P21-activated protein kinase (PAK2)-mediated c-Jun phosphorylation at 5 threonine sites promotes cell transformation

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The oncoprotein c-Jun is one of the components of the activator protein-1 (AP-1) transcription factor complex. AP-1 regulates the expression of many genes and is involved in a variety of biological functions such as cell transformation, proliferation, differentiation and apoptosis. AP-1 activates a variety of tumor-related genes and therefore promotes tumorogenesis and malignant transformation. Here, we found that epidermal growth factor (EGF) induces phosphorylation of c-Jun by P21-activated kinase (PAK) 2. Our data showed that PAK2 binds and phosphorylates c-Jun at five threonine sites (Thr2, Thr8, Thr9, Thr93 and Thr286) in vitro and ex vivo. Knockdown of PAK2 in JB6 Cl41 (P+) cells had no effect on c-Jun phosphorylation at Ser63 or Ser73 but resulted in decreases in EGF-induced anchorage-independent cell transformation, proliferation and AP-1 activity. Mutation at all five c-Jun threonine sites phosphorylated by PAK2 decreased the transforming ability of JB6 cells. Knockdown of PAK2 in SK-MEL-5 melanoma cells also decreased colony formation, proliferation and AP-1 activity. These results indicated that PAK2/c-Jun signaling plays an important role in EGF-induced cell proliferation and transformation.

Introduction

The activator protein-1 (AP-1) transcription factor is implicated in many diverse biological functions including proliferation, differentiation and apoptosis, as well as transformation. AP-1 comprises dimers composed of members of the Fos, Jun and ATF protein families in which c-Jun is the most essential component. Knockout studies in mice showed that mice lacking c-Jun die within 10 days. The activation of c-Jun directly affects AP-1 activity suggesting that c-Jun is a positive regulator of proliferation and transformation (1). Total c-Jun and phosphorylated c-Jun proteins are highly abundant in many carcinoma cells (2,3) and are also involved in tumor invasion and metastasis (4). The transactivation activity of c-Jun is induced by extracellular signals including growth factors, transforming oncogenes, chemokines and extracellular stress (5,6). The transactivation activity of c-Jun is believed to be mainly dependent on its phosphorylation at Ser63/73 by c-Jun NH2-terminal kinase (5–8). However, other kinases, including extracellular signal-regulated protein kinases (ERKs), protein kinase C, DNA-dependent protein kinase, c-Abl tyrosine kinase and cyclin-dependent kinase 3 (9), have also been shown to phosphorylate c-Jun to stimulate its activity. c-Jun has emerged as a promising therapeutic target in clinical cancer treatment (10).

Therefore, the identification of additional novel c-Jun kinases could further clarify its activation mechanism and role in cancer. Herein, we found that P21-activated kinase (PAK) 2 is a novel kinase of c-Jun when induced by epidermal growth factor (EGF). PAKs are serine/threonine kinases that are activated by binding with small G proteins, such as cell division control protein 42 (Cdc42) and Rac, and plays an important role in Cdc42/Rac signaling (11). PAK family kinases are activated when stimulated by extracellular stresses such as hyperosmolarity, ionizing radiation or DNA damage and are involved in cellular activities including growth, morphology, movement, survival and apoptosis (12,13). PAKs are also involved in cell transformation and tumor development (14). At present, six PAK family members have been identified in mammals, and they are divided into two groups comprised PAK1, PAK2 and PAK3 (group 1) and PAK4, PAK5 and PAK6 (group 2) according to sequence similarity (15). PAKs have a highly conserved C-terminal kinase domain and a variable NH2-terminal area. The binding region of Cdc42/Rac Rho kinase (P21-Rho-binding domain; PBD) and an autoinhibitory domain are present in the NH2-terminal and part of the PBD overlaps with the autoinhibitory domain (16). Binding can occur between both groups of PAK proteins and Cdc42/Rac, but only the first group of PAKs can be activated after binding (17). The C-terminal kinase domain of PAK1, PAK2 and PAK3 share 80–90% homology. The PAKs in group 1 are structurally similar to each other but are distributed in different organs. For example, PAK1 is mainly distributed in the brain, muscle and spleen; PAK2 is widely distributed throughout the body and PAK3 is expressed only in brain tissue (11). In the PAKs family, PAK2 is unique because it is not only activated by binding with the small G protein complex Cdc42/Rac, but it is also cleaved and activated by caspase-3 and similar proteases (12,18–20). After the binding of PAK2 and Cdc42/Rac, the autoinhibitory activity of PAK2 is attenuated, which results in PAK2 autophosphorylation and activation (15). During apoptosis, caspase 3 cleaves PAK2 into two fragments referred to as p27 and p34. The p27 fragment includes most of the regulatory region, whereas the p34 fragment encompasses the entire kinase domain and a few of the regulatory regions (21). PAK2 has eight autophosphorylation sites, with seven serine sites located in the NH2-terminal domain and one threonine site in the kinase domain (22). After cleavage by caspase 3, PAK2 is autophosphorylated at Ser141 and Thr402 and then activated (23). These two activation patterns are associated with different cell signaling transduction pathways. Caspase-activated PAK-2-p34 induces a cell death response. Activation of full-length PAK2 by Cdc42/Rac stimulates cell survival and protects cells from death. PAK2 plays an important role in cell proliferation, but the direct downstream proteins and precise regulatory mechanism involved in mediating PAK2’s role in proliferation are not yet clear. In this work, we found that PAK2 phosphorylates c-Jun and promotes cell transformation stimulated by EGF. A role for PAK2 in transformation was revealed and the possibility that PAK2 might be a new target for cancer prevention or treatment was explored.

Materials and methods

Reagents and antibodies
The Checkmate mammalian two-hybrid system was from Promega (Madison, WI). PAK2 and JNK1 active kinases were from Upstate Biotechnology (Charlottesville, VA). Antibodies for western blotting were purchased from Cell Signaling Technology (Beverly, MA), Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate Biotechnology. The JetPEI reagent was purchased from Qiogene (Montreal, Quebec, Canada) and EGF was from BD Biosciences. Control cell arrays were from the American Type Culture Collection.
Cell culture and transfection

JB6 C141 mouse skin epidermal cells were cultured at 37°C in a 5% CO₂ incubator with 5% fetal bovine serum (FBS) in Minimum Essential Medium Eagle (MEM). The 293T cells were cultured at 37°C in a 5% CO₂ incubator in 10% FBS in Dulbecco's Modified Eagle Medium. SK-MEL-5 and SK-MEL-28 human melanoma cells were cultured at 37°C in a 5% CO₂ incubator in 10% FBS–MEM. Cells were split at 80–90% confluence and media changed every 3 days. For transfection experiments, the expression plasmids were transfected into cells using JetPEI according to the manufacturer’s suggested protocol.

Western blotting

Cells were harvested at 80–90% confluence and proteins extracted with RIPA cell lysis buffer. Protein concentration was determined by a protein assay kit (Bio-Rad, Hercules, CA). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blocked with phosphate buffered saline between 20 containing 5% nonfat milk and incubated in a 1:1000 dilution of the primary antibody 1h at 4°C. After three washes with phosphate buffered saline between 20, membranes were incubated with a 1:5000 dilution of the corresponding secondary antibody. Proteins were detected with ECL Plus western blotting detection reagents (GE Healthcare, Piscataway, NJ).

Proliferation assay

To assess proliferation, JB6 cells were infected with an sh-mock or sh-PAK2 plasmid to form stable cells and were seeded into 96-well plates in 100 μl of 5% FBS–MEM and cultured in a 5% CO₂ incubator at 37°C. Cells were cultured for various times (24, 48, 72 or 96 h) and then 20 μl of the CellTitre 96 Aqueous One Solution (Promega) were added to each well and cells were placed into a 37°C, 5% CO₂ incubator for 1 h. Absorbance was measured at 490 nm with a plate reader (Labsystems Multiskan MS, Analytical Instruments, LLC, Golden Valley, MN).

Anchorage-independent transformation assay

To examine the role of PAK2 and c-Jun in EGF-induced transformation, JB6 cells were infected with sh-mock or sh-PAK2 plasmids and selected with 2 μg/ml puromycin. JB6 cells were also stably transfected with a pcDNA4-wt-c-jun or pcDNA4-mut-c-jun plasmid and SK-MEL-5 melanoma cells were stably infected with a sh-mock or sh-PAK2 plasmid. Each cell (8 x 10⁵) type above was exposed to EGF (10 ng/ml) in 1 ml of 0.3% basic medium Eagle agar with 10% FBS. Cultures were maintained in a 5% CO₂ incubator at 37°C for 7–14 days and then colonies were counted by microscope and the Image-Pro PLUS computer software program (v.4; Media Cybernetics, Bethesda, MD).

AP-1 activity assay

JB6 cells stably transfected with an AP-1 luciferase reporter plasmid were transfected with the sh-mock or sh-PAK2 plasmid, pcDNA4-wt-c-jun or pcDNA4-mut-c-jun plasmid, and then, the cells were transiently transfected with the PRL-SV40 plasmid (10 ng). SK-MEL-5 melanoma cells stably infected with sh-mock or sh-PAK2 were transiently transfected with the AP-1 luciferase reporter plasmid (2 μg) together with the PRL-SV40 (10 ng) plasmid. Cells were starved in 0.1% FBS–MEM for 24 h, followed by stimulation with EGF (10 ng/ml) for 16 h. Then, the cells were disrupted with lysis buffer and luciferase activity was measured by luminometer (Monolight 2010, San Diego, CA).

In vitro kinase assay

A purified c-Jun fusion protein or histone H4 was used as substrate for active PAK2 (100 ng; Upstate Biotechnology) in an in vitro kinase assay. The reaction was conducted in 1× kinase buffer with 50 μmol/1 ATP or [γ-32P]ATP at 30°C for 30 min. Then, the reaction was stopped and proteins resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the [32P]-labeled c-Jun protein was visualized by autoradiography.

Immunoprecipitation

To determine whether PAK2 can bind with c-Jun under ex vivo conditions, 293T cells (5 x 10⁵) were seeded in 60 mm dishes. After 14 h of culture, the pcDNA4-wt-c-jun and pcDNA3.1-pak2 plasmids were transiently co-transfected into these cells. The cells were cultured for 36–48 h in a 5% CO₂, 37°C incubator, harvested and disrupted with NP-40 lysis buffer (300 μl). After measuring protein concentration, the V5 antibody was used for immunoprecipitation of the protein mixture (300 μg) at 4°C overnight and proteins were visualized by western blotting with anti-Xpress.

Tissue array

Two human malignant melanoma skin tissue arrays (U.S. Biomax, Rockville, MD) were prepared and analyzed according to the provided protocol. The samples were blocked with 5% goat serum albumin in 600 μl 1× phosphate-buffered saline/0.03% Triton X-100, (pH 6.0) in a humidified chamber for 1 h at room temperature and then incubated with PAK2 goat antibody (1:25 dilutions in 500 μl 1× phosphate-buffered saline/0.03% Triton X-100, pH 6.0) at 4°C in a humidified chamber overnight. The slides were washed and hybridized 2 h at room temperature in the dark with the secondary antibody (anti-goat, donkey antibody) conjugated with Cy2 (Jackson Immunoresearch Laboratories, West Grove, PA) (1:200 dilution). Slides were washed with phosphate-buffered saline (2×, 5 min). Expression of PAK2 was observed by laser scanning confocal microscopy (Nikon C1® Confocal Spectral Imaging System; Nikon Instruments Co., Melville, NY). Confocal Z-sections of 0.6 μm thickness were imaged.

Results

Knockdown of PAK2 inhibits JB6 cell transformation induced by EGF

EGF is a well-known skin cancer promoter in vivo and ex vivo (24–26). We examined whether EGF can activate PAK2 in the mouse skin epidermal JB6 C141 (F⁻) cell line. JB6 cells were treated with EGF and harvested at different times and protein levels were determined by western blotting (Figure 1A). Phosphorylation of PAK2 was detected at 15 min after EGF treatment and gradually increased with the strongest phosphorylation occurring at 180 min (Figure 1A, top). ERK1/2 phosphorylation induced by EGF is used as a positive control and total PAK2, ERK1/2 and β-actin verified equal protein loading. These results showed that PAK2 is activated in JB6 cells by the tumor promoter EGF. Next, we examined the effect of PAK2 knockdown on JB6 cell proliferation, colony formation in soft agar and AP-1 activity. PAK2 knockdown stable cells, JB6-sh-Mock and JB6-sh-PAK2, were established by infection with sh-mock or sh-pak2. Results indicated that the abundance of the endogenous PAK2 protein was decreased by ~90% compared with PAK2 in JB6-sh-Mock cells (Figure 1B, left). Expression of PAK1 and PAK3 are not affected by knockdown of PAK2 indicating that the results observed in PAK2 knockdown cell lines are specific to PAK2 and cannot be attributed to PAK1 or PAK3.

To determine the role of PAK2 in proliferation, growth curves were generated for JB6-sh-Mock and JB6-sh-PAK2 cells and the results showed that proliferation of JB6-sh-PAK2 cells was significantly slower than that of JB6-sh-Mock cells (Figure 1B, right). Importantly, we found that PAK2 knockdown inhibited JB6 cell transformation in soft agar (Figure 1C) and AP-1 luciferase activity (Figure 1D). Overall, these results indicated that PAK2 might play an important role in EGF-induced JB6 cell proliferation and transformation by affecting AP-1 activity.

PAK2 knockdown has no effect on c-Jun phosphorylation at Ser63 or Ser73

In order to investigate the mechanism explaining the decrease in AP-1 activity after PAK2 knockdown in JB6 cells, we examined the effect on various proteins known to be associated with AP-1 activation and proliferation in JB6-sh-Mock and JB6-sh-PAK2 cells treated with EGF. c-Jun is a major protein component of AP-1 and the most well-characterized phosphorylation sites of c-Jun are Ser63 and Ser73 located near the NH2 terminus (27). Our results indicate that knockdown of PAK2 had no effect on either phosphorylation site and had no effect on phosphorylation of ERKs or ribosomal S6 kinase or total c-Jun or c-Fos abundance (Figure 2). These data indicated that even though PAK2 had no effect on phosphorylation of c-Jun at Ser63 or 73, knockdown of PAK2 blocked AP-1 activity. Therefore, we hypothesized that PAK2 might play a role in suppressing AP-1 activity through modification of other c-Jun phosphorylation sites.

PAK2 binds and phosphorylates c-Jun

To test our hypothesis, we first determined whether PAK2 could bind and phosphorylate c-Jun. Using the mammalian two-hybrid assay, PAK2 and JNK1 were each cloned into the pBIND vector and c-Jun was cloned into pACT vector, and then, each plasmid was transfected alone or co-transfected into NIH3T3 cells. Results indicated that when pBIND-pak2 and pACT-c-jun were co-transfected, the luciferase activity was ~35 times higher than that of the control group.

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Fig. 1. Knockdown of PAK2 inhibits EGF-induced JB6 P+ cell transformation. (A) EGF induces phosphorylation of PAK2 (Ser141, Ser144). Cells were treated with EGF (10 ng/ml) for various times and then harvested for western blot analysis. β-Actin was used to verify equal protein loading. (B) Knockdown of PAK2 in JB6 cells inhibits proliferation. Efficiency of PAK1, PAK3 or PAK2 knockdown (JB6-sh-PAK2) is shown (left). Cell proliferation was measured using the absorbance (A492nm) assay (right) and data are shown as means ± SDs of values from triplicate samples. The asterisk (*) indicates a significant decrease (P < 0.005) in proliferation of JB6-sh-PAK2 cells compared with JB6-sh-Mock cells. (C) EGF-induced transformation is suppressed in JB6-sh-PAK2 cells compared with JB6-sh-Mock cells. Representative photographs of colony formation are shown (upper). Colonies were counted using a microscope and the Image-Pro Plus software program (v4; lower). Data are shown as means ± SDs of triplicate samples and the asterisk (*) indicates a significantly (P < 0.005) lower number of colonies formed by JB6-sh-PAK2 cells. (D) Knockdown of PAK2 suppresses AP-1 activity in JB6 cells. JB6 cells stably expressing an AP-1 luciferase promoter and sh-mock or sh-PAK were subjected to a luciferase assay as described in Materials and Methods. The AP-1-luciferase activity was normalized against Renilla luciferase activity (phRL-SV40). Data are shown as mean ± SD of values from triplicate samples. The asterisk (*) indicates a significantly (P < 0.005) lower level of AP-1 activity in JB6-sh-PAK2 cells compared with JB6-sh-Mock cells.
transfected with pG5lu (Figure 3A, lane 1 versus 4). Co-transfection of pBIND-jnk1 and pACT-c-jun as a positive control showed activity that was ~65 times higher than the control (Figure 3A, lane 5). These results indicated that PAK2 could bind with c-Jun in NIH3T3 cells. To confirm that the binding can also occur in cells ex vivo, the pcDNA4-wt-c-jun and pcDNA3.1-pak2 plasmids were transiently transfected into 293T cells. At 40 h post-transfection, the V5-tagged PAK2 was immunoprecipitated with anti-V5 and the Xpress tagged c-Jun protein was detected by western blot using anti-Xpress (Figure 3B). Results confirmed that c-Jun could be co-immunoprecipitated with PAK2, which is consistent with the in vitro experimental results. The next question addressed was whether the binding resulted in phosphorylation of c-Jun by PAK2. A His-c-Jun fusion protein was purified from BL21 bacteria (4) and used as the substrate for commercially available active PAK2 in the presence of [\(^{32}\)P]ATP in an in vitro kinase assay. Results indicated that PAK2 could phosphorylate c-Jun in vitro (Figure 3C). Histone H4 is a known substrate for active PAK2 and was used a positive control (Figure 3C). Overall, these results indicated that PAK2 could bind and phosphorylate c-Jun in vitro and ex vivo.

PAK2 phosphorylates c-Jun at five threonine sites

PAK2 is a serine/threonine protein kinase, and therefore we used a phospho-serine and phospho-threonine primary antibody and western blotting to determine whether PAK2 phosphorylates c-Jun at serine and/or threonine residues. The results revealed that PAK2 strongly phosphorylates c-Jun on threonine sites (Figure 4A, top) but not on serine residues (Figure 4A, bottom). The reaction of JNK1 and c-Jun served as a positive control (Figure 4A, bottom). The next question addressed was the identity of the specific threonine (Thr) sites that PAK2 phosphorylates on c-Jun. The potential c-Jun threonine sites that might be phosphorylated by c-Jun were predicted using the software program NetPhos 2.0 (28) (Figure 4B). Based on the software prediction, we designed eight different c-Jun peptides to serve as substrates for PAK2 with [\(^{32}\)P]ATP in an in vitro kinase assay. The results indicated that Thr2, Thr8, Thr89, Thr93 and Thr286 had relatively strong signals, suggesting that these 5 c-Jun threonine sites are possible PAK2 phosphorylation sites (Figure 4C, lanes 2, 3, 4, 6 and 9). To further confirm our results, wild-type full-length c-Jun and different threonine site c-Jun mutants were used as substrates for active PAK2 and [\(^{32}\)P]ATP. The reaction mixture was resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and [\(^{32}\)P]-labeled His-c-Jun was visualized by autoradiography. The reaction of PAK2 and histone H4 served as a positive control. (C) PAK2 phosphorylates c-Jun in vitro. The His-c-Jun fusion protein served as substrate for active PAK2 in the presence of [\(^{32}\)P]ATP. The reaction mixture was resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and [\(^{32}\)P]-labeled His-c-Jun was visualized by autoradiography. The reaction of PAK2 and histone H4 served as a positive control.

Mutation of five threonine sites in c-Jun decreases transforming ability

c-Jun is an important component of AP-1 and can promote cell transformation. We determined the effect on proliferation and

![Fig. 2. Knockdown of PAK2 has no effect on c-Jun phosphorylation at Ser63 or Ser73. JB6-sh-PAK2 cells or JB6-sh-Mock cells were treated with EGF (10 ng/ml) for various times and subjected to western blotting with different specific antibodies. β-Actin was used to verify equal protein loading.](https://academic.oup.com/carcin/article-abstract/32/5/659/2896610)

![Fig. 3. PAK2 can bind and phosphorylate c-Jun. (A) PAK2 interacts with c-Jun. The interaction between the GAL4-PAK2 (pBIND-pak2) and VP16-c-Jun (pACT-c-jun) fusion constructs (lane 4) resulted in a significant (*P < 0.005) increase in luciferase activity compared with the negative controls (lanes 1, 2 and 3). JNK1 (pBIND-jnk1; lane 5) was used as a positive control. Data are shown as means ± SDs of three independent experiments. (B) PAK2 binds c-Jun ex vivo. pcDNA3.1-pak2 (V5-tagged) and pcDNA4-c-jun (Xpress tagged) were transiently transfected into 293T cells. After 40 h, PAK2 was immunoprecipitated with anti-V5 and c-Jun was detected with anti-Xpress by western blotting. (C) PAK2 phosphorylates c-Jun in vitro. The reaction of PAK2 and histone H4 served as a positive control.](https://academic.oup.com/carcin/article-abstract/32/5/659/2896610)
PAK2 mediates c-Jun phosphorylation

AP-1 activity of each group was measured. Results indicated when wt-c-jun and pak2 were co-transfected into JB6 cells, AP-1 activity was dramatically increased compared with other cell types (Figure 5D, lane 11). However, when mut-c-jun (T5A) and pak2 were co-transfected into JB6 cells, AP-1 activity was decreased compared with cells co-transfected with pak2 and wt-c-jun (Figure 5D, lane 12). This indicated that c-Jun phosphorylation by PAK2 at five threonine sites can promote AP-1 activity.

Knockdown of Pak2 causes decreased colony formation by SK-MEL-5 melanoma cells

Finally, in order to obtain more conclusive evidence that PAK2 can promote transformation, we examined PAK2 protein abundance in several skin cell lines by western blot. We found that Pak2 is highly expressed in SK-MEL-5 and SK-MEL-28 melanoma cells compared with normal human HaCaT or mouse JB6 cells (Figure 6A, left). In addition, results of a human melanoma tissue array analysis indicated that the abundance of Pak2 was greater in malignant melanoma tissues compared with normal tissues (Figure 6A, right). We chose the SK-MEL-5 cell line for further study. First, we determined whether knockdown of Pak2 in SK-MEL-5 cells can affect proliferation, transformation and AP-1 activity. We infected sh-mock and sh-pak2 into SK-MEL-5 melanoma cells and established stable cells. The efficiency of Pak2 knockdown was examined by western blot and the results show that the endogenous Pak2 protein level was suppressed by ~80% by sh-pak2 compared with sh-mock control (Figure 6B, left). We also examined the level of other proteins in both of the cell types. We found that p-ERK1/2 and p-c-Jun (Ser63, Ser73) were not affected by knockdown of Pak2 (Figure 6B, middle), which further confirms that the function of Pak2 is not related to c-Jun phosphorylation at Ser63 or Ser73. We next examined proliferation in SK-MEL-5-sh-Mock and SK-MEL-5-sh-pak2 cells, and results showed that SK-MEL-5-sh-pak2 suppressed proliferation compared with the mock cells (Figure 6B, right). In addition, results of the anchorage-independent cell transformation assay indicated that SK-MEL-5-sh-pak2 cells displayed about a 60% reduction in colony formation in soft agar compared with mock cells (Figure 6C). Accordingly, AP-1 activity was also reduced in SK-MEL-5-sh-pak2 cells (Figure 6D). These data further confirm that Pak2 plays an important role in cell proliferation and transformation.

Discussion

The c-jun gene is an early response proto-oncogene and its protein product c-Jun is a major component of the AP-1 transcription factor complex. AP-1 plays an important role in tumor formation, metastasis and invasion (29). The development of anticancer drugs based on the inhibition of AP-1 activity is in progress (10). c-Jun is involved in the transcription of many growth factor and cytokine genes and plays an important role in the regulation of proliferation, survival and apoptosis (30). In tumor cells, the c-Jun protein expression level and activity are increased. The EGF can induce AP-1 activity and c-Jun is phosphorylated by various upstream kinases and exhibits increased activity with EGF stimulation and thus performs its functions by increasing AP-1 activity. Cyclin-dependent kinase-3 is reported to phosphorylate c-Jun and its phosphorylation results in increased AP-1 activity induced by EGF (9). Similarly, c-Jun is phosphorylated at Ser63 and Ser73 by JNKs with ultraviolet stimulation to promote AP-1 activity (31,32). Pak2 activity is increased in a number of late stage tumors including breast, brain, pancreatic, ovarian and colon cancers (14). In MCF-7 breast cancer cells, increased Pak1 activity can promote proliferation and anchorage-independent growth (34). The ability to promote cell proliferation and transformation is shared all PAKs and cells stably

![Fig. 4. PKA2 phosphorylates c-Jun at five threonine sites. (A) PKA2 phosphorylates c-Jun at five threonine sites as determined by an in vitro kinase assay with detection by western blotting. (B) c-Jun-phosphorylated threonine sites as predicted by NetPhos 2.0. Score indicates the possibility that the site might be phosphorylated ex vivo; Pred indicates prediction. (C) Peptide mapping of the c-Jun threonine sites phosphorylated by PKA2. (D) Confirmation of the peptide mapping results using wild-type (wt) or mutant (mut) GST-c-Jun as substrate in an in vitro kinase assay in the presence of [γ-32P]ATP as visualized by autoradiography.](https://academic.oup.com/carcin/article-abstract/32/5/659/2896610)
transfected with PAK4 produce more colonies in soft agar (35). PAK4 overexpression is observed in 78% of all tumor cell lines (35). These findings indicate that PAK4 plays a prominent role in oncogenic transformation (35). PAK2 is a serine/threonine kinase and previous results confirmed that phosphorylation of the oncoprotein Myc by PAK2 can prevent Myc/Max/DNA complex formation, causing Myc to lose its...
ability to induce cell proliferation and transformation (36). PAK2 activation can inhibit cell proliferation in many species (16,37). These results seemed to indicate that PAK2 is a negative regulator of tumorigenesis. However, some reports suggest that PAK2 can promote neoplastic transformation and promote fibroblast proliferation and transformation mediated by transforming growth factor-B independently of Smad2 and Smad3 (38). Here, we show that PAK2 phosphorylates c-Jun at five threonine sites, thereby promoting...
proliferation and transformation of JB6 cells when stimulated by EGF. We also found that PAK2 was highly abundant in SK-MEL-5 and SK-MEL-28 melanoma cells. JB6-sh-PAK2 and SK-MEL-5-sh-PAK2 stable cells displayed a dramatic reduction of colony formation in soft agar compared with sh-mock control cells. Accordingly, AP-1 activity was also reduced in either sh-PAK2 stable cell types. These results indicated that PAK2 might be a tumor-promoting agent.

In summary, overall results show that PAK2/c-Jun signaling plays an important role in tumorigenesis and provides an increased understanding of the signalling transduction mechanism of skin tumor cells and suggest that PAK2 might be a new chemopreventive or chemotherapeutic target.

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References

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