Pak1 Phosphorylation on T212 Affects Microtubules in Cells Undergoing Mitosis

Monisha Banerjee,[1] Daniel Worth,[1]
David M. Prowse,[2] and Margareta Nikolic[1,3]
[1]Molecular and Developmental Neurobiology
   MRC Centre
New Hunt's House
King's College London
Guy's Campus
London SE1 1UL
[2]Cancer Research UK
London Research Institute
Lincoln's Inn Fields Laboratories
London WC2A 3PX
United Kingdom

Summary

The Pak kinases are targets of the Rho GTPases Rac and Cdc42, which regulate cell shape and motility [1–5]. It is increasingly apparent that part of this function is due to the effect Pak kinases have on microtubule organization and dynamics. Recently, overexpression of *Xenopus* Pak5 was shown to enhance microtubule stabilization, and it was shown that mammalian Pak1 may inhibit a microtubule-destabilizing protein, Op18/Stathmin [6, 7]. We have identified a specific phosphorylation site on mammalian Pak1, T212, which is targeted by the neuronal p35/Cdk5 kinase [8, 9]. Pak1 phosphorylated on T212, Pak1-T212(PO_4), is enriched in axonal growth cones and colocalizes with small peripheral bundles of microtubules. Cortical neurons overexpressing a Pak1A212 mutant display a tangled neurite morphology, which suggests that the microtubule cytoskeleton is affected [9]. Here, we show that cyclin B1/Cdc2 phosphorylates Pak1 in cells undergoing mitosis. In the developing cortex and in cultured fibroblasts, Pak1T212(PO_4) is enriched in microtubule-organizing centers and along parts of the spindles. In living cells, a peptide mimicking phosphorylated T212 accumulates at the centrosomes and spindles and causes an increased length of astral microtubules during metaphase or following nocodazole washout. Together these results suggest that similar signaling pathways regulate microtubule dynamics in a remodeling axonal growth cone and during cell division.

Results and Discussion

Pak1 Phosphorylation Is Seen in Neuronal Precursors Undergoing Mitosis

The p35/Cdk5 kinase exclusively functions in postmitotic cells, and the highest levels and activity are seen in neurons of the central nervous system (CNS), where they regulate their migration and morphology [10, 11]. While examining the temporal and spatial pattern of Pak1 phosphorylation in the developing cortex, we ob-

[3]Correspondence: margareta.nikolic@kcl.ac.uk

served that many cells lining the lateral ventricle were positive for Pak1T212(PO_4), especially during the early stages of development. At mouse embryonic stage 12 (E12), the cortical region primarily consists of proliferating neuroepithelial cells in an area termed the ventricular zone (VZ) [12, 13]. In the VZ, the neuronal precursors change the position of their soma depending on which stage of the cell cycle they reside in. Thus, cells in G1 and G2 reside in the middle of the VZ, while S and M phase cells are at the upper and lower boundary of the VZ, respectively [13]. The position and morphology of the Pak1T212(PO_4)-positive cells indicated that they were undergoing mitosis. Neuronal precursors in other stages of the cell cycle were largely devoid of Pak1-T212(PO_4), despite having high expression levels of the Pak1 protein (Figure 1A and data not shown). The mitotic marker, histone H3 phosphorylated on S10, revealed a similar distribution of positively labeled cells (Figure 1B). The specificity of the anti-Pak1T212(PO_4) antibody, PK-18, was verified for paraffin-embedded tissue by preincubation with a phosphorylated peptide, which effectively abolished all positive staining (Figure 1C).

To confirm these observations, we costained cryostat sections of E19 rat cortices for Pak1T212(PO_4) and phosphorylated histone H3. In all cases, mitotic cells were positive for both markers (Figure 1D). DNA labeling with TOPRO3 revealed high levels of Pak1T212(PO_4) during prophase, metaphase, and anaphase (Figure 1E). In addition to cortical progenitors, other cells in the CNS were strongly positive for PK-18, particularly in the early stages of development. Their positions, for instance, lining the lateral ventricle in the striatal, pallidal, and hippocampal neuroepithelium, or surrounding the third ventricle in the thalamic and hypothalamic neuroepithelium (Figure 1A), frequently correlated with the mitotic phase of the cell cycle. In the cortex, cells with upregulated Pak1T212(PO_4) were occasionally observed in more superficial areas, where mitotic neuronal progenitors do not reside. These cells may be dividing radial glia, since a similar frequency and distribution of phosphohistone H3-stained cells was evident (Figures 1A and 1B). PK-18-positive cells were also seen in other parts of the developing embryo where cells were proliferating, such as the epithelial cells of the eye and gut (Zhong and M.N., unpublished data), and their presence suggests that Pak1T212(PO_4) is not restricted to the nervous system but occurs in all mitotic cells.

Pak1T212(PO_4) Accumulates in Microtubule-Organizing Centers

Swiss3T3 fibroblasts in different stages of the cell cycle were examined for the presence of endogenous T212-phosphorylated Pak1. Interphase cells had very low levels of predominantly nuclear Pak1T212(PO_4), which increased as cells entered prophase, initially in the nucleus and subsequently throughout the cell (Figure 2A). High levels of Pak1T212(PO_4) persisted during metaphase, anaphase, and telophase and were subsequently
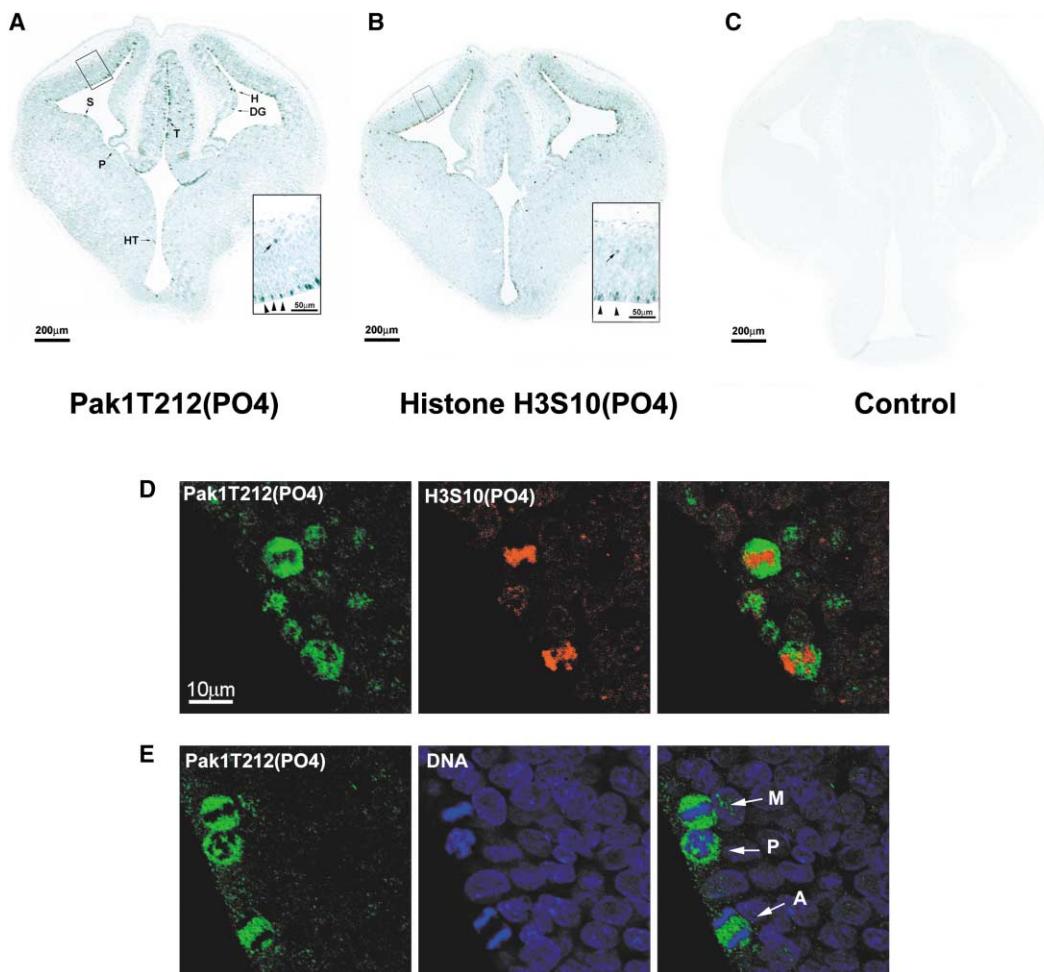
Figure 1. Neuroepithelial Cells Accumulate Pak1T212(PO$_4$) in M Phase

(A) A representative coronal section of paraffin-embedded mouse E12 brain, revealing positive cells lining the lateral and third ventricles, was immunostained with an anti-Pak1T212(PO$_4$) antibody, PK-18. The arrows point to striatal (S), pallidial (P), hippocampal (H), dentate gyrus (DG), thalamic (T), and hypothalamic (HT) neuroepithelial cells in M phase.

(B) An adjacent section to (A) that reveals a similar distribution pattern of cells that are strongly positive for histone H3 phosphorylated on S10. In (A) and (B), the insets show closer views of the developing cortex where M phase cortical progenitors and other mitotic cells are marked with arrowheads and arrows, respectively.

(C) Preincubation of PK-18 with a phosphorylated peptide against which the antibody was raised, efficiently blocked all staining.

(D) A coronal section of a frozen E19 rat cortex that reveals neuronal progenitors in M phase that have upregulated both Pak1T212(PO$_4$) and the mitotic marker histone H3S10(PO$_4$).

(E) Cortical progenitors in prophase (P), metaphase (M), and anaphase (A) that show high levels of Pak1T212(PO$_4$). Staining with the DNA marker TOPRO3 revealed their stage in mitosis. Images in (D) and (E) were obtained with an Olympus confocal microscope.

downregulated (Figure 2A). The distribution of Pak1-T212(PO$_4$) was always punctate, similar to the pattern seen in hippocampal neurons, especially their growth cones [9]. Interestingly, in all mitotic cells, Pak1-T212(PO$_4$) accumulated in two distinct points that appeared to be microtubule-organizing centers (MTOCs). This was confirmed by colocalization with a centrosomal marker, γ-tubulin (Figure 2B). In vivo, in E19 rat cortices, Pak1T212(PO$_4$) also accumulated in MTOCs at either side of the spindles and colocalized with γ-tubulin (Figure 2C).

Cyclin B1/Cdc2 Catalyzes Pak1
Phosphorylation on T212

Despite its ubiquitous expression, Cdk5 kinase activity is primarily restricted to postmitotic neurons and has never been detected in proliferating cells [14–17]. Cdk5 is closely related to the cell cycle-promoting kinases Cdc2 and Cdk2 with which it shares similar substrate specificity [18–22]. The cyclin B1/Cdc2 kinase is essential for the G2/M transition and passage through the first half of M phase [23]. In interphase cells, it is largely cytoplasmic, while, in prophase, it rapidly translocates into the nucleus. It also accumulates in centrosomes [24]. We compared the timing of Pak1 phosphorylation and cyclin B1 expression in Swiss3T3 fibroblasts that were arrested in G0 and following the addition of serum (4, 10, 16, and 26 hr when a fraction enriched with M phase cells was harvested). The cell cycle profile of the samples was determined by flow cytometry. The levels of both cyclin B1 and Pak1T212(PO$_4$) were low in G1 and increased as more cells entered the G2 and M phases
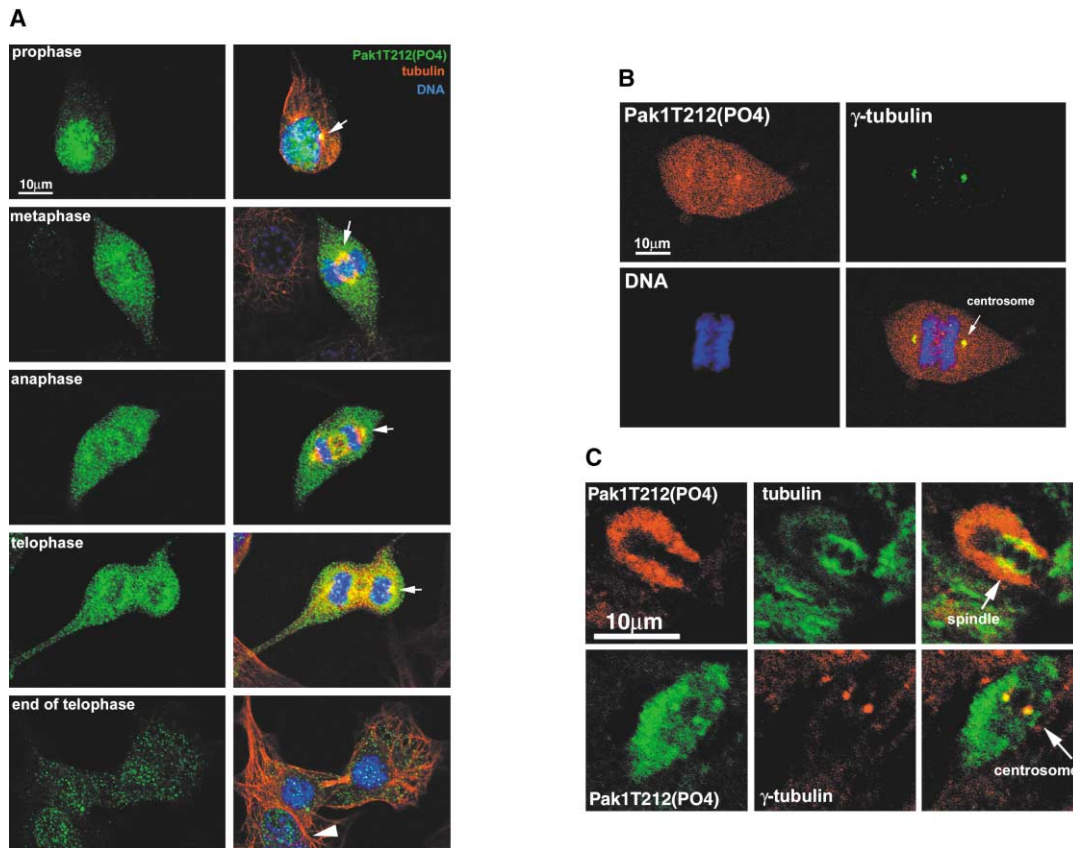
Figure 2. Pak1T212(PO$_4$) Accumulates in MTOCs

(A) Swiss3T3 fibroblasts in prophase accumulated Pak1T212(PO$_4$) in the nucleus and in two spots on either side of it. By metaphase, Pak1T212(PO$_4$) had spread out throughout the cytoplasm and was concentrated at the spindle poles. Its distribution remained the same throughout anaphase and telophase, when it was downregulated. PK-18 (green), tyrosinated α-tubulin (red), and TOPRO3 (blue). The arrows point to MTOCs, and the arrowhead points to the nucleus of a cell in interphase.

(B) A representative Swiss3T3 fibroblast costained with PK-18 (red), the centrosomal marker γ-tubulin (green), and the DNA stain TOPRO3 (blue). The arrow points to the centrosome.

(C) Representative M phase neuronal progenitors in frozen E19 rat cortices that show accumulation of Pak1T212(PO$_4$) in the area of the spindle and in MTOCs, where it colocalizes with γ-tubulin. The arrows point to the spindles and centrosomes. All images were obtained with an Olympus confocal microscope.

of the cell cycle, while Pak1 levels remained constant (Figure 3A).

To determine if cyclin B1/Cdc2 catalyzes Pak1 phosphorylation on T212, we carried out in vitro kinase assays using a fragment of Pak1 (147–231) as a substrate. Cyclin B1 immunoprecipitates from Cos7 cells phosphorylated the 147–231 fragment, not the same region containing a T212A mutation (Figure 3B). Cdk2 immunoprecipitates failed to phosphorylate 147–231, despite the clear presence of kinase activity, as determined by histone H1 phosphorylation. p35 immunoprecipitates were used as a positive control. Cyclin B1/Cdc2 also phosphorylated full-length Pak1 on T212, as determined by a nonradioactive kinase assay (Figure 3C).

T212 Phosphorylation Targets Pak1 to MTOCs

To address the biological role of Pak1T212(PO$_4$) during mitosis, we generated small peptides containing 15 amino acids that were identical to the sequence surrounding the T212 site of Pak1, and one of the peptides was synthesized with a phosphate group on T212. These were fused to a stretch of arginines and tryptophans (termed penetratin) that have previously been shown to enter living cells due to their hydrophobic properties and charge distribution [25, 26]. N-terminally conjugated biotin enabled us to examine the subcellular localization of the peptides using FITC-conjugated streptavidine. In interphase cells, the peptides distributed uniformly throughout the cell. However, in mitosis, the phosphorylated peptide accumulated along the length of the spindles and in their poles (Figure 4A). This was especially evident in metaphase cells. The unphosphorylated peptide was less efficiently transported to the spindles, while cells that were not exposed to peptides had no detectable streptavidine staining. These results suggest that phosphorylated T212 is part of a protein binding site that targets Pak1 to areas of microtubule organization.

Pak1T212(PO$_4$) Regulates the Organization of Dynamic Microtubules

We used the peptides to address the functional significance of Pak1 phosphorylation during mitosis. Swiss3T3 fibroblasts were treated for 3 hr with the mi-
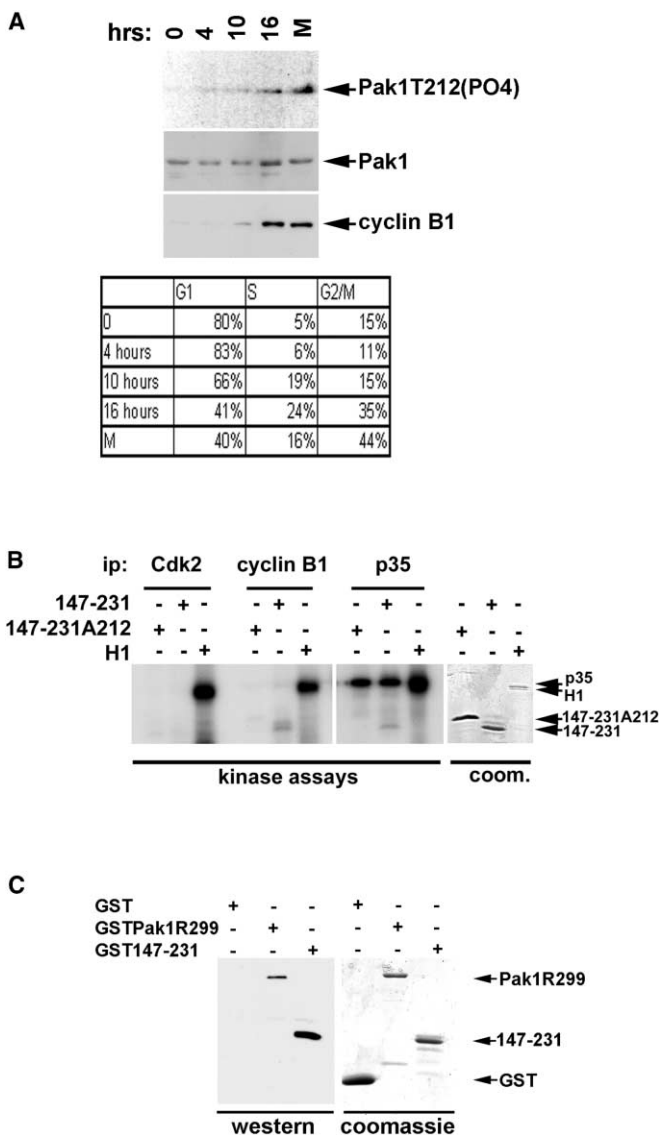
A

	G1	S	G2/M
0	80%	5%	15%
4 hours	83%	6%	11%
10 hours	66%	19%	15%
16 hours	41%	24%	35%
M	40%	16%	44%

B

C

crotubule-depolymerizing drug nocodazole. The phosphorylated or unphosphorylated peptides were added during the last 30 min of nocodazole treatment, subsequent to which the cells were washed and allowed to recover for 2 min. In the controls, an antibody to tyrosinated α-tubulin revealed newly formed microtubules that extended from one or two MTOCs located close to the nucleus toward the cell peripheries (Figure 4B). Remarkably, in cells treated with the phosphorylated peptide, microtubules often appeared to be disorganized and extended much further toward the cell edges (Figure 4B). Since it was not possible to accurately estimate their length, the area within a cell that contained microtubules was compared to its overall size. A total of 100 cells were examined for each treatment group, and all scoring was performed blind. Similar results were obtained from three separate experiments. Following 2 min of recovery, 22% of the untreated and 33% of cells exposed to the unphosphorylated peptide contained microtubules that had spread over more than half of the cell area. In contrast, 55% of the Swiss3T3s treated with

the phosphorylated peptide had more than half of their areas filled with microtubules. The fixation of cells at earlier times after nocodazole washout (30 s or 1 min) revealed that most cells in all the treatment groups were able to nucleate microtubules. However, in many cases, exposure to the phosphorylated peptide induced extra microtubules outside the main nucleation center and the appearance of many free, nontethered microtubules located at the cell peripheries (46% of cells) (Figure 3B). This was far less common in the control samples (no peptide, 17%; and unphosphorylated peptide, 13%). A peptide with a T212A mutation gave a similar phenotype to the unphosphorylated peptide (Figure 3B and data not shown).

Pak1T212(PO$_4$) Regulates Astral Microtubules in Metaphase Cells

We next determined if the distribution of microtubules was affected by the phosphorylated peptides during cell division. Swiss3T3 fibroblasts approaching mitosis were treated for 2 hr with the peptides prior to fixation. Expo-
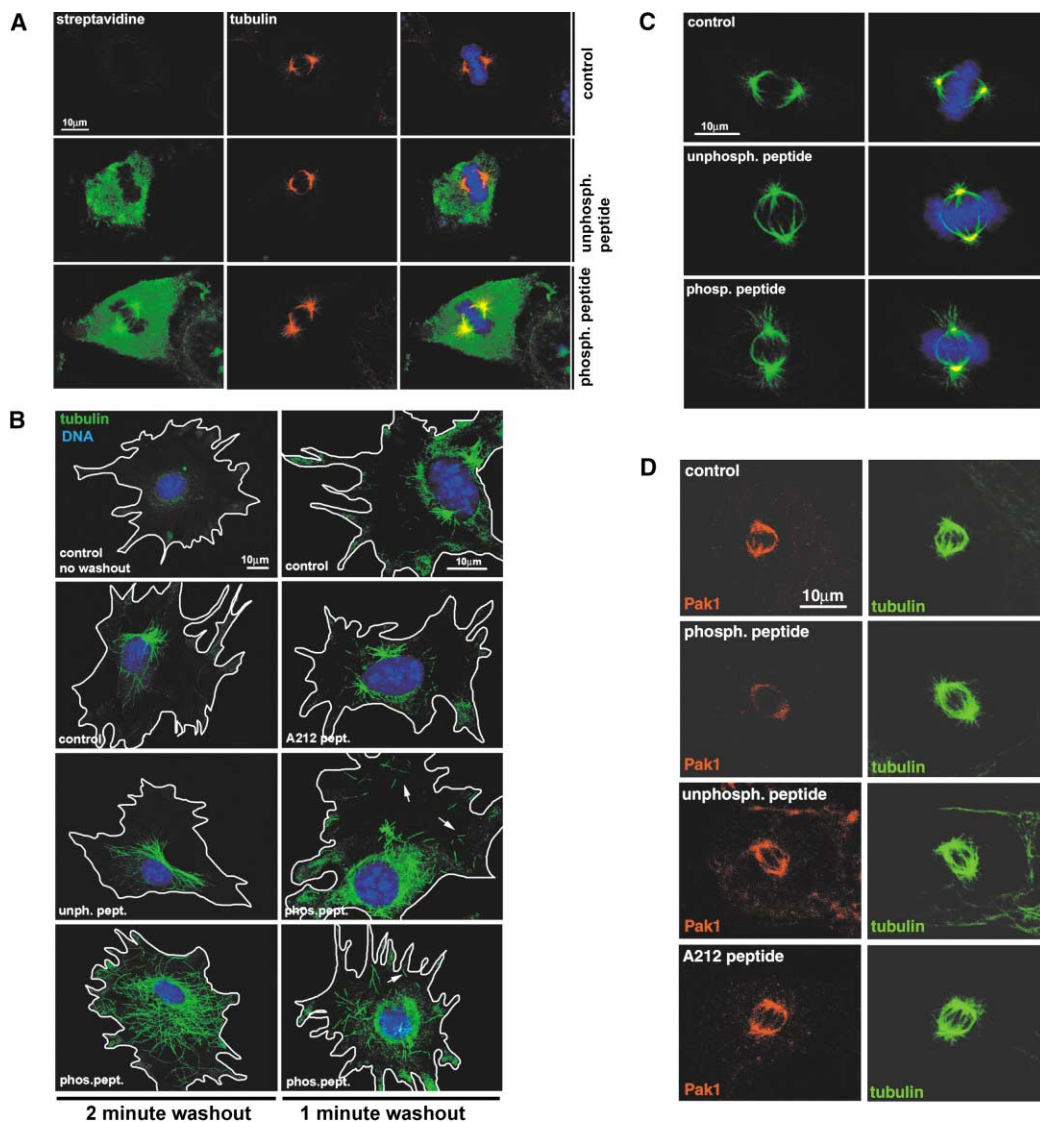
Figure 4. Pak1T212(PO₄) Affects Microtubule Organization during Mitosis

(A) The subcellular localization of biotin-conjugated penetratin peptides containing 15 amino acids surrounding T212 was examined with FITC-streptavidine. The top panel shows no background staining, and the middle panel shows the localization of the nonphosphorylated peptide. Phosphorylation of T212 directs the peptides to the spindles and MTOCs of metaphase cells (lower panel).

(B) Swiss3T3 fibroblasts that have been treated with nocodazole have completely depolymerized microtubules (top left panel). A total of 1 or 2 min after nocodazole removal, microtubules extended from an organization center in the controls (no peptide), in the presence of A212 or unphosphorylated peptides. Exposure to the phosphorylated peptide induced the formation of nonattached microtubules (arrows at 1 min of recovery) and greater length and disorganization (2 min). A white line outlines the cells.

(C) A Swiss3T3 fibroblast in metaphase that shows normal spindle organization in untreated cells (top panel) and after incubation with the unphosphorylated peptide (middle panel). The bottom panel shows the increase in the length of astral microtubules that is seen after exposure to the phosphorylated peptide. The right panel is an overlay showing microtubules (green), centrosomes (γ-tubulin; red), and DNA (blue).

(D) Swiss3T3s in metaphase that have been extracted with 0.5% Triton X-100 prior to fixation to remove cytoplasmic proteins. A proportion of Pak1 remains associated with the spindles in the control (no peptide), A212, and unphosphorylated peptide treatments. Exposure to the phosphorylated peptide reduced the levels of spindle-associated Pak1. All images were obtained with an Olympus confocal microscope.

sure to the phosphorylated peptide increased the number and length of astral microtubules that extended from the MTOCs (Figure 4C). The areas containing astral microtubules were measured for each metaphase cell, and the average size of the measured areas was divided by the overall cell size. Many cells (38%) that had been treated with the phosphorylated peptide had prominent astral microtubules that extended throughout more than a tenth of the soma. This proportion was reduced to

16% and 8% of cells exposed to unphosphorylated or no peptides, respectively. Equivalent measurements of the spindles revealed no significant differences between the three treatments. Similar results were obtained from four separate experiments. Prolonged exposure to the peptides (up to 4 hr) did not appear to affect cell division.

Since the phosphorylated peptide is targeted to the spindles, it is probable that it acts as a dominant negative by competing with endogenous Pak1. To date, an

association between Pak1 and microtubules has not been shown. We therefore extracted Swiss3T3 fibroblasts with detergent prior to fixation in order to remove cytoplasmic proteins. Subsequent staining with anti-Pak1 antibodies revealed that a small subpopulation of this kinase remained tightly bound to the mitotic spindles. A 1-hr treatment with unphosphorylated or A212 peptides had no effect on the amount of Pak1 retained on the spindles. In contrast, exposure to the phosphorylated peptide reduced the pool of microtubule-bound Pak1 (Figure 4D). We have also observed significant levels of the phosphorylated peptide tightly bound to the cytoskeleton, and this finding suggests that it competes with endogenous Pak1 for microtubule association (data not shown).

In summary, our data show that the phosphorylation of Pak1 on T212 is not restricted to postmitotic neurons but also plays a role during mitosis. By utilizing a novel technique that mimics protein phosphorylation, we support a role of Pak kinases in controlling microtubule dynamics. Overexpression of *Xenopus* Pak5 and an as yet unknown member of the Pak kinase family was recently proposed to induce microtubule stabilization either by direct association or indirectly by phosphorylating the microtubule-destabilizing protein, Op18/Stathmin [6, 7]. Overexpression of a constitutively active Pak1 mutant was reported to cause centrosomal abnormalities in 10% of breast epithelial cells [27]. Our data suggest that Pak1T212(PO$_4$) may act to destabilize microtubules and indicate diverse and possibly opposing roles of different Pak kinase family members. We also propose that the region surrounding phosphorylated T212 contains a protein binding site, since the phosphorylated peptide was enriched in spindles and MTOCs and competed with endogenous Pak1 for this location. Further work in our laboratory is directed at determining how Pak1T212(PO$_4$) affects the stability of dynamic microtubules during mitosis and axonal outgrowth.

Experimental Procedures

Reagents
The following antibodies were used: PK-18, γ-tubulin, and cyclin B1 (Western blots) (Sigma); tyrosinated α-tubulin (Oxford Biotechnology); histoneH3S10(PO$_4$) (Upstate Biotechnology); cyclin B1 (GNS1; kinase assays), Cdk2 (M2), Pak1 (C-19), and p35 (C-19) (Santa Cruz); TOPRO3 and Alexa-conjugated secondary antibodies (Molecular Probes); biotin-conjugated anti-mouse antibody and AB reagents (DAKO).

Cell Cultures and Manipulations
Swiss3T3s were grown in DMEM containing 0.1% FBS for at least 72 hr to arrest them in G0. Microtubule depolymerization was induced by incubation with 5 μg/ml nocodazole. HPLC-purified penetratin peptides (Biotin-Aminohexaonic acid-RRWRRWWRRWWRR WRR-VIEPLPVT(PO$_4$)PTRDVAT-OH, -VIEPLPVTPTRDVAT-OH, or -VIEPLPVAPTRDVAT-OH) were used at 350 μM (Chiron Technologies).

Indirect Immunohistochemistry
Indirect immunohistochemistry was carried out on rodent brains following deparaffination and heating in citrate buffer or, for frozen tissue, directly after cutting. For immunocytochemistry, cells were washed in warm PBS and fixed in ice-cold methanol for 10 min. Cytoplasmic extraction was performed for 1 min in 100 mM PIPES, 25 mM HEPES, 1 mM EGTA, 2 mM MgCl$_2$, 0.5% Triton X-100 (pH 7.0). Background staining was blocked in 0.2% fish skin gelatin,

and antibodies were diluted in 2% normal goat serum in PBS containing 0.2% Tween 20.

Cell Scoring
Scoring was carried out blind with a Zeiss Axioscop microscope at 40× and a macro provided by Imaging Associates. The macro enabled the outlining of individual cells and areas within as well as the calculation of the surface areas.

Kinase Assays
Kinase assays were carried out as previously described for p35/Cdk5 [8]. The substrates were histone H1, or fragments of Pak1 that had been purified from *Escherichia coli* and the GST tag was removed by cleavage with thrombin, or GST-Pak1R299 [16].

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