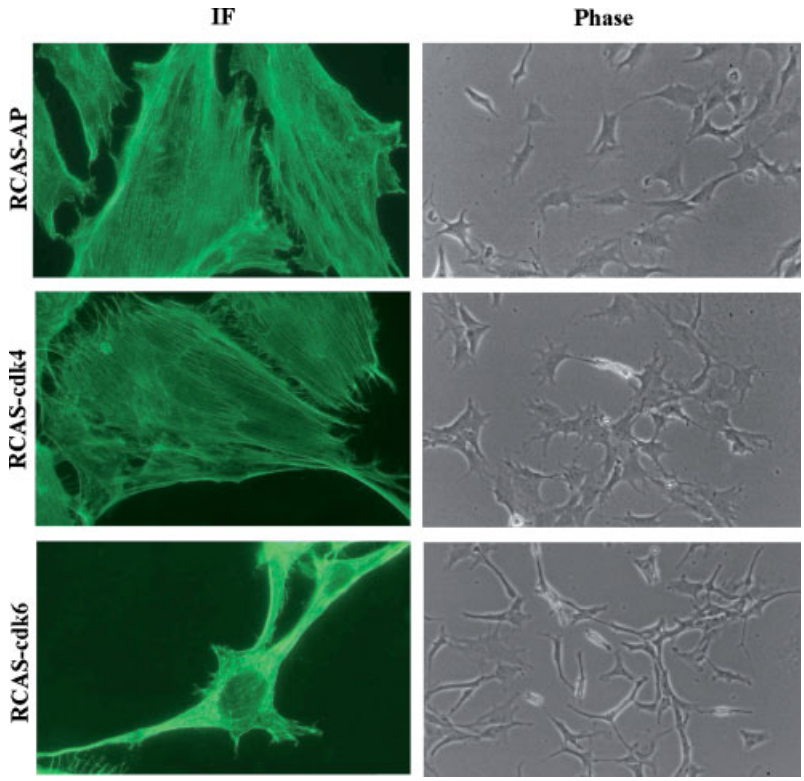


**Fig. 2.** Microscopy of infected astrocytes. Astrocytes infected with RCAS vectors, as noted, were stained with Alexa 488 conjugated phalloidin and imaged at magnification 63× (**left column**). Phase-contrast images of cultured monolayers of primary mouse astrocytes infected with RCAS vectors, as noted (**right column**).

changes observed in the morphology of the cytoplasm, phalloidin staining revealed distinctly different actin cytoskeletal arrangements in these cells. Figure 2 demonstrates that astrocytes infected with AP and those infected with cdk4 displayed well-ordered stress fibers throughout the cells. In contrast, cells infected with cdk6 displayed a punctate, broken or choppy pattern of actin distribution and lacked the long extended fibers observed in AP- and cdk4-infected cells. Thus, cdk6-infected astrocytes displayed aberrant morphology, altered actin dynamics, and differentially regulated expression of actin-related genes. The lack of stress fibers in cdk6 astroctyes combined with the upregulation of several genes encoding actin-binding proteins prompted a closer examination of one pathway that regulates stress fiber formation.

### Disruption of the ROCK/LIMK/Cofilin Pathway to Stress Fiber Formation

Members of the Rho family of GTPases are important in the signal transduction pathway that ends in actin polymerization. The RhoA/ROCK/LIMK/cofilin pathway is known to regulate the presence of stress fibers in astrocytes and to be involved in the process of differentiation (For review see [Ridley, 2001]. Components of the pathway were examined in an attempt to determine if the signaling pathway to stress fiber formation had been disrupted in cdk6 cells. Immunoblots of Rho pathway proteins were examined and results are presented in Figure 3. In these studies, AP-, cdk4-, and cdk6-infected cells were lysed, an equal amount of total protein was loaded and proteins were separated by gel electrophoresis. Each immunoblot was probed with specific antibodies, then stripped and reprobed with actin antibody to provide a control for protein loading. Figure 3A clearly demonstrates an increase in RhoA protein in cdk6-infected cells as compared to AP or cdk4 cells, and titration experiments (3A bottom panel) further supported the observed increase in cdk6 cells compared to cdk4 cells. This observed increase in RhoA protein correlated well with a significant increase in ROCK II

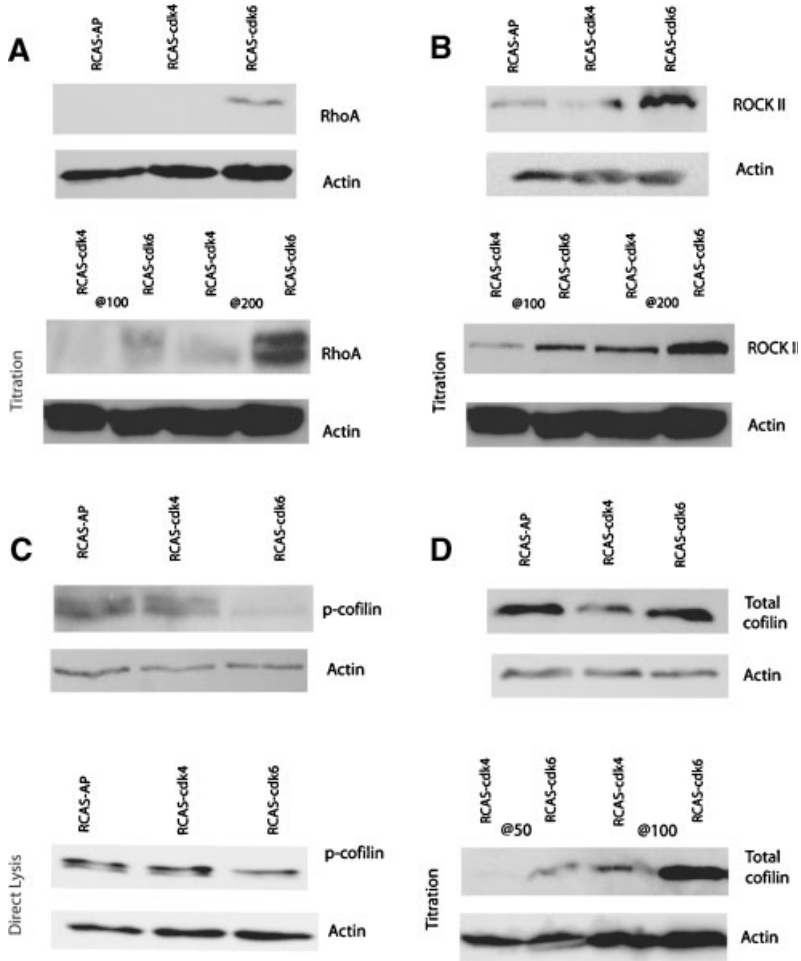


**Fig. 3.** Immunoblots of proteins in the cofilin signaling pathway. Immunoblots of infected astrocytes probed with indicated anitibody to identify proteins in the RhoA signaling pathway to stress-fiber formation. Hundred micrograms protein was loaded in RhoA, ROCK II, and total cofilin immunoblots. Twenty micrograms protein was loaded in p-cofilin immunoblot. Titration immunoblots were loaded as noted in the figure. Direct lysis of extracts did not allow the quantitation of protein extracts. All immunoblots were stripped and re-probed with actin as a loading control. Results are representative of a least three independent experiments.

protein levels that were observed in cdk6-infected cells as compared to AP or cdk4 infected cells and in titration experiments with cdk4 and cdk6 cells (Fig. 3B top and bottom panel, respectively). ROCK II is known to phosphorylate LIMK to yield p-LIMK, which phosphorylates cofilin to generate p-cofilin. The increased levels of ROCK II observed in cdk6-infected cells would predict that these cells would also contain increased levels of p-cofilin. However, using a cofilin antibody that detects only phosphorylated cofilin, decreased amounts of p-cofilin were seen in cdk6-infected cells. This decrease was observed in extracts that were lysed in RIPA buffer and also in those lysed directly in SDS/PAGE loading buffer (Fig. 3C, top and bottom panel, respectively). Direct lysis of extracts highlighted a difference in the banding pattern of p-cofilin of cdk6 cells and cdk4 or AP cells. Cdk6 cells displayed only one p-cofilin band compared to two bands of p-cofilin observed in the blots of the other cell lines. Further investigation revealed that the reduced levels of p-cofilin in cdk6 cells were not due to a lack of total cofilin protein. In fact, the total cofilin levels were elevated in cdk6 cells compared to cdk4 cells (Fig. 3D, top and bottom panels), suggesting that the lack of p-cofilin is due to a decrease in the phosphorylation of cofilin, likely by LIMK. Unfortunately, repeated attempts using several different antibodies to detect endogenous LIMK or p-LIMK in these cells were unsuccessful. Regardless, the reduced levels of phosphorylated, inactive, cofilin

observed in cdk6 cells predicts a reduction in stress fibers, correlating well with the lack of stress fibers observed in these cells.

Together, these data suggest that the overexpression of cdk6 is blocking the RhoA/ROCK/LIMK/cofilin pathway, leading to a reduction in p-cofilin. The disruption of this pathway in cdk6-infected cells is consistent with the altered morphology of these cells. The inhibition of RhoA/ROCK signaling has been shown to induce an elongated morphology, and dephosphorylation of cofilin has been shown to correlate with increased cellular protrusions and increased motility of cells [Carlier et al., 1999; Yamazaki et al., 2005].

### Cdk6 Expressing Cells are Motile

Cell migration is one of several acquired capabilities of cancer cells and is important in the process of differentiation. Cell migration occurs through dynamic reorganization of the actin cytoskeleton. Rho-family GTPases are known to activate pathways that affect cell motility, and adhesion (mediated by stress-fiber-associated focal adhesions) inhibits cell migration. In addition, upregulation of cdc2 has been shown to result in increased cell motility [Manes et al., 2003]. Data presented here show that cdk6-infected astrocytes display a decrease in stress fibers, an increase in RhoA and ROCK protein levels, an increase in de-phosphorylated coflin, and a slight increase in cdc2 homolog transcripts. All of these characteristics predict an increased motility of cdk6-expressing cells.

To determine motility of these astrocytes, wound assays were performed on monolayers of AP-, cdk4-, and cdk6-infected astrocytes. In these experiments, the confluent monolayer was scraped to generate a cell-free linear zone and the wound was monitored at regular intervals. Wound assays were performed on standard tissue culture plates (data not shown) and on collagen-coated plates, where motility was enhanced. As shown in Figure 4, cdk6-infected cells migrated into the wound and completely reconnected the disrupted monolayer by 24 h. Both the AP- and cdk4-infected cells did not close the wound at 24 h. These differences in cell migration were observed as early as 6 h post-wounding and drastic differences were observed at 10 h. Importantly, migration of cdk6-infected cells occurred before a significant effect on cell proliferation would be observed, indicating that the observed difference in migration was not simply due to a difference in proliferation rates. Detailed studies on proliferation rates of these cells have been performed and these studies indicated that cell lines used in these experiments did not have significant differences in rate of proliferation [Ericson et al., 2003]. To ensure that the enhanced motility of cdk6 cells was not due to an increase in proliferation rates, Mitomycin C was added to the cells to inhibit cell proliferation (data not shown). The addition of Mitomycin C showed no effect on the ability of cdk6 cells to close the wound, demonstrating that the results of the wound assays were due to an increase in cdk6 motility, and not an enhanced rate of proliferation. The migration of cells in this assay was also quantified to provide a measure of cell motility. These data are presented in Figure 4B and demonstrate a greater than two-fold increase in migration of cdk6 cells at 6 h and greater than three-fold increase at 10 and 24 h. The results of the wound assay clearly show that cdk6-infected cells display enhanced motility.

## DISCUSSION

Infection of primary mouse astrocytes with cdk6, but not cdk4, induced morphological change and changes in gene expression that are consistent with glial progenitor cells [Ericson et al., 2003]. Results shown here demonstrate that cdk6-infected cells (1) upregulate the expression of genes encoding proteins that modify cytoskeletal architecture and influence differentiation, (2) lack organized stress fibers (3) contain disruptions of the RhoA/ROCK/LIMK/cofilin signal transduction pathway, and (4) demonstrate enhanced motility. This is the first paper correlating cdk6 expression with changes in cytoskeletal dynamics and cell motility, both processes associated with differentiation.

Data presented here suggest a cytoplasmic role for cdk6, a known nuclear cell cycle regulatory factor. There is precedent for a dual-role cyclin dependent kinase. Cdc2 targets substrates in the cytoplasm and in the nucleus to synchronize these subcellular compartments during cell cycle [Lowe et al., 1998]. In the cytoplasm, cdc2 has been shown to influence cell motility [Manes et al., 2003] and in the nucleus it functions in cell cycle regulation [Lowe et al., 1998; Schwartz and Assoian, 2001]. The CIP/KIP family of proteins that regulate cdk activity

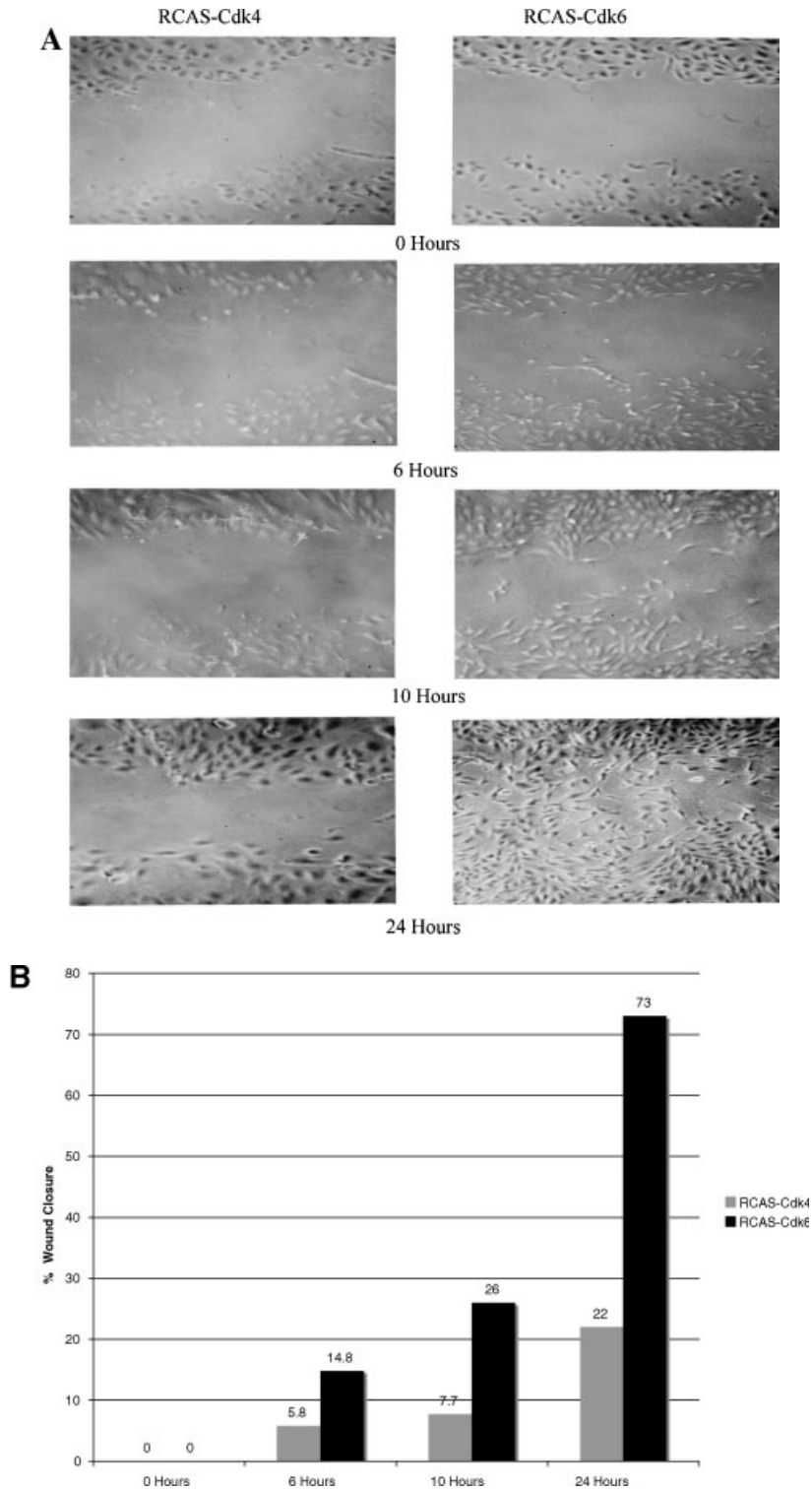


**Fig. 4.** Wound assays of RCAS-cdk4, and RCAS-cdk6 cells. **A**: Cells were plated on collagen-coated plates and grown to confluence. A wound was introduced into the monolayer at 0 h and the migration of the cells was monitored by digital imagining at 0, 6, 10, and 24 h time points. Results are representative of at least three independent experiments. **B**: Bar graph depicting quantitation of wound closure. Images from A were superimposed with a $0.3 \times 0.3 mm^2$ grid to quantify wound closure. Percent wound closure was calculated by comparing the number of grids not occupied by cells after each time interval.

have also been shown to regulate cytoskeletal dynamics as well as cell proliferation. For instance, p21 disrupts the ROCK pathway to stress fiber formation, regulates cell division in $G_1$ phase, and is induced during the process of differentiation [Lee and Helfman, 2004]. Likewise, the p57 protein has been shown to influence stress fiber formation and modulate the subcellular localization of LIMK [Yokoo et al., 2003]. The third member of this CIP/KIP family, p27, was shown to induce migration of cultured hepatocytes and p27-deficient MEFs failed to migrate in cell culture assays [McAllister et al., 2003]. The role of p27 in migration appears to be distinct from its function as a regulator of cell division [McAllister et al., 2003]. Finally, the yeast cdk inhibitor, Far1, causes re-orientation of the actin cytoskeleton in response to alpha factor treatment [Gulli and Peter, 2001]. Data presented here predict that cdk6, like cdc2 and the CIP/KIP proteins, has dual functions in the nucleus and cytoplasm.

To regulate cell cycle progression, $G_1$ phase cyclin dependent kinases are known to integrate signals from growth factor receptors and from integrins. Integrins mediate adhesion to the extracellular matrix and transmit direct signals to the $G_1$ cyclin-cdk machinery. Integrin signaling can decrease p21 and p27 levels and increase levels of cyclin D1 (For review see [Schwartz and Assoian, 2001]). Expression of $\alpha_v\beta_3$ integrin has been shown to upregulate cdc2 and to modulate cell migration via a mechanism involving cyclin B2-associated cdc2 kinase activity on the substrate caldesmon [Manes et al., 2003]. Interestingly, caldesmon was shown to be phosphorylated by cdk6 when complexed with viral cyclin K [Cuomo et al., 2005] suggesting one mechanism by which cdk6 may influence cell motility. Another mechanism may involve integrins; $\alpha_v\beta_3$ integrin-mediated cell spreading can be inhibited by $p16^{INK4a}$, this inhibition can be overcome by the expression of cdk6, but not cdk4 [Fahraeus and Lane, 1999]. The authors of this study localized cdk6 to the ruffling edge of spreading fibroblasts and suggested a role for cdk6 in cell spreading [Fahraeus and Lane, 1999]. Consistent with cytoplasmic localization of cdk6, several groups have noted cdk6 in the cytoplasm of multiple cell types, including the primary mouse astrocytes studied here [Mahony et al., 1998; Fahraeus and Lane, 1999; Ericson et al., 2003].

In the past three years, several independent studies in multiple cell types have indicated a novel role for cdk6 in differentiation. Interestingly, astrocyte dedifferentiation has been shown to occur during tumorigenesis to high-grade gliomas; the authors of this work suggested that a dynamic state may exist between a fully dedifferentiated cell and the commitment to terminal differentiation of glial lineages [Bachoo et al., 2002]. This work also suggested an important role for $p16^{INK4a}$ and $p19^{ARF}$ in restricting the dedifferentiation of astrocytes [Bachoo et al., 2002]. While the mechanism of p16 was not uncovered, it is tempting to speculate that it involves inhibiting cdk6 function. A role for cdk6 in glioblastomas has been described; cdk6 expression was increased in 12 of 14 glioblastomas but not detected in any of the lower grade matched tumor samples from the same patients [Lam et al., 2000]. Glioma cells characteristically migrate from the main tumor mass [Holland, 2000] and migration of glioma cells involves cell surface receptors, integrins and the activation of the PKC pathway [Besson et al., 2001]. Given that cdk6 over-expression affects differentiation status, enhances cell motility, and overcomes p16-mediated inhibition of integrin-mediated cell spreading, perhaps increased expression of cdk6 is an important contributor to the oncogensis of gliomas.

Data presented here suggests that cdk6 may function to synchronize cytoplasmic and nuclear functions throughout the complex processes of cellular proliferation and differentiation. Because differentiation of cells involves both cell cycle arrest and changes in the cytoskeleton, cdk6 is uniquely positioned to integrate these events.

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