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 tumorigenesis 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, 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 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 Qbiogene (Montreal, Quebec, Canada) and EGF was from BD Biosciences (San Jose, CA). The tissue array was from US Biomax (Rockville, MD).
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 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% confluency 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 tween-20 containing 5% nonfat milk and incubated in a 1:1000 dilution of the primary antibody 16 h at 4°C. After three washes with phosphate buffered saline tween-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 CellTiter 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 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 × 10⁵) type above was exposed to EGF (10 ng/ml) in 1 ml of 0.3% basal 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).

PAK1 and PAK3 are not affected by knockdown of PAK2

To determine the role of PAK2 in proliferation, growth curves were generated for JB6-sh-mock and JB6-sh-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-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 of the protein (27). Our results indicated 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 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.
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 [\(\gamma\)-32P]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 as 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 [\(\gamma\)-32P]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 [\(\gamma\)-32P]ATP in an in vitro kinase assay. Interestingly, the results indicated that PAK2 phosphorylation of c-Jun still persisted even when 2, 3 or 4 of the threonine sites were all mutated (Figure 4D, lanes 3–7). Only when all five threonine sites were mutated did the phosphorylation signal disappear (Figure 4D, lane 8). This confirms that all five of these threonine sites of c-Jun are phosphorylated by PAK2.

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
PAK2 mediates c-Jun phosphorylation

Confirmation of the peptide mapping results using wild-type (wt) or mutant Peptide mapping of the c-Jun threonine sites phosphorylated by PAK2. (D) that the site might be phosphorylated threonine sites as predicted by NetPhos 2.0. Score indicates the possibility B kinase assay with detection by western blotting. (in vitro of c-Jun [mut-c-Jun (T5A)]. We transfected wt-c-jun 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 at Ser63 and Ser73, which are the two most well-studied sites, and their 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).

PAKs were originally discovered and defined as effectors of Rho GTPases (33), but in recent years, PAK2 was found to be a downstream substrate of many oncoprotein signal transduction pathways and is an important regulator of tumor cell signaling networks (14). PAK1 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 PKAs and cells stably

Fig. 4. PAK2 phosphorylates c-Jun at five threonine sites. (A) PAK2 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 PAK2. (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.
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

Fig. 5. Mutation of the five threonine sites in c-Jun phosphorylated by PAK2 decreases transforming ability. (A) wt-c-Jun and mut-c-Jun(T5A) were each stably transfected into JB6 cells and expression levels were confirmed by western blot with anti-Xpress (left). Proliferation of JB6-wt-c-Jun and mut-c-Jun (T5A) cells was measured by MTS assay and data are shown as means ± SDs of values from three replicates. The asterisk (*) indicates a significant decrease (P < 0.005) in proliferation of JB6-mut-c-Jun (T5A) cells compared with JB6-wt-c-Jun cells (right). (B) Representative photographs of colony formation by JB6-wt-c-Jun and JB6-mut-c-Jun (T5A) cells (left). The average number of colonies formed by JB6-wt-c-Jun or JB6-mut-c-Jun (T5A) cells was determined by microscope and the Image-Pro Plus software program (v4; right). 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-mut-c-Jun (T5A) cells. (C) JB6-mut-c-Jun (T5A) inhibits AP-1 activity in JB6 cells. JB6 cells stably expressing the AP-1 luciferase promoter and wt-c-jun or mut-c-jun(T5A) were assessed for EGF-induced luciferase activity. Data are shown as means ± SDs of values from triplicate samples. The asterisk (*) indicates a significantly (P < 0.005) lower level of AP-1 activity in JB6-mut-c-Jun (T5A) cells compared with JB6-wt-c-Jun cells. (D) PAK2 can promote JB6-wt-c-Jun-mediated AP-1 activity. JB6 cells stably expressing an AP-1 luciferase promoter were seeded into 12-well dishes and cultured overnight. Luciferase activity was analyzed as described in Materials and Methods. The AP-1-luciferase activity was normalized against Renilla luciferase activity (phRL-SV40). Data are shown as means ± SDs of values from triplicate samples. The asterisk (*) indicates a significantly (P < 0.005) higher level of AP-1 activity with overexpression of pak2 and c-jun.
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 cell proliferation and transformation.

Fig. 6. Knockdown of PAK2 causes decreased cancer cell colony growth. (A) Left, PAK2 is highly expressed in human melanoma cells (SK-MEL-5 and SK-MEL-28) compared with normal skin cells (JB6 and HaCaT). (A) Right, PAK2 protein levels are higher in malignant melanoma human skin tissue compared with matched normal skin tissue ($P < 0.0001$). Data are expressed as means ± SDs of two independent experiments. (B) Knockdown of PAK2 in melanoma SK-MEL-5 cells inhibits proliferation. Efficiency of PAK2 knockdown in SK-MEL-5 cells (left); knockdown PAK2 has no effect on c-Jun phosphorylation (Ser63 or Ser73; middle); cell proliferation was measured using the MTS assay and data are shown as means ± SDs of values from three replicates (left). The asterisk (*) indicates a significant decrease ($P < 0.005$) in proliferation of SK-MEL-5-sh-PAK2 cells compared with SK-MEL-5-sh-Mock cells. (C) Knockdown of endogenous PAK2 in SK-MEL-5 cells inhibits colony formation. Representative photograph of SK-MEL-5-sh-Mock and SK-MEL-5-sh-PAK2 colony formation (left); number of colonies formed by SK-MEL-5-sh-PAK2 or SK-MEL-5-sh-Mock cells were counted using a microscope and the Image-Pro Plus software program (v4; right). Data are shown as means ± SDs of triplicate samples and the asterisk (*) indicates a significantly ($P < 0.005$) lower number of colonies formed by SK-MEL-5-sh-PAK2 cells. (D) Knockdown of PAK2 inhibits AP-1 activity in SK-MEL-5 cells. SK-MEL-5 cells stably expressing sh-mock or sh-pak2 were transfected with the AP-1 luciferase and the Renilla luciferase promoters. The AP-1-luciferase activity was normalized against Renilla luciferase activity (phRL-SV40) and data are shown as means ± SDs of values from triplicate samples. The asterisk (*) indicates a significantly ($P < 0.005$) lower level of AP-1 activity in SK-MEL-5-sh-PAK2 cells compared with SK-MEL-5-sh-Mock cells.
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 signaling transduction mechanism of skin tumor cells and suggest that PAK2 might be a new chemopreventive or chemotherapeutic target.

**Funding**
The Hormel Foundation and National Institutes of Health (CA111356, CA111536, CA120588, R7CA081064, ES016548).

**Acknowledgements**
We thank Dr Dirk Bohman (Department of Biomedical Genetics, University of Rochester, 601 Elmwood Avenue, Box 633, Rochester, NY 14642, USA) for the c-Jun plasmids.

**Conflict of Interest Statement:** None declared.

**References**

Received May 19, 2010; revised October 4, 2010; accepted December 11, 2010.