PAK4 Functions in Tumor Necrosis Factor (TNF) α-induced Survival Pathways by Facilitating TRADD Binding to the TNF Receptor*

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PAK4 is a member of the group B family of p21-activated kinases. Its expression is elevated in many cancer cell lines, and activated PAK4 is highly transforming, suggesting that it plays an important role in tumorigenesis. Although most previous work was carried out with overexpressed PAK4, here we used RNA interference to knock down endogenous PAK4 in cancer cells. By studying PAK4 knockdown HeLa cells, we demonstrated that endogenous PAK4 is required for anchorage-independent growth. Because cell survival is a key part of tumorigenesis and anchorage-independent growth, we studied whether PAK4 has a role in protecting cells from cell death. To address this, we studied the role for PAK4 downstream to the tumor necrosis factor (TNF) α receptor. Although overexpressed PAK4 was previously shown to abrogate proapoptotic pathways, here we demonstrate that endogenous PAK4 is required for the full activation of prosurvival pathways induced by TNFα. Our results indicate that PAK4 is required for optimal binding of the scaffold protein TRADD to the activated TNFα receptor through both kinase-dependent and kinase-independent mechanisms. Consequently, activation of several prosurvival pathways, including the NFκB and ERK pathways, is reduced in the absence of PAK4. Interestingly, constitutive activation of the NFκB and ERK pathways could compensate for the lack of PAK4, indicating that these pathways function downstream to PAK4. The role for PAK4 in regulating prosurvival pathways is a completely new function for this protein, and the connection between PAK4 and cell survival under stress helps explain its role in tumorigenesis and development.

Tumor necrosis factor (TNF)α was originally discovered as an anticancer cytokine that can induce apoptosis in certain tumor cells (1). TNFα was also shown to play important roles in regulating cell proliferation and differentiation, inflammatory responses, and immune functions (1–3). The mechanisms by which different signaling transduction pathways activated by TNFα interact with each other are only recently being defined.

It is well established that binding of TNFα to the TNFR1 (TNFα receptor 1) on the cell membrane leads to activation of prosurvival pathways followed by proapoptosis pathways. According to recent models (4), the prosurvival pathways are activated by a rapid recruitment of a protein complex, known as complex I, to the cytosolic portion of the activated TNFR1. Formation of complex I, including TNFR1, TRADD, RIP, and TRAF2 proteins, leads to activation of the NFκB pathway, as well as mitogen-activated protein kinase pathways such as the ERK, JNK, and p38 pathways (3). The NFκB pathway is considered to be the major prosurvival pathway (5).

The proapoptosis pathways are activated by a second complex, known as complex II or DISC (death-inducing signaling complex), which includes TRADD, RIP, and FADD proteins (4). The molecular mechanism by which complex I transitions to complex II is still not clear, and it is not certain whether TNFR1 is even included in complex II (4, 6). FADD is the essential component of this complex. FADD can recruit and activate the apoptosis initiators caspase 8 and 10, leading to the activation of two different apoptosis pathways (7). The intrinsic mitochondria-independent apoptosis pathway is activated through directly cleavage and activation of executor caspases 3 and 7 by caspases 8 and 10. Activated caspases 3 and 7 then regulate the activities of target proteins that play important roles in various aspects of apoptosis (8).

The intrinsic mitochondria-dependent apoptosis pathway is mediated by cleavage and activation of the Bcl-2 family protein BID by activated caspase 8 (9). The resulting cleaved BID translocates to mitochondria, where it interacts with other Bcl-2 family members to promote cytochrome c release (10). Released cytochrome c leads to activation of caspase 9, followed by cleavage and activation of caspase 3 and apoptosis (9, 11).

Upon activation of the TNFα receptor, the cell responds either by undergoing apoptosis or by activating survival pathways. For the cell to survive, full activation of the survival pathways triggered by complex I is critical. Activation of the NFκB pathway leads to increased expression of several antiapoptotic proteins such as FLIP and c-IAP, which can bind to complex II. If the NFκB pathway is fully activated and sufficient amounts of FLIP and C-IAP are presented in complex II, the activation of caspase 8 will be blocked, and the cell will survive (4). Because the NFκB-mediated survival depends on the production of new proteins, it is disrupted by drugs such as the translation inhibitor cycloheximide (CHX). The combination of TNFα and CHX therefore favors activation of the apoptosis pathway, leading to cell death (4).

The p21-activated kinase family of serine/threonine kinases were originally identified as targets for the Rho GTPases Cdc42 and Rac (12). Although originally identified as proteins that regulate cell morphology (13), the six mammalian PAKs have also been shown to play important roles in regulating cell survival and apoptosis. For example, apoptotic stimuli can induce rapid activation of full-length PAK2 to promote cell survival (14). At later time points, PAK2 is also cleaved, probably by caspase 3, and the resulting activated fragment is associated with DNA fragmentation and membrane changes that occur during apoptosis (15).
PAK1, PAK4, and PAK5 were also reported to protect cells from apoptosis by directly phosphorylating the proapoptotic protein Bad (16, 18, 19), which prevents cytochrome c release in the mitochondrial pathway (20).

Along with PAK5 and PAK6, PAK4 is a member of the group B family of PAKs. PAK4 has important roles in embryonic development, and deletion of PAK4 in mice leads to embryonic lethality, along with defects in both cardiac and neural development (21). In cultured cells, PAK4 regulates cell adhesion, cytoskeletal organization, and transformation (22, 23). Overexpression of wild type or activated PAK4 was shown to protect cells from apoptosis induced by UV irradiation and serum withdrawal. This is associated with direct phosphorylation and inactivation of BAD by PAK4 (19). Overexpressed PAK4 also protects cells from apoptosis induced by a fusion of TNFR1 and the Fas receptor. In this case the protective response appears to be kinase-independent and is associated with an abrogation of the recruitment and activation of caspase 8 to DISC (24).

Although PAK4 is expressed at low levels in most normal tissues, PAK4 mRNA levels are greatly up-regulated in many cancer cell lines from various origins (25). This led us to study whether PAK4 is important for oncogenesis and cell survival in cancer cells. Whereas our previous work was carried out in cell lines that overexpress exogenous PAK4, here we took advantage of RNAi technology to knock down endogenous PAK4 expression in a cancer cell line that expresses high levels of PAK4. We found that endogenous PAK4 is required for anchorage-independent growth and for the full activation of the survival pathways induced by TNFα in HeLa cells. Specifically, we found that PAK4 is required for optimal binding of TRADD to the activated TNFα receptor. The kinase activity is not absolutely required but can further enhance the interaction between TNFα and TRADD. Consequently, activation of the NFκB pathway by TNFα was reduced in the absence of PAK4, as well as the ERK and JNK pathways. Interestingly, constitutive activation of the NFκB and ERK pathways could over-ride the loss of PAK4 and restore cell survival, indicating that these pathways operate downstream to PAK4. Thus, PAK4 acts not only as a direct inhibitor of the proapoptotic pathway, but it is also required for the activation of the survival pathways. Because increased cell survival is an important part of oncogenesis, our results help explain why PAK4 is highly transforming and why its expression is associated with oncogenic transformation.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum. All of the media were supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin, and 4 mM glutamine. 1.5 μg/ml puromycin was added for stable cell lines. Transient transfection assays were carried out in HeLa cells using the calcium phosphate precipitation or Lipofectamine method.

Construction of PAK4 Knockdown Stable Cell Lines—Two small interfering RNA (siRNA) oligonucleotides were synthesized to target two different regions in the PAK4 cDNA: PAK4-RNAi-1 targeting a linker region between the regulatory domain and the kinase domain AACCTCATCAAGATTGCGGAG and PAK4-RNAi-2 targeting a sequence within the kinase domain AACGAGGCTGTAATCATGAGG. Transient transfection of both siRNAs could disrupt PAK4 expression in HeLa cells, whereas a scrambled double-stranded RNA did not affect PAK4 expression.

pSuper vector was kindly provided by the lab of Dr. Ron Prywes, and 64-mer oligonucleotide DNA nucleotides targeting the same region as PAK4-RNAi-1 were designed according to the specifications recommended (26) and synthesized by Invitrogen. pSuper-PAK4 was constructed by ligation of the annealed nucleotides into the BglII and HindIII sites of the pSuper vector. Stable cell lines were made by co-transfection of pSuper or pSuper-PAK4 together with the pLPC vector (containing a puromycin-resistant gene) into HeLa cells using the calcium phosphate precipitation method. The cells were selected with puromycin (1.5 μg/ml), and the colonies were picked ~2 weeks after selection. 20 clones from both transfections were picked, and expression of PAK4 was determined by Western blot. The expression of PAK4 was successfully knocked down in 4 of the 20 clones from the pSuper-PAK4 transfection.

Reagents and Antibodies—Human recombinant TNFα and goat polyclonal anti-TNFRI antibody were from R & D Systems. CHX was from Sigma. Mouse anti-human PARP, anti-phospho-SAPK/JNK (Thr185/Tyr185) (G9), anti-phospho-p44/42 (Thr202/Tyr204) E10, anti-BID, and anti-caspase 8 (1C12) antibody were from New England Biolabs. Mouse anti-TNFFR1 (H-5), rabbit anti-IκBα (C-21), anti-IKKα, and anti-ERK2 antibody were from Santa Cruz; mouse anti-TRADD (clone 37) and anti-RIP (clone 38) were from BD Transduction Labs. Rabbit anti-phosphorylated IκBα (pS32pS36) was from BIOSOURCE International. Mouse anti-human JNK1 antibody was from Pharmingen. Secondary antibodies conjugated to horseradish peroxidase were from Sigma.

Growth Curve—To estimate the growth rate of stably transfected cell lines, equal numbers of each stable cell line were seeded in growth medium in six-well plates. Each day after the seeding, one set of cells was collected using trypsin and counted. Each point on the curve is the average of three duplicates.

Flow Cytometry—After UV or TNFα + CHX treatment, both floating and attached cells were collected by low speed centrifugation, washed in PBS, and fixed in ice-cold methanol for overnight. The cells were then stained with propidium iodide (50 mg/ml) in the presence of 50 mg/ml RNase A for 30 min at room temperature. The DNA content indicated by propidium iodide staining was analyzed using a FACSCalibur flow cytometer (Becton Dickinson).

Apoptosis Assay—For the apoptosis assay, equal numbers of cells were seeded in 6-cm plates. For TNFα + CHX treatment, the cells were washed once with PBS, and then medium containing 10 ng/ml TNFα and 10 μg/ml CHX either alone or together were added. For UV irradiation, the cells were washed twice with PBS and then exposed to 50 J/m² UV light in a UV cross-linker (Fisher) followed by the addition of fresh medium. After stimulation the cells were collected at the indicated time points (both attached and floating dead cells unless otherwise indicated). Two different methods were used to quantify the apoptosis level in the treated cells: quantitation of the sub-G1 population using FACS analysis and the detection of the 85-kDa proteolytic product of PARP by Western blot. The percentage of the PARP cleavage was quantified using NIH Image J.

Soft Agar Assay—2 ml of 0.6% Baco agar in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, antibiotics, and glutamine was plated into six-well plates. Stable cell lines suspended in 2 ml of 0.3% Baco agar in the same medium were seeded into these six-well plates at 5,000 or 10,000 cells/well. Each cell line was tested in duplicates. After 2–3 weeks, the colonies were visualized under an inverted light microscope. Digital pictures were also taken for each well, and the number and size of the colonies were measured using NIH Image J.

TdT-mediated dUTP Nick End Labeling Assays—Wild type and PAK4 knockout embryos were obtained as shown previously (21). TdT-mediated dUTP nick end labeling staining was performed on paraffin-embedded sections using an ApopTag peroxidase in situ
apoptosis detection kit (Integrin), according to the manual from the manufacturer.

**Immunoprecipitation**—The immunoprecipitation protocol was adapted from those as described previously (24, 27). In brief, the cells were washed by cold PBS twice before being lysed in lysis buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA), supplemented with proteinase and phosphatase inhibitors (2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotonin, 20 mM β-glycerophosphate, 1 mM Na3VO4). The cell lysates were collected, rotated at 4 °C for an hour, and cleaned by centrifugation to obtain whole cell extracts. For immunoprecipitation, 50 μl of protein G-agarose slurry (Santa Cruz) preloaded with antibodies or goat serum was added to equal amounts of cell extracts and rotated overnight at 4 °C. The immune complexes were precipitated by centrifugation, washed two times with lysis buffer, then washed one time with lysis buffer plus 250 mM NaCl and then washed two times again with lysis buffer. The precipitated proteins were denatured in SDS loading buffer, separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and analyzed by Western blotting. The results of the Western blots were quantified using NIH Image J.

For the PAK4 rescue experiments, control and PAK4 knockdown (RNAi) cells were left untreated (0 min) or treated with TNFα+CHX for 5 min. Equal amounts of cell lysate were combined with equal amount of lysate from HeLa cells stably overexpressing hemagglutinin-tagged wild type PAK4 (PAK4WT) (19), Myc-tagged kinase-dead PAK4 (PAK4KM) (24), or Myc-tagged constitutively active PAK4 (PAK4NE) (19). Equal amounts of the cell lysates were then incubated with protein G-agarose beads loaded with anti-TNFR1 antibody. TRADD protein bound to the TNFR1 was analyzed by Western blot and was also quantified using NIH Image J. The expression levels of TRADD protein in lysates from the HeLa cells stably overexpressing wild type PAK4 (PAK4WT), kinase-dead PAK4 (PAK4KM), or constitutively active PAK4 (PAK4NE) and from the control and PAK4-RNAi cells was assessed by Western blot. The blots were also probed with PAK4 antibody to show the expression of endogenous PAK4, as well as hemagglutinin-tagged PAK4WT, Myc-tagged PAK4NE, and Myc-tagged PAK4KM.

**Western Blot Analysis**—Western blots were carried out as described in Ref. 28. The results of the Western blots were quantified using NIH Image J.

**RESULTS**

**PAK4 Is Required for Anchorage-independent Growth in HeLa Cells**—Overexpression of activated PAK4 has been shown to induce anchorage-independent growth on soft agar (23, 25). To determine whether endogenous PAK4 is also required for anchorage-independent growth in cancer cell lines, we used siRNA to knockdown PAK4 expression in HeLa cells. HeLa cells were chosen because they are an example of a cancer cell line (derived from a cervical carcinoma) expressing high

![FIGURE 1. PAK4 is required for anchorage-independent growth in HeLa cells. A, Western blot analysis shows PAK4 levels in control and HeLa cells transiently expressing two siRNA oligonucleotides targeting two different regions in PAK4. Both siRNA sequences knocked down PAK4 expression in HeLa cells, whereas a scrambled double-stranded RNA did not affect PAK4 expression. B, Western blot analysis showing PAK4 levels in control and PAK4 knockdown stable cell lines: The expression of PAK4 was successfully knocked down in cells stably transfected with pSuper-PAK4, as shown by Western blot. Expression of PAK1 and PAK2 were not affected. The pSuper vector alone (V) does not affect the expression of PAK4. The same blot was probed with anti-actin antibody as a loading control. C, anchorage-independent growth is abrogated in PAK4 knockdown cells: Soft agar assays were carried out to test for anchorage-independent growth in parental HeLa cells, control cells (transfected with empty vector), and PAK4 knockdown (RNAi) cells (clones 1 and 20). Sample pictures of colonies are shown. PKA knock down stable cells formed fewer and smaller clones on soft agar after 2 weeks. D, PAK4 knockdown cells grow at the same rate as control cells. Growth curves were carried out to plating equal numbers of cells on plastic dishes. Parental HeLa cells, control cells (transfected with empty vector), and PAK4 knockdown cells (clones 1 and 20) were counted each day after plating. PAK4 knockdown cells had a normal growth rate compared with the control cells. E, PAK4 knockdown cells had the same cell cycle progression profile compared with the control cells. FAC analysis was carried out to quantitate the number of cells in the different phases of the cell cycle. The cell cycle profile for PAK4 knockdown cells was similar to that of control cells.**
levels of PAK4 (25). Two siRNAs were synthesized to target two different regions in the PAK4 cDNA: PAK4-RNAi-1 (AACCTTCATCAA-GATTGGCCGAG) targeting a linker region between the regulatory domain and kinase domain and PAK4-RNAi-2 (AACGAGGTGGTA-ATCATGAGG) targeting a sequence within the kinase domain. Transient transfection of both siRNAs could disrupt PAK4 protein expression in HeLa cells, whereas a scrambled double-stranded RNA did not affect PAK4 expression, as shown by Western blot in Fig. 1A.

An expression vector (pSuper-PAK4) targeting the same region as PAK4-RNAi-1 was constructed and used to generate stable cell lines. Expression of PAK4 in control (empty vector alone) or PAK4 knockdown (pSuper-PAK4) HeLa cell lines was assessed by Western blot. As shown in Fig. 1B, the expression of PAK4 was successfully knocked down in cells containing pSuper-PAK4 (subsequently referred to as PAK4 knockdown cells), whereas the control pSuper vector alone did not affect PAK4 expression. PAK1 and PAK2 expression levels were not affected by the PAK4 RNAi (Fig. 1B).

Wild type HeLa cells, control stable cell lines, and PAK4 knockdown cells were plated on soft agar. After 2 weeks, PAK4 knockdown stable cells formed significantly fewer and smaller colonies on soft agar compared with control cells, as shown in Fig. 1C and as quantified in TABLE ONE. These data indicate that PAK4 plays an essential role in the anchorage-independent growth of HeLa cells. To determine whether the decreased growth in soft agar was due to an overall decrease in anchorage-independent growth of HeLa cells, whereas a scrambled double-stranded RNA did not affect PAK4 expression, as shown by Western blot in Fig. 1A.

### TABLE ONE

|                | Total colonies | Distribution of colony size |
|----------------|----------------|----------------------------|
|                |                | 1–5 | 5–10 | 10–15 | >15 |
| Control        | 187            | 73  | 64   | 28    | 22  |
| PAK4-RNAi Clone 1 | 53            | 36  | 15   | 2     | 0   |
| PAK4-RNAi Clone 20 | 18            | 16  | 2    | 0     | 0   |

PAK4 knockdown HeLa Cells Are More Sensitive to Both UV- and TNFα-induced Apoptosis.—PAK4 knockdown cell lines formed fewer and smaller colonies on soft agar, whereas their growth rates were normal; one possible explanation for this is that PAK4 knockdown cells are more sensitive to apoptosis. Overexpression of PAK4 was previously shown to protect cells from apoptosis (24). Furthermore, depletion of PAK4 in mice leads to a pronounced increase in apoptosis in certain parts of the PAK4 knockout embryos (21). This suggests that PAK4 is required for protecting cells from apoptosis in some tissues during development.

To determine whether PAK4 is also required for protecting cancer cells from apoptosis, knockdown and control cells were treated with either TNFα+CHX or UV irradiation. Results from FACS analysis indicate that there were more sub-G₁ (apoptotic) cells in the PAK4 knockdown cells compared with the control cells after 4 h of TNFα+CHX treatment (Fig. 2A). Likewise, PAK4 knockdown cells were also more sensitive to UV-induced apoptosis (Fig. 2A). We also analyzed another apoptosis indicator, the cleavage of PARP protein by the executor caspase 3. Western blot analysis indicates that PARP cleavage occurred earlier in the PAK4 knockdown cells, as indicated in Fig. 2B. The cleavage states of apoptosis initiator caspase 8 and its downstream effector BID were also analyzed by Western blot. As shown in Fig. 2C, both caspase 8 and BID were cleaved and activated earlier in PAK4 knockdown cells compared with the control cells. Our results indicate that the absence of PAK4 leads to an earlier onset of apoptosis, indicating that PAK4 plays an essential and early role in protecting cells from cell death.

### Activation of NFκB, ERK, and JNK Pathways by TNFα Are Reduced by the Absence of PAK4—In HeLa cells, TNFα treatment can activate survival pathways so that apoptosis is blocked. Activation of survival signals depends on the transcription and translation of new proteins. Therefore, if the survival pathways are disrupted by translation inhibitor CHX, the apoptosis pathway predominates, and the cell dies (4). Interestingly, we found that when treated with TNFα alone for 24 h, the PAK4 knockdown cells underwent apoptosis even in the absence of CHX, whereas control cells, as expected, did not undergo apoptosis in the absence of CHX (Fig. 3A). These results suggest that one way PAK4 may protect cells from apoptosis is by activating the survival pathway rather than merely blocking the proapoptotic pathway. Activation of the survival pathway is triggered by recruitment of complex I to the TNFR1 (4). In response to formation of this complex, the NFκB pathway is activated, along with other pathways, including the ERK, JNK, and p38 signaling pathways (3). Interestingly, we found that activation of three of these pathways, ERK, JNK, and NFκB, were reduced in PAK4 knockdown cells (Fig. 3B–D), whereas the activation of p38 pathway was not significantly affected by the absence of PAK4 (data not shown).

### Constitutive Activation of the NFκB and ERK Pathways Can Rescue the Sensitivity to Apoptosis That Is Induced by the Absence of PAK4—Among the three pathways affected by the absence of PAK4, the NFκB pathway is the major survival pathway to antagonize apoptosis in cells treated with TNFα (5). The ERK pathway, however, also has a well-established role in cell proliferation and survival (29), and although JNK has different roles depending on the cell type, it was shown to be antiapoptotic in tumor cells (30, 31). To determine whether any of these pathways have an essential role in PAK4-mediated survival, they were restored in the knockdown cells by expression of constitutively active mutants. A constitutively activated Iκκα (EM) mutant (32) and a constitutively active Raf (Raf CAAX) mutant (33) were introduced into the PAK4 knockdown cells and control cells to activate the NFκB or ERK signaling pathways, respectively, followed by treatment of TNFα+CHX (1KKκα (EM) has both EE mutants (S177E, S181E) and M10 mutations, which have 10 alanine mutations in the C-terminal serine cluster of the protein). As shown in Fig. 4, constitutive activation of either the NFκB or ERK signaling rescued the sensitivity to TNFα-induced apoptosis in the PAK4 knockdown cells. Activation of the JNK pathway, however, via expression of activated JNKK-JNK, did not rescue the apoptosis-sensitive phenotype (data not shown). These results suggest that the ERK and...
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FIGURE 2. PAK4 knockdown cells are more sensitive to both UV- and TNFα+CHX-induced apoptosis. A, quantitation of the sub-G1 population after TNFα + CHX or UV treatment. Control and PAK4 knockdown (RNAi) cells were treated with TNFα + CHX or UV to induce apoptosis. The cells were collected 4 h after treatment and then fixed and stained with propidium iodide. DNA content was analyzed by FACS flow cytometry. The percentage of the apoptotic cells (sub-G1 population) in response to UV to induce apoptosis. The percentage of the apoptotic cells (sub-G1 population) in response to TNFα treatment is shown. PAK4 knockdown cells were more sensitive to both UV and TNFα + CHX induced apoptosis. B, PARP cleavage occurs earlier in PAK4 knockdown cells compared with control cells, following TNFα + CHX treatment. Control and PAK4 knockdown (RNAi) cells were left untreated (0 h) or treated with TNFα + CHX for 2, 3, or 4 h to induce apoptosis. Whole cell lysates were then analyzed by Western blot. The same blot was probed with anti-actin antibody as a loading control. The cleavage of PARP in three experiments were mixed with cell lysate from either control or PAK4-RNAi cells before pulling down TNFR. As shown in Fig. 5, PAK4 knockdown stable cell lines and control cell lines. TRADD binding to the activated TNF receptor was immunoprecipitated from both PAK4 knockdown stable cell lines and control cell lines. TRADD binding to the activated TNF receptor was then analyzed by Western blot. As shown in Fig. 6, PAK4WT and PAK4NE both increased the binding of TRADD to TNFR in PAK4-RNAi cells. PAK4NE increased binding even more than wild type PAK4, even though it was expressed at a much lower level. Interestingly, however, even PAK4KM led to a slight increase in TRADD binding. Our results suggest that PAK4 can facilitate TRADD binding to TNFR1 via both kinase-independent and kinase-dependent mechanisms, although binding is enhanced when a kinase active PAK4 is used.

DISCUSSION

PAK4 was first identified for its role in regulating the organization of the actin cytoskeleton (22). Recent work indicates that it also has important roles in oncogenesis. PAK4 is overexpressed in cancer cells (25), and we previously showed that its overexpression promotes cell survival (19) as well as anchorage-independent growth (23), important hallmarks of oncogenic transformation. Although previous work relied largely on overexpression studies, here we used siRNA technology to block endogenous PAK4. We found that PAK4 is actually required for anchorage-independent growth in a tumor cell line. Furthermore, we found that blocking PAK4 made the cells hypersensitive to apoptosis. However, rather than affecting only proapoptotic pathways, as suggested by overexpression studies (19), our results indicate that endogenous PAK4 is required for activation of TNFα-induced survival pathways. Specifically, PAK4 is required for normal binding of TRADD to the TNFR1. TRADD is a key component of complex I, which leads to activation of several distinct signaling pathways, including the NFκB, ERK, and JNK pathways. This suggests that PAK4 functions upstream from all of these pathways. The pathways indicated above are all activated rapidly in response to recruitment of plasma membrane-bound complex I (which includes TRADD, RIP, and TRAF2) to the activated TNF receptor. Our results suggest that PAK4 may function early in the TNFα pathway, possibly by affecting the recruitment of components of complex I to the TNFα receptor. To test this, the TNFα receptor was immunoprecipitated from both PAK4 knockdown stable cell lines and control cell lines. TRADD binding to the activated TNFα receptor was then analyzed by Western blot. As shown in Fig. 6, PAK4WT and PAK4NE both increased binding of TRADD to TNFR1. Our results suggest that PAK4-RNAi cells (p43/p41 and p18 are cleaved forms of caspase 8).

NFκB pathways function downstream from PAK4 to antagonize the apoptosis pathway.

The Binding of TRADD to the TNFR1 Was Attenuated in PAK4 Knockdown Cells—Our results indicate that the absence of PAK4 affects the activation of several distinct signaling pathways, including the NFκB, ERK, and JNK pathways. This suggests that PAK4 functions upstream from all of these pathways. The pathways indicated above are all activated rapidly in response to recruitment of plasma membrane-bound complex I (which includes TRADD, RIP, and TRAF2) to the activated TNF receptor. Our results suggest that PAK4 may function early in the TNFα pathway, possibly by affecting the recruitment of components of complex I to the TNFα receptor. To test this, the TNFα receptor was immunoprecipitated from both PAK4 knockdown stable cell lines and control cell lines. TRADD binding to the activated TNFα receptor was then analyzed by Western blot. As shown in Fig. 6, PAK4WT and PAK4NE both increased binding of TRADD to TNFR in PAK4-RNAi cells. PAK4NE increased binding even more than wild type PAK4, even though it was expressed at a much lower level. Interestingly, however, even PAK4KM led to a slight increase in TRADD binding. Our results suggest that PAK4 can facilitate TRADD binding to TNFR1 via both kinase-independent and kinase-dependent mechanisms, although binding is enhanced when a kinase active PAK4 is used.
turn promotes the proapoptosis pathway and inhibits the survival pathway (34). Because of this positive feedback mechanism, a small amount of TRADD binding to the TNFR1 may be sufficient for activation of apoptosis yet not sufficient to fully activate the survival pathways. It is still not clear how PAK4 functions in the formation of complex I. One possibility is that PAK4 itself is a part of the complex. Although PAK4 did not co-immunoprecipitate with either TNFR or TRADD antibody (data not shown), it remains possible that PAK4 can bind to...
other unknown proteins in the complex to facilitate TRADD binding. Another possibility is that one or more of the proteins in the complex, or proteins that may facilitate formation of the complex, need to be phosphorylated by PAK4. Our results suggest that PAK4 can operate by both mechanisms. The fact that kinase-dead PAK4 can promote a slight increase in the binding of TRADD to TNFR indicates that this process is not completely dependent on the kinase activity of PAK4. However, the fact that wild type and constitutively activated PAK4 further increased TRADD binding to TNFR implies that an additional mechanism requiring PAK4 kinase activity of PAK4 can also further strengthen TRADD binding to TNFR. These results are consistent with previous results in which we have found that PAK4 can have both kinase-dependent and kinase-independent functions in cell survival (19, 24). Furthermore, members of the group A PAKs have also been shown to have both kinase-dependent and kinase-independent functions (35).

Following complex I formation at the TNFR1, the NFκB pathway, which is the major survival pathway, is activated. NFκB promotes survival by promoting the expression of genes encoding many anti-apoptotic proteins such as FLIP and IAP to protect cells from apoptosis (5). In addition to the NFκB pathway, other pathways, including the ERK, JNK, and p38 pathways, are also activated downstream to complex I (3). Interestingly, the NFκB, ERK, and JNK pathways were all abrogated in TNFα-treated PAK4 null cells. This suggests that PAK4 functions at a level upstream to all of these pathways during cell survival. This is consistent with our finding that PAK4 is required for binding of TRADD to the TNFR1 and hence the formation of complex I, which lies upstream to all three pathways.

Both the NFκB and the ERK pathways play important roles in cell survival and growth (5, 29, 36), and it is interesting that adding back activators of these pathways rescued the apoptosis-sensitive phenotype in PAK4 knockdown cells. These results suggest that the ERK and NFκB pathways lie downstream to PAK4 in TNFα signaling. It is interesting that although both pathways were abrogated in the PAK4 knockdown cells, activation of either pathway on its own was sufficient to rescue the apoptosis-sensitive phenotype. Because these studies were carried out by overexpression of constitutively active mutants, one possible explanation is that high levels of activation of one pathway may compensate for the loss of the other pathway. More work will be needed to determine whether one or both of these pathways are actually required for PAK4-mediated survival.

Like the ERK and NFκB pathways, the JNK pathway was also abrogated in TNFα-stimulated PAK4 knockdown cells. Interestingly, however, activation of the JNK pathway, via expression of activated JNKK-JNK, not only did not rescue the apoptosis-sensitive phenotype, it actually made the cells more sensitive to TNFα-induced apoptosis (data not shown). The JNK pathway has been shown to play different roles in the apoptosis pathway depending on the cell type, the stimulus, and the duration of its activation (37–40). Although transient activation of JNK suppresses apoptosis via phosphorylation of the proapoptotic Bcl-2 family protein BAD (41), prolonged JNK activation has been shown to
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promote apoptosis via a caspase 8-independent cleavage of Bid (42). Activation of the NFκB pathway has been shown to prevent the prolonged activation of JNK pathway (43), and thus activation of JNK pathway by TNFα treatment favors cell survival. In tumor cells where there may be abnormal activation of NFκB pathway, JNK has been shown to be anti-apoptotic (30, 31), whereas in primary mouse embryonic fibroblast cells, JNK has been shown to be proapoptotic (44). The different effects of prolonged versus transient expression of JNK may explain why prolonged JNK activation induced by expressing constitutively activate JNK-K-JNK promotes apoptosis rather than cell survival in our system.

Unlike the ERK, JNK, and NFκB pathways, activation of the p38 pathway by TNFα was not affected by the knockdown of PAK4. This suggests that other pathways in addition to, or instead of, the complex I-mediated pathway can lead to p38 activation in response to TNFα. The role for p38 in the TNFα pathway is still not clearly defined (45, 46), and it has been shown to be antiapoptotic or proapoptotic depending on the cell type or the stimulus (45, 47). Our results suggest that p38 does not play an essential role in TNFα-induced cell survival pathways in HeLa cells.

FIGURE 5. The binding of TRADD protein to the TNFR was attenuated in PAK4 knockdown cells. Control and PAK4 knockdown (RNAi) cells were left untreated (0 min) or treated with TNFα + CHX for 5, 15, and 30 min. The same amount of cell lysate was incubated with protein G-agarose beads loaded with anti-TNFR1 antibody or control (goat serum). Proteins that bound to the TNFR or control immunoprecipitates were analyzed by Western blot. TRADD binding to TNFR was also quantified using NIH Image J, and the results of three experiments were averaged and plotted. TRADD binding to the TNFR1 was greatly reduced (but not completely abolished) in the PAK4 knockdown cells. No PAK4 was seen in the complex. The total amount of TNFR1 and PAK4 in the lysates is shown in the bottom panel. IP, Western blot of immunoprecipitates; WB, direct Western blot of whole cell lysates; WCL, whole cell lysates.

FIGURE 6. Restoring PAK4 increased TRADD binding to TNFR. A, control and PAK4 knockdown (RNAi) cells were left untreated (0 min) or treated with TNFα + CHX for 5 min. Equal amounts of cell lysate were combined with equal amount of lysate from HeLa cells stably overexpressing hemagglutinin-tagged wild type PAK4 (PAK4WT), Myc-tagged kinase-dead PAK4 (PAK4KM), or Myc-tagged constitutively active PAK4 (PAK4NE). The mixture of cell lysate was incubated with protein G-agarose beads loaded with anti-TNFR1 antibody. TRADD protein bound to the TNFR1 was analyzed by Western blot and was also quantified using NIH Image J. All three different PAK4 proteins increased TRADD binding to TNFR in PAK4 knockdown cells, although PAK4KM showed the least stimulation and constitutively activate PAK4 mutant increased the binding the most, even though it was expressed at low levels. Both kinase-dead and constitutively activated PAK4 also led to a slight increase in TRADD binding in control cells. B, Western blot analysis showing the expression of TRADD protein and PAK4 in whole cell lysate from the HeLa cells stably overexpressing wild type PAK4 (PAK4WT), kinase-dead PAK4 (PAK4KM), or constitutively active PAK4 (PAK4NE) and from the control and PAK4-RNAi cells. IP, immunoprecipitation.

The fact that PAK4 is required for the full activation of survival pathways and for anchorage-independent growth sheds new light on the potential role for this protein in oncogenesis. Anchorage-independent growth requires the ability of cells to survive under conditions where they would normally stop growing or undergo apoptosis. We found that PAK4 is required for full activation of survival pathways in response to TNFα. If PAK4 also promotes survival in response to other stimuli, this could help explain why it is required for anchorage-independent growth and why its overproduction is associated with transformation.

In addition to its potential role in the oncogenic process, PAK4 was also shown to be required for normal embryonic development (21). The present study gives us some insight into the role of PAK4 during normal development. Deletion of PAK4 in mice results in embryonic lethality, and we have found that there are regional increases in apoptosis in certain parts of the PAK4 null embryos. The fact that the increase in apoptosis is regional is very interesting and at first glance difficult to explain, because PAK4 expression is ubiquitous. Our new data provide an intriguing possible explanation. We found that PAK4 is not required for normal cell growth but is required for the full activation of the survival pathways under stressful conditions such as exposure to TNFα. Our results raise the possibility that in PAK4 null embryos, apoptosis is increased only in regions that are being exposed to specific cellular stresses that require activation of PAK4-mediated survival pathways to develop normally.
PAK4 Facilitates TRADD Binding to TNFR

In summary, in this paper, we propose a completely new role for PAK4 in the promotion of survival in response to cell stress. Using HeLa cells as a model system, we found that PAK4 is required for anchorage-independent growth in cancer cells. We also found that PAK4 is required for the full activation of survival pathways in response to TNFα. PAK4 functions early in the TNFα signaling pathways by facilitating the binding of TRADD to TNFR1, which is necessary for the rapid formation of complex I followed by activation of survival pathways, especially the NFκB pathway. Our data give insight of the role of PAK4 in tumorigenesis as well as mouse embryonic development.

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