The Serine/Threonine Kinase PAK4 Prevents Caspase Activation and Protects Cells from Apoptosis*

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The serine/threonine kinase PAK4 was identified first as an effector molecule for the Rho GTPase Cdc42. PAK4 differs from other members of the PAK family both in sequence and function. Previously we have shown that an important function of this kinase is to mediate the induction of filopodia in response to activated Cdc42. Studies with a constitutively active PAK4 mutant have shown that it also has a role in promoting anchorage-independent growth, an important hallmark of oncogenic transformation. Here we show that another function of PAK4 is to protect cells against apoptotic cell death. Expression of wild-type or constitutively active PAK4 delays the onset of apoptosis in response to tumor necrosis factor α stimulation, UV irradiation, and serum starvation. Consistent with an antiapoptotic function, expression of PAK4 leads to an increase in phosphorylation of the proapoptotic protein Bad and an inhibition of caspase activation.

Normal development requires a carefully controlled balance between cell survival and cell death. Throughout development excess cells are eliminated by the process of programmed cell death, or apoptosis. A number of different stimuli can trigger apoptosis in cells in culture including Fas ligand, UV irradiation, serum deprivation, and cytokines such as tumor necrosis factor α (TNFα).[1] Exposure to apoptosis-inducing agents triggers a series of events, often involving the caspase cysteine proteases. For example, Fas ligand induces the activation and cleavage of initiating caspases such as caspase-8, which in turn leads to activation of downstream caspases including caspases-3 and -9. Caspase-3 as well as other effector caspases in turn cleave a number of different target proteins that play important roles in mediating the apoptotic response (1, 2).

Caspase-8 can also activate a signaling pathway that leads to cytochrome *c* release from the mitochondria. Release of cytochrome *c* leads to activation of caspase-9 followed by cleavage and activation of caspase-3, leading to apoptosis (2, 3). The release of cytochrome *c* from the mitochondria is governed largely by Bid and members of the Bcl-2 family. Members of the

Bcl-2 family can have both proapoptotic and antiapoptotic activities. For example Bcl-2, Bcl-x$_L$, Mcl-1, A1, and Bag-1 promote survival, whereas Bcl-x$_S$, Bad, Bax, and Bak promote apoptosis (4). The interactions between the different BCL-2 family members play an important role in determining cell fate. For example Bcl-2 and Bcl-x$_L$, which are located at the mitochondrial outer membrane, promote cell survival by inhibiting cytochrome *c* release. In contrast Bad can interact with Bcl-2 and Bcl-x$_L$ and prevent their inhibitory activities. Thus binding of Bad to Bcl-2 and Bcl-x$_L$ stimulates the release of cytochrome *c* from the mitochondria. One way that the activities of the Bcl-2 family members are regulated is by phosphorylation. When Bad is phosphorylated on serines 136 and 112 it can no longer interact with Bcl-2 or Bcl-x$_L$, cytochrome *c* release is inhibited, and apoptosis is prevented (5). A number of survival signals including growth factors can lead to phosphorylation of Bad at both serines 136 and 112. Some of these signals are mediated by the survival factor Akt, which phosphorylates Bad on serine 136 (6). In addition to Akt other protein kinases can phosphorylate Bad. For example constitutively active PAK1, a member of the PAK family of serine/threonine kinases, was shown recently to stimulate Bad phosphorylation at both serines 136 and 112 (7, 8).

The PAK family members were identified originally as molecular targets for the Rho GTPases Rac and Cdc42 (Refs. 9–14; for review see Refs. 15–17). At present four major members of the PAK family have been identified in mammalian cells, which seem to fall into two categories based on their structures. The first category includes the closely related human PAK1, human PAK2, and mouse PAK3 and the corresponding rat homologues αPAK, γPAK, and βPAK, respectively (9, 11–13). Each of these proteins contains an amino-terminal regulatory domain and a carboxyl-terminal kinase domain. Within the regulatory domain is a GTPase binding domain (GBD) that mediates binding to Cdc42 and Rac. Also within the regulatory domain are two to five proline-rich regions that bind to SH3 domain-containing proteins such as the adaptor protein Nck (18, 19) and the exchange factors of the PIX/COOL families (20, 21). Carboxyl-terminal to the kinase domain is a motif that can interact with yeast G protein βγ subunits, suggesting that the PAKs might be regulated by heterotrimeric G proteins in mammalian cells (22, 23). The most recently identified member of the PAK family is PAK4, which falls into a second category of PAKs (14). Like the other PAKs, PAK4 contains an amino-terminal GBD and a carboxyl-terminal kinase domain. Unlike other PAKs, however, PAK4 does not have SH3-domain recognition sites or a G protein βγ binding domain, and it does not bind to PIX or Nck (Ref. 14).[2] Furthermore the GBD and kinase domains of PAK4 have only ~50% identity with

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[1] The abbreviations used are: TNFα, tumor necrosis factor α; GST, glutathione *S*-transferase; CHX, cycloheximide; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; GBD, GTPase binding domain; PBS, phosphate-buffered saline; PARP, Poly(ADP-ribose) polymerase.

[2] C. Dan and A. Minden, unpublished results.

those of PAKs 1, 2, and 3, and the regulatory domain of PAK4 outside of the GBD is completely different from the other PAKs.

One important function of the PAK proteins is the regulation of cytoskeletal architecture (24–27). PAK1 has been reported to induce filopodia and membrane ruffles by a mechanism that is independent of its ability to bind the Rho GTPases and partially independent of its kinase activity (28, 29). Others have found that αPAK and PAK2 do not induce filopodia or lamellipodia but instead have a role in the dissolution of stress fibers, down-regulation of focal adhesions, and cell retraction (30). More recently the PAKs have been shown also to have important roles in regulating the apoptotic response, although the different PAKs have different functions. For example γPAK and PAK2 get cleaved during apoptosis most likely by caspase-3. This leads to their activation, and the activated kinases in turn are thought to contribute to morphological and membrane changes that occur during apoptosis (31–33). In contrast PAK1, which is not cleaved by caspases, was reported to protect cells from apoptosis induced by serum withdrawal in fibroblasts and interleukin-3 withdrawal in lymphoid cells (7, 8). The survival signal induced by PAK1 is apparently due to phosphorylation of Bad on both serines 112 and 136 (7, 8).

PAK4 was identified originally on the basis of its role as a cytoskeletal regulatory protein. It is the first member of the PAK family that was shown to be a link between Cdc42 and filopodia formation (14). More recently we have found that PAK4 also has a role in regulating cell growth. In fact a constitutively active mutant of PAK4 can induce anchorage-independent growth, one of the hallmarks of oncogenic transformation.[3] Here we have investigated another role for PAK4: the induction of cell survival pathways in response to apoptotic stimuli. We have found that cells overexpressing either wild-type or constitutively active PAK4 have a survival advantage during apoptosis. PAK4 protects cells from apoptosis not only in response to serum withdrawal but also in response to TNFα treatment or UV irradiation. Expression of PAK4 inhibits activation of caspase-3-like enzymes and specifically promotes the phosphorylation of Bad on serine 112.

EXPERIMENTAL PROCEDURES

Plasmids—To construct pLPC-HA-PAK4wt, HA-PAK4wt (14) was removed form pBluescript KS II(+) as a *Hin*dIII-*Stu*I fragment and inserted into the *Hin*dIII-*Eco*RI (blunted) site of the pLPC vector. The pLPC vector is a retroviral expression vector with a puromycin resistance marker (a gift from R. Prywes). pCAN-Myc-PAK4(S445N,S474E) is cloned into the *Eco*RI site of the pCAN-Myc2 vector as described.[3] This constitutively active PAK4 mutant contains a serine to glutamate mutation at amino acid 474, the putative autophosphorylation site, and a serine to asparagine mutation at amino acid 445. Both mutations were generated using site-directed mutagenesis (Stratagene Quick-Change kit). The mutation at amino acid 474 is thought to mimic a phosphorylated amino acid, and the mutation at amino acid 445 is thought to stabilize the catalytic loop as described.[3] The Bad-glutathione *S*-transferase (GST) fusion mammalian expression plasmid (pEBG-Bad) was obtained from New England Biolabs.

Reagents and Antibodies—Cycloheximide (CHX), propidium iodide, and Hoechst 33258 reagent (bis-benzimide) were from Sigma. Human recombinant TNFα was from R&D Systems. Alexa™488 conjugated phalloidin (A-12379) was from Molecular Probes, Inc. Histone H4 was from Roche Molecular Biochemicals. Bacterially expressed, purified recombinant GST fusion-human Bad was kindly provided by T. Franke. Mouse monoclonal anti-HA antibody (HA.11, clone 16B12) was from Covance, and mouse monoclonal anti-c-Myc antibody (sc-40, clone 9E10) was from Santa Cruz Biotechnology. Mouse monoclonal anti-human PARP (clone 4C10–5) was from PharMingen. Rabbit polyclonal antibodies to phosphorylated Bad were from New England Biolabs. Mouse monoclonal anti-GST antibody (clone GST-2) and secondary antibodies conjugated to horseradish peroxidase were from Sigma.

Cell Culture and Transfection—NIH3T3 stably transfected with either pLPC, PAK4, or PAK4(S445N,S474E) as described[3] were grown at 37 °C in 5% CO_2 and cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% bovine calf serum in the presence of 2 μg/ml puromycin. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in the presence of 1 μg/ml puromycin for stably transfected PAK4 and pLPC clones. All media were supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin, and 4 mM glutamine. Transient transfection assays were carried out in HeLa cells using the calcium phosphate precipitation method. HeLa stable cell lines were generated by retroviral infection. Briefly, ΦNX cells were transfected with pLPC empty vector, or pLPC-HA-PAK4, by the calcium phosphate precipitation method. Supernatants containing the released viruses were collected from cells 2 days after transfection and filtered through 0.45-μm filters. The virus was used then to infect HeLa cells. Cells were selected with puromycin (1 μg/ml), and colonies were picked ~2 weeks after selection. Expression of PAK4 was determined by Western blot and immunofluorescence microscopy using a monoclonal antibody against the HA tag.

Survival and Apoptosis Assays—To estimate apoptosis and survival of stably transfected cell lines, equal numbers of cells were seeded in growth medium in 3.5-, 6-, or 10-cm plates. Two days later, cells were stimulated as follows: for UV irradiation cells were washed twice in phosphate-buffered saline (PBS). After removal of the PBS, cells were exposed to 50 J/m² UV-light in a UV cross-linker (Fisher) followed by addition of fresh medium. For TNFα-CHX treatment cells were washed once with fresh medium that was replaced by medium containing TNFα and CHX either alone or together at a concentration of 10 ng/ml and 10 μg/ml, respectively. For serum deprivation experiments cells were washed once with medium without serum, followed by addition of fresh medium containing 0.1, 0.5, or 10% serum for 24 h.

After stimulation cells were collected at the indicated time points (attached and floating dead cells unless otherwise indicated) and fixed for flow cytometry analysis or used to prepare total cell extracts. To determine the percentage of cell death by flow cytometry analysis cells with DNA content lower than the G_1 peak (sub-G_1) were considered to be apoptotic. Caspase-3-like activity was examined by Western blot analysis of the caspase-3 substrate PARP in equal amounts of cell lysate. Detection of the M_r 85,000 proteolytic product of PARP was used as an indication of caspase activity.

To examine nuclear condensation, cells stained with Hoechst 33258 were analyzed by fluorescence microscopy. Cells that displayed condensed chromatin and blebbed nuclei were considered apoptotic. To determine the amount of cell death in each clone apoptotic cells were counted in the same number of viewing fields.

To determine survival rates cells were seeded in 6-well plates and treated as described above. At the indicated time point the medium was aspirated, and floating cells were removed by washing twice with PBS. Attached cells were collected and counted. The survival rate is expressed in percentage of surviving cells in treated cells compared with the untreated control.

Flow Cytometry—After stimulation cells were detached from the plates and combined with the floating cells present in the growth medium, collected by low speed centrifugation, washed in PBS, and fixed in ice-cold methanol for 30 min. After washing in PBS, DNA was stained with propidium iodide (50 μg/ml) in the presence of 50 μg/ml RNase A for 30 min at room temperature.

The DNA content was analyzed using a FACSCalibur flow cytometer (Becton Dickinson).

Fluorescence Microscopy—Cells were seeded in 6-well plates containing 22-mm glass coverslips and treated with TNFα-CHX as described. At the indicated time point cells were fixed by adding 1 volume of 4% paraformaldehyde (in PBS) to the medium and stored for 16 h at 4 °C. Cells were washed in PBS and permeabilized with 0.1% Triton X-100 in PBS for 20 min at room temperature. Permeabilized cells were treated then with 10 μg/ml Hoechst 33258 in PBS (to stain the DNA) and Alexa™488-phalloidin (to visualize F-actin structures) for 45 min at room temperature. Nuclear and cellular morphology was then determined using a Carl Zeiss, Inc. fluorescence microscope with appropriate filters. Images of representative fields were taken with a digital imaging device.

Western Blots—Cell extracts were obtained in M2 lysis buffer (20 mM Tris, pH 7.6, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM β-glicerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin) (34) and equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidene difluoride membranes (Immo-

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[3] Qu, J., Cammarano, M. S., Shi, Q., Ha, K., de Lanerolle, P., and Minden, A., (2001) *Mol. Cell. Biol.* in press.

bilon P, Millipore Corp.), and incubated with antibodies as described in Ref. 14.

Protein Kinase Assays—Cell lysates and immunoprecipitations for kinase assays were performed as described in Ref. 14 using histone H4 as a substrate. For the detection of phosphorylated Bad, cells were lysed in M2 buffer (34), and equal amounts of proteins were used for immunoprecipitation. Kinase assays were then performed essentially as described in Ref. 35 with slight modifications: immunopurified proteins were washed four times in lysis buffer, once in ice-cold water, and once with Akt kinase buffer (20 mM Hepes, pH 7.4, 10 mM MgCl$_2$,10 mM MnCl$_2$; Ref. 35). Kinase reactions were performed in kinase buffer with 1 mM dithiothreitol and 5 μM ATP for 20 min at 30 °C in the presence of 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol). [γ-^{32}P]ATP was omitted when phosphorylation was examined by Western blotting. Reactions were stopped by the addition of SDS-

FIG. 1. **Expression and activity of PAK4 and PAK4(S445N,S474E) in HeLa and NIH3T3 stable cell lines.** Whole-cell lysates were prepared from HeLa stable cell lines containing either empty vector (*pLPC*) or HA-PAK4 wt (*left panels*) or NIH (*right panels*) stable cell lines containing empty vector (*pLPC*), Myc-PAK4wt, or Myc-PAK4(S445N,S474E). Equal amounts of lysates were analyzed for the expression of PAK4 proteins by Western blotting (*WB*) using anti-HA antibodies (*left panels*) or anti-Myc antibodies (*right panels*). To measure kinase activity, immunocomplex kinase assays were carried out using histone H4 (*HH4*) as a substrate. Phosphorylated histone H4 was detected by autoradiography after SDS-PAGE.

loading buffer, and denatured proteins were separated by SDS-PAGE. Gels were dried and exposed to x-ray films for the detection of radioactive-labeled proteins or processed for Western blotting.

RESULTS

Expression of PAK4 Results in a Delay in Caspase Activation and Apoptosis in TNFα/Cycloheximide-treated Cells—To examine the function of PAK4 in cell survival pathways NIH3T3 cells and HeLa cells were generated in which either wild-type PAK4, constitutively active PAK4(S445N,S474E),[3] or empty vector were stably expressed. Even wild-type PAK4 had kinase activity in these cells without prior stimulation by extracellular factors. The expression and activity of PAK4 in the stable cell lines are illustrated in Fig. 1. To examine cell survival in the stable cell lines HeLa cells were treated with TNFα and CHX to induce apoptosis. (CHX was used to block the NF-κB-mediated survival pathway induced by TNFα; Ref. 36.) As shown in Fig. 2*A*, in control cells, TNFα and CHX induced caspase activation within 2 h after treatment, as demonstrated by the partial cleavage of the caspase-3 substrate PARP. By 4 h PARP was cleaved almost completely. In contrast in cells expressing wild-type PAK4, PARP remained completely uncleaved 2 h after treatment and was cleaved only partly at 4 h, indicating that induction of caspase-3-like activity was delayed in the PAK4-overexpressing cells. At 2 h a significant proportion of the control cells displayed condensed nuclei indicating that they were undergoing apoptosis, as determined by Hoechst staining (see Fig. 2*B*). At this time point approximately five times more control cells had condensed nuclei as compared with the PAK4-expressing cells. At later time points many of the control cells had detached from the plate, and viability was assessed by survival assays in which the number of living cells that remained attached to the surface of the dish was determined after TNFα/CHX treatment. The survival assays revealed that ~5-fold more PAK4-expressing cells from two independent cell lines were surviving 6 h after TNFα and CHX treatment,
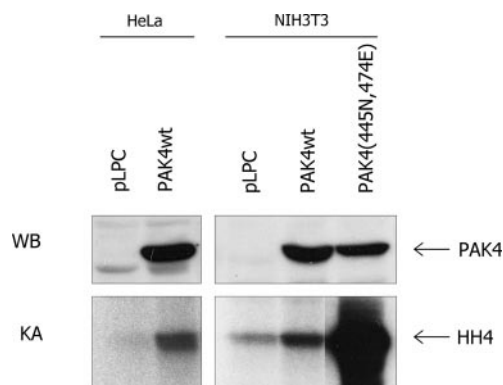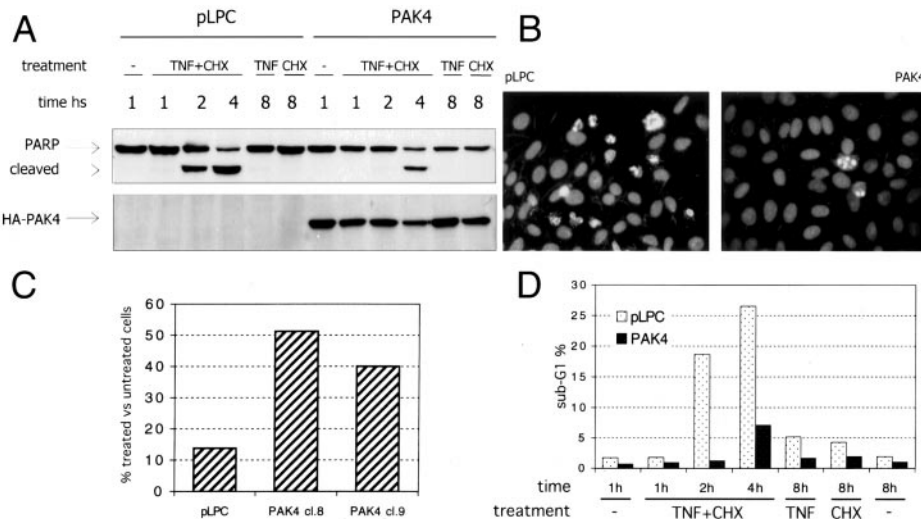
FIG. 2. **Stable cell lines expressing PAK4 are resistant to TNFα-induced apoptosis.** *A*, delay in caspase activation in PAK4 cells treated with TNFα: HeLa pLPC (control) and PAK4 clones were treated with TNFα (10 ng/ml) and CHX (10 μg/ml) alone or together as indicated or left untreated (−). Cells were harvested at the indicated number of hours. Whole-cell lysates were prepared and analyzed for PARP cleavage by immunoblotting with anti-PARP antibodies (*top panel*). Caspase activation is indicated by the appearance of the M_r 85,000 PARP cleavage product. The same lysates were probed with anti-HA antibodies (*bottom panel*) to visualize HA-PAK4 expression levels. *B*, nuclear condensation is reduced in TNFα-treated PAK4 clones. Nuclear condensation after 2 h of TNFα and CHX treatment was detected by fluorescence microscopy analysis of pLPC and PAK4 cells stained with Hoechst 33258. Alexa™488-phalloidin was used to stain F-actin structures to help visualize cell shape. Fields with representative numbers of apoptotic cells are shown. *C*, PAK4 cells show higher survival after TNFα treatment. HeLa pLPC cells and two independent PAK4 cell lines (PAK4 clones 8 and 9) were either treated with TNFα and CHX for 6 h or left untreated. Floating dead cells were washed away, and the cells that remained attached to the plates were trypsinized and counted. The graph shows (in percentage) the relative numbers of cells in the treated *versus* untreated samples. *D*, the sub-G$_1$ population is reduced in TNFα-treated PAK4 cells. HeLa pLPC (*light bars*) and PAK4 (*dark bars*) cells were treated with TNFα and CHX as described above, cells were collected at the indicated time points, fixed, and stained with propidium iodide, and DNA content was analyzed by flow cytometry. The percentage of cells displaying DNA content lower than the G$_1$ peak (*sub-G1%*) is shown in the graph.
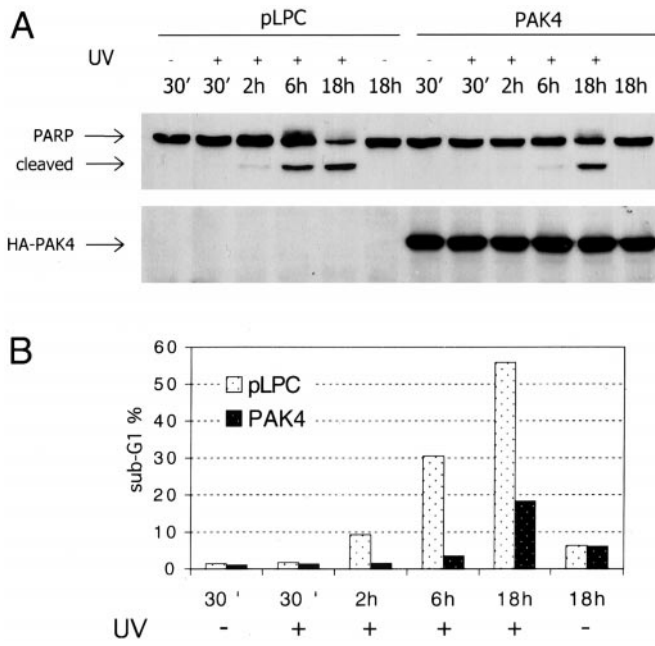
FIG. 3. **Stable cell lines expressing PAK4 are resistant to apoptosis induced by UV irradiation.** *A*, delay in caspase activation in UV-treated PAK4 cells. HeLa pLPC and PAK4 clones were exposed (+) to 50 J/m² UV radiation (*UV*) or left untreated (−) and harvested at the indicated time points. Whole-cell lysates were prepared and analyzed for PARP cleavage (*top panel*). PAK4 expression levels were assessed by Western blot analysis using anti-HA antibody (*bottom panel*) as described in Fig. 2*A*. *B*, the sub-G_1 population is reduced in UV-treated PAK4 cells. HeLa pLPC and PAK4 clones were UV-treated as described above and collected at the indicated time points. DNA content was analyzed by flow cytometry as described in Fig. 2*D*. The percentage of cells displaying DNA content lower than the G_1 peak (*sub-G1%*) is shown in the graph.

FIG. 4. **NIH3T3 cell lines expressing PAK4 are resistant to apoptosis induced by serum deprivation.** The sub-G_1 population is reduced in serum-deprived PAK4 cells. NIH pLPC (*light bars*), PAK4wt (*dark bars*), or PAK4(S445N,S474E) (*gray bars*) clones was cultured for 24 h in medium containing 10, 0.5, or 0.1% serum. Cells were collected, and DNA content was analyzed by flow cytometry as described in Fig. 2*D*. The percentage of cells displaying DNA content lower than the G_1 peak (*sub-G1%*) is shown in the graph.

compared with the control cells (Fig. 2*C*). Another assay for apoptosis is DNA fragmentation, which can be assessed by flow cytometry analysis of propidium iodide-stained cells. A significant proportion of control cells displayed a sub-G_1 DNA content after TNFα and CHX treatment, indicative of DNA fragmentation and apoptosis, whereas only a small percentage of PAK4-expressing cells displayed a sub-G_1 DNA content (Fig. 2*D*).

Delay in Apoptosis in UV-treated HeLa Cells—To see whether the PAK4 protective effect could be extended to other stimuli cell survival and apoptosis were analyzed after exposure of cells to UV irradiation. UV irradiation induced PARP cleavage in control cells by 6 h after exposure to UV, and near complete cleavage of PARP was observed by 18 h. In contrast in the PAK4-expressing cells there was no PARP cleavage after 6 h, and only partial cleavage was detectable after 18 h (see Fig. 3*A*). Thus similar to the TNFα-treated cells, caspase activation seemed to be delayed in the PAK4-expressing cells. Also similar to the TNFα-treated cells, the percentage of cells that displayed a sub-G_1 DNA content was significantly lower for the PAK4-expressing cells compared with control cells (Fig. 3*B*).

Delay in Apoptosis in Serum-deprived NIH3T3 Cells—Another trigger of apoptosis in many cells is serum withdrawal. Serum withdrawal induces apoptosis most likely by preventing Akt activation and thereby preventing Bad phosphorylation (37). To see whether PAK4 protects cells from serum withdrawal-induced apoptosis NIH3T3 cells expressing either empty vector, wild-type PAK4, or a hyperactive PAK4 mutant, PAK4(S445N,S474E)[3] were grown in either complete medium or medium containing low serum. DNA content was examined after 24 h. Significantly fewer cells expressing PAK4 wild type displayed a sub-G_1 DNA content compared with the control
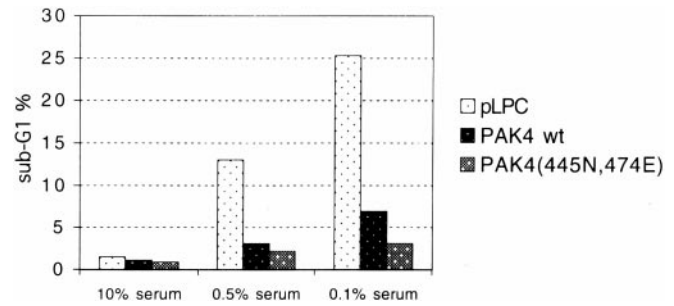
cells, and even fewer of the PAK4(S445N,S474E)-expressing cells had a sub-G_1 DNA content (Fig. 4).

TNFα-induced Dephosphorylation of Bad Is Delayed in Cell Lines Expressing PAK4—Activated PAK1 was shown recently to induce a protective effect against serum deprivation and cytokine withdrawal by phosphorylating the apoptotic regulatory protein Bad on serines 112 and 136. We found that the basal level of Bad phosphorylation on both serines 112 and 136 was higher in PAK4-expressing HeLa cells compared with control cells when the cells were grown in low serum (see Fig. 5, *left panels*). When cells were grown in the presence of 10% serum this difference was less noticeable because in this case the basal levels of Bad phosphorylation were quite high even in the control cells. However, even in the presence of serum a significant difference between the two HeLa clones was detected when the cells were treated with TNFα and CHX. When control cells were treated with TNFα and CHX Bad phosphorylation on both serines 112 and 136 decreased over time (Fig. 5, *right panels*), suggesting that as cells undergo apoptosis Bad phosphorylation decreases, cytochrome *c* is released, and caspase-3 is activated. In cells expressing PAK4, however, there was a significant delay in the decrease in Bad dephosphorylation on both sites, suggesting that one way that PAK4 can inhibit apoptosis is by causing an increase in Bad phosphorylation.

PAK4 Phosphorylates Bad—To analyze Bad phosphorylation by PAK4, additional cells were transfected with either empty vector or activated PAK4(S445N,S474E). *In vitro* kinase assays then were carried out in which recombinant Bad was used as a substrate for immunopurified PAK4. The results revealed that PAK4 phosphorylates Bad specifically on serine 112 (see Fig. 6). Phosphorylation of serine 136 that we observed in PAK4 cell lines therefore is more likely to be an indirect effect rather than direct phosphorylation by PAK4.

DISCUSSION

The PAK4 serine/threonine kinase was identified originally as a molecular target of the Rho GTPase Cdc42, and one function of PAK4 is to mediate the induction of filopodia in response to Cdc42 (14). Here we have shown that in HeLa and NIH3T3 cells, another function of PAK4 is to protect cells against apoptosis. We have found that overexpression of PAK4 protects cells against apoptosis induced by three different stimuli: serum withdrawal, TNFα/CHX treatment, and UV irradiation. PAK4 causes a delay in caspase activation, a corresponding delay in apoptosis, and an increased overall cell survival rate in response to these stimuli. The antiapoptotic function of PAK4 seems to be correlated with its kinase activity because a con-

stitutively active PAK4 mutant has a stronger protective effect than wild-type PAK4 (see Fig. 4), although even wild-type PAK4 has kinase activity and protects cells from apoptosis.

Although all members of the PAK family are thought to be involved in the apoptotic response, the different family members have quite different roles. During apoptosis γPAK and PAK2 are activated by caspase cleavage and are thought to be involved in promoting the apoptotic response because activated forms induce morphological changes that are typical of the onset of apoptosis (31–33, 38). In contrast PAK1 is not cleaved by caspases, and expression of activated PAK1 protects cells from apoptosis triggered by serum and interleukin-3 withdrawal (7, 8). We have found that PAK4 cleavage is not induced upon apoptotic stimulation and that it is not cleaved by caspase-3 *in vitro* (data not shown). Likewise, we have found that both wild-type and activated PAK4 protects cells from apoptosis. Not only does PAK4 protect cells from serum withdrawal, but it also protects cells from TNFα- or UV irradiation-induced apoptosis.

Because PAK4-overexpressing cell lines were resistant to apoptotic stimuli we have examined a number of different signaling pathways that have important roles in cell growth
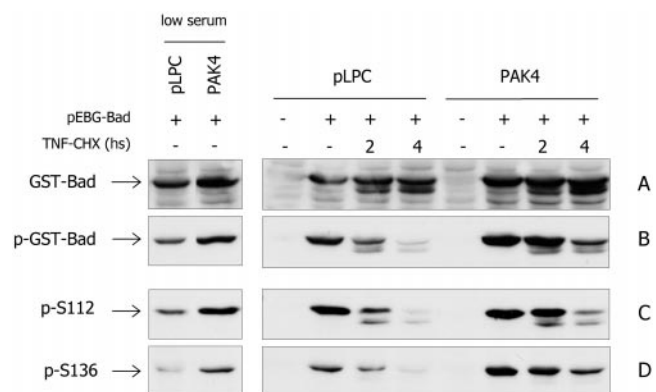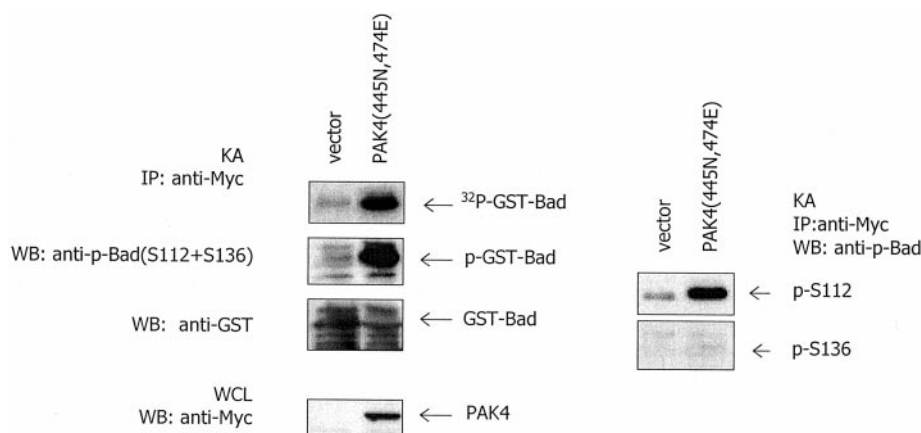
and apoptosis including the c-Jun NH$_2$-terminal kinase, p38, extracellular signal-regulated kinase, and NF-κB pathways (Refs. 39 and 40; data not shown). However, none of these pathways were significantly elevated or inhibited in the PAK4-overexpressing cells. In contrast phosphorylation of the pro-apoptotic protein Bad was elevated in the cell lines expressing PAK4. Phosphorylation of Bad on two critical sites, serines 112 and 136, inhibits its activity and therefore protects cells against apoptosis (5). A number of different stimuli including growth factors lead to phosphorylation of Bad via a pathway that requires the serine/threonine kinase Akt, which phosphorylates Bad on serine 136 (5). Recently PAK1 was shown to be activated by Akt and to phosphorylate Bad on both serines 112 and 136 (7, 8). In contrast to PAK1 we have found that PAK4 phosphorylates Bad specifically on serine 112 in *in vitro* kinase assays. In the stable PAK4-expressing cell lines, however, Bad phosphorylation on both serines 112 and 136 are increased. The reason for the increase in phosphorylation of serine 136 in the PAK4-expressing cell lines is not clear but is more likely to be an indirect effect rather than direct phosphorylation by PAK4. One possibility is that Akt may be responsible for Bad phosphorylation on this residue. In fact we have found that in contrast to PAK1-expressing cells (7, 8) cells expressing PAK4 show an increase in Akt phosphorylation (data not shown), suggesting a possible increase in Akt activity. Thus although expression of PAK4 leads to Bad phosphorylation on both serines 112 and 136, the two sites are most likely phosphorylated by different mechanisms. Although serine 112 can be phosphorylated directly by PAK4, serine 136 is most likely phosphorylated by an indirect mechanism.

Caspase activation triggered by either TNFα or UV irradiation is prevented or delayed in PAK4 cell lines, as detected by PARP cleavage. This is the first demonstration that caspase activity can be regulated by expression of a PAK protein. Because PAK4 phosphorylates Bad the most likely explanation for this is that PAK4 prevents the release of cytochrome *c* from the mitochondria by inhibiting Bad activity and thereby inhibits the activation of caspase-3, an effector caspase in this pathway. Another possibility, however, is that initiator caspases are affected. Death ligands such as TNFα activate initiator caspases such as caspase-8, which in turn can trigger apoptosis by either mitochondria-dependent or -independent mechanisms (41). Therefore protection from TNFα-induced cell death by PAK4 could also potentially occur at the level of initiator caspases such as caspase-8 by a mechanism that does not strictly require Bad phosphorylation. Although the mechanism by which TNFα induces apoptosis is relatively well understood (41), the molecular events that mediate UV irradiation-induced apoptosis are quite complex. UV irradiation can stimulate

FIG. 5. **TNFα induced activation of Bad is delayed in cell lines expressing PAK4.** *Left panels*, levels of phospho-Bad in low serum: HeLa pLPC and PAK4 clones, transfected with GST-Bad expression vector (*pEBG-Bad*), were cultured in 0.5% serum for 24 h. Equal amounts of cell extracts were analyzed by Western blotting with antibodies directed against Bad that is phosphorylated on Ser-112 or Ser-136 either together (*B*) or separately (*C* and *D*, respectively). GST-Bad expression levels were detected with anti-GST antibodies (*A*). *Right panels*, TNFα-induced dephosphorylation of Bad is delayed in PAK4 cells. HeLa pLPC and PAK4 clones were transfected with a control plasmid (−) or GST-Bad expression vector (+). After 36 h, cells were treated with TNFα and CHX (*TNF-CHX*) for the indicated number of hours or left untreated (−). Equal amounts of protein cell extracts were analyzed for Bad phosphorylation by Western blotting as described above.

FIG. 6. **PAK4 phosphorylates Bad on serine 112.** HeLa cells were transfected either with empty vector or Myc-PAK4(S445N,S474E), as indicated, and equal amounts of cell extracts were used for *in vitro* kinase assays (*KA*): immunoprecipitates (*IP*) were used in kinase reactions with recombinant GST-Bad as a substrate. After SDS-PAGE, phosphorylated Bad was detected by autoradiography (*32P-GST-Bad*) or by Western blotting (*WB*) using phospho-specific antibodies (*anti-p-Bad*) recognizing residue Ser-112 (*p-S112*) or Ser-136 (*p-S136*) separately or combined (*S112+S136*). Equal amounts of substrate (*GST-Bad*) were used in each reaction, as detected with anti-GST antibodies. PAK4(S445N, S474E) levels were detected in whole-cell lysates (*WCL*) by Western blotting with anti-c-Myc antibodies.

apoptosis by triggering the activation of death receptors such as TNF receptor and thereby activate a pathway similar to the one described above for TNFα (42). However, UV can also induce apoptosis by other mechanisms such as by DNA damage-dependent pathways (43, 44). Thus although we have found that PAK4 inhibits caspase cleavage induced by UV irradiation, the exact role for PAK4 in UV-induced apoptosis remains to be fully clarified.

The finding that PAK4 can cause a protective effect in cells is especially interesting because PAK4 is a target for Cdc42. Cdc42 and other members of the Rho GTPase family have important roles in the regulation of cell growth, proliferation, and oncogenic transformation (45–49). Recently we have found that constitutively active PAK4(S445N,S474E) can induce anchorage-independent growth[3] an important hallmark of oncogenic transformation similar to activated mutants of Cdc42 (50–52). Furthermore, a dominant negative PAK4 mutant can inhibit transformation by oncogenic Dbl, an exchange factor for Cdc42[3] These results suggest that similar to Cdc42, PAK4 also has an important role in oncogenic transformation. The mechanisms by which PAK4 can regulate oncogenic transformation are not entirely clear. Although changes in cytoskeletal architecture and cell adhesion are likely to be involved,[3] another important aspect of transformation is the inhibition of apoptosis. It will be interesting to determine whether the antiapoptotic function of PAK4 plays a direct role in the oncogenic process.

The regulation of apoptosis also has important implications in normal development, where extra cells are being discarded continuously. This process is especially pronounced during neuronal development. It is interesting that the closest known homologue to PAK4 is a *Drosophila* protein called Mushroom Body Tiny (MBT; Ref. 53). *Drosophila* lacking the *mbt* gene show a reduced number of Kenyon cells in the mushroom body, a structure in *Drosophila* brain. These studies suggest that MBT has a role in promoting either proliferation or survival of cells in the mushroom body. Although PAK4 is ubiquitously expressed, it will be interesting to see whether PAK4 and MBT share a common role in regulating cell survival either in neuronal or nonneuronal cells.

Finally, the extracellular stimuli that regulate the activity of endogenous PAK4 are not yet known. Although PAK4 is a target for Cdc42, we do not rule out the possibility that it can also be regulated by Cdc42-independent stimuli. Other members of the PAK family can be regulated in fact by different types of stimuli and can be activated by Rho GTPase-dependent and -independent mechanisms (15, 16). In future work it will be important to determine what types of stimuli can activate kinase activity in endogenous PAK4, and specifically whether it is activated by stimuli that have a protective effect against apoptosis.

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