p21-activated Protein Kinase γ-PAK Suppresses Programmed Cell Death of BALB3T3 Fibroblasts*

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In response to stress stimulants, cells activate opposing signaling pathways for cell survival and programmed cell death. p21-activated protein kinase γ-PAK is involved in both cell survival and cell death pathways. Many stress stimulants activate γ-PAK as a full-length enzyme and as a proteolytic fragment. Caspase-mediated proteolytic activation parallels cell death and appears to be a pro-apoptotic factor in stress-induced cell death. Here, we show that activation of full-length γ-PAK promotes cell survival and suppresses stress-induced cell death. Expression of constitutively active γ-PAK-T402E, which mimics activated full-length γ-PAK, stimulates cell survival of BALB3T3 fibroblasts in response to tumor necrosis factor α, growth factor withdrawal, and UV light. This stimulation of cell survival is mainly due to protection of cells from cell death rather than by stimulation of proliferation. Expression of γ-PAK-T402E increases phosphorylation of the pro-apoptotic Bcl-2 family protein Bad and protects from apoptosis. Our results indicate that the ubiquitous γ-PAK may have a crucial function in cell survival by regulating the pro-apoptotic activity of Bad and the stress-induced activation of ERK, JNK, and p38.

Cells respond to stress stimulants by activating two opposing processes; cell growth and survival pathways are activated for protection and repair, whereas programmed cell death pathways are activated to eliminate damaged cells. The decision between cell survival and cell death depends on the balance of constitutive and extracellular signal-induced pro- and anti-apoptotic factors. Disturbances in the balance of pro- and anti-apoptotic factors can lead to either increased cell death or increased cell survival and are involved in the development of diseases such as AIDS and cancer. Programmed cell death can occur by multiple pathways with the apoptotic and the necrotic pathways at the extremes (1). Some cells are competent to follow one cell death pathway but lack required components for another or contain survival factors that block a particular pathway. In addition, different cell death pathways can co-exist within the same cell and be activated by different stimulators. The regulation of cell survival and cell death involves many signaling pathways, and the decision between cell survival and cell death requires cross-talk between these pathways and checkpoints where pro- and anti-apoptotic signals converge (2).

p21-activated protein kinases (PAK) are a growing family of serine/threonine protein kinases, which are activated by binding of monomeric (p21) G-proteins such as Cdc42 and Rac and subsequent autophosphorylation at a threonine residue in the activation loop (3–8). PAKs have been implicated in a wide range of biological functions such as cell morphology and motility, stress response, programmed cell death, and malignant transformation. At least three isoforms of PAK exist in mammals. α-PAK (PAK1), a 68-kDa protein, is present in brain, muscle, and spleen (9, 10). β-PAK (PAK3), a 65-kDa protein, is also present in brain but in different areas than α-PAK (11, 12). γ-PAK (PAK2, PAK I, PAK65), a 58- to 62-kDa protein, is present ubiquitously in all tissues and cell types (10, 13–15).

PAKs have been linked to mitogen-activated protein kinase and stress-activated protein kinase pathways. PAKs can stimulate JNK and p38 activity in some cell types but the degree of stimulation is modest and may reflect indirect effects (4, 16–18). γ-PAK phosphorylates and positively regulates c-Raf in the ERK pathway (19). (The reference states that PAK-3 (β-PAK) phosphorylates c-Raf, but it is actually PAK-2 (γ-PAK) (20). This error in nomenclature is due to temporary mistakes in the GenBank™ annotations for PAK.) Phosphorylation of c-Raf by γ-PAK is required for activation of the ERK pathway in response to growth factors or by oncogenic Ras (19). MEK-1, another protein kinase in the ERK pathway, has been shown to be phosphorylated by α-PAK (21). In addition to a role in mitogenic stimulation PAKs are also involved in cell transformation. Catalytically inactive mutants of PAK inhibit Ras transformation of rat Schwann cells and cooperative transformation of Rat-1 cells by Ras, Rac, and Rho (22–24). Hyperactive forms of α-PAK and γ-PAK have been detected in highly proliferative breast cancer cell lines (25). Hyperactivity of PAK is required to convey the highly proliferative phenotype. Recently, α-PAK has been shown to mediate signals from Ras through phosphoinositide 3-kinase and Akt to sustain cell transformation (26). Activated Akt stimulates α-PAK activity through a p21 G-protein-independent mechanism. One of the downstream targets of Akt is the pro-apoptotic Bcl-2 family

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1 The abbreviations used are: PAK, p21-activated protein kinase; JNK, c-Jun N-terminal kinase; BrdUrd, bromodeoxyuridine; FBS, fetal bovine serum; TNF, tumor necrosis factor; BHA, butylated hydroxyanisol; EGFP, enhanced green fluorescent protein; ROS, reactive oxygen species; UVC; ultraviolet C; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; MEKK, MEK kinase; CHO, Chinese hamster ovary; GTPyS, guanosine 5′-3-O-(thio)triphosphate; PCR, polymerase chain reaction; TRE, Tet-response element; CMV, cytomegalovirus; ZVAD-FMK, Z-Val-Ala-Asp(OMe)-CH2F.
protein Bad. Bad dimerizes with anti-apoptotic Bcl-2 or Bcl-XL and inhibits their ability to block the release of cytochrome c from mitochondria (27). Phosphorylation of Bad at Ser-112 and Ser-136 results in dissociation from Bcl-2 or Bcl-XL and association with 14-3-3. α-PAK has been shown to phosphorylate Bad in vitro and in vivo at Ser-112 and Ser-136, and phosphorylation at these residues correlates with stimulation of cell survival (28). The authors also report that γ-PAK phosphorylates Bad in vitro, but phosphorylation and inhibition of Bad in vivo has not yet been demonstrated for γ-PAK.

The mammalian PAK isoforms have a high degree of sequence homology, especially within their catalytic domain (3–8). The regulatory domains contain regions of high sequence homology but also nonconserved regions that may reflect important differences in functions. A unique characteristic of the ubiquitous γ-PAK is the existence of a cleavage site for caspase 3 or a caspase 3-like protease within the regulatory domain (29, 30). Cleavage by caspase 3 removes most of the regulatory domain and results in the irreversibly activated γ-PAK phosphorylase fragment. Caspase cleavage of γ-PAK is correlated with Fas- and ceramide-induced cell death of Jurkat cells, TNF-α-induced cell death of MCF-7 cells, heat shock-induced cell death of BALB3T3 and Hep 3B cells, and UVC light-induced cell death of A431 cells (29, 31, 32). Ectopic expression of γ-PAK induces morphological changes, which are typical for the initial stages of apoptosis, and is sufficient to trigger apoptosis in CHO, HeLa, and Jurkat cells (33, 34). Therefore, caspase-activated γ-PAK appears to have a pro-apoptotic role in programmed cell death. Here, we show that the ubiquitous γ-PAK can also act as a survival factor and protect cells from programmed cell death. Ectopic expression of constitutively active full-length γ-PAK stimulates survival of BALB3T3 fibroblasts in response to stress stimulants such as tumor necrosis factor α (TNF-α), growth factor withdrawal, and UVC light. Cell survival is stimulated, because constitutively active γ-PAK protects BALB3T3 cells from cell death. Phosphorylation and inhibition of the pro-apoptotic Bcl-2 family protein Bad is one mechanism by which γ-PAK mediates stimulation of cell survival. Other potential survival mechanisms mediated by γ-PAK include regulation of ERK, JNK, and p38 activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—The inducible retroviral expression system pRevTet-On was obtained from CLONTECH. The constitutive retroviral expression vector pBMNZ was a gift from Dr. Gary Nolan at Stanford University. Cloned Pfs DNA polymerase was purchased from Stratagene. An ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit was obtained from PE Applied Biosystems. Restriction enzymes, T4 DNA ligase, phospho-p44/42 MAPK (Thr-202/Tyr-204) and phospho-p38 MAPK (Thr-180/Tyr-182) antibodies, and the PhosphoPlus Bad (Ser-112/Ser-136) antibody kit were purchased from New England BioLabs. The anti-γ-PAK antibodies γ-PAK (N-19) and γ-PAK (V-19), the anti-α-PAK antibody α-PAK (N-20), and the phospho-specific anti-p-JNK antibody were from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated secondary antibodies, ECL reagents, and protein G-Sepharose were from Amersham Pharmacia Biotech. Alexa Fluor 588-conjugated secondary antibody was from Molecular Probes. Brodoxyuridine (BrduRd), monoclonal anti-bromodeoxyuridine antibody, ATP, GTP, γS, and histone 4 were purchased from Roche Molecular Biochemicals. Dulbecco’s modified Eagle’s medium, fetal bovine serum (FBS), LipofectAMINE 2000, and customized primers were obtained from Life Technologies. Tumor necrosis factor (TNF)-α and caspase inhibitor ZVAD-FMK were from Calbiochem. The CellTiter 96 AQueous One Solution cell proliferation assay was purchased from Promega. M-PER Mammalian Protein Extraction Buffer and Gelcode Blue staining reagent were from Pierce. Butylated hydroxyanisol (BHA), propidium iodide, and myelin basic protein were from Sigma Chemical Co. γ-32P]ATP was purchased from PerkinElmer Life Sciences.

**Subcloning and Site-directed Mutagenesis**—cDNAs encoding the protein coding region of wild-type γ-PAK and the kinase-deficient mutant γ-PAK-K278R were subcloned into pKoz/EGFP to form a fusion with enhanced green fluorescent protein (EGFP). Site-directed mutagenesis according to the megaprimer PCR method (35, 36) was performed with Pfu DNA polymerase to obtain the constitutively active mutant γ-PAK-T402E, where Thr-402 was replaced with glutamic acid to mimic a consensus caspase cleavage site. This group incorporation was subsequently confirmed by DNA sequencing. The resulting mutant was subcloned into pKoz/EGFP and sequenced to confirm the T402E mutation and to ensure the absence of accidental mutations due to misincorporation during PCR. EGFP fusion constructs for wild-type γ-PAK, kinase-deficient γ-PAK-K278R, and constitutively active γ-PAK-T402E were subcloned into the inducible retroviral expression vector pRevTet-On. Mouse Bad plasmid was subcloned by PCR using a unique BglII site and mBad plasmid from New England BioLabs as a template into the constitutive retroviral expression vector pBMNZ.

**Cell Culture, Transfection, and Retroviral Transduction**—The eukaryotic 293T-cell-derived packaging cell line Phoenix Eco (37), the normal mouse fibroblast cell line BALB3T3, and the K-Ras sarcoma virus-transformed K-BALB cell line (38, 39) were obtained from American Type Culture Collection (ATCC). Phoenix Eco, BALB3T3, and K-BALB cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated FBS, 2 munits, and 100 units/ml penicillin/100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO2. For long term storage, cells were frozen over at −80 °C and stored in liquid nitrogen. Ecotropic retroviruses for the transduction of murine cells were obtained by transfection of the retroviral vectors into the packaging cell line Phoenix Eco. Phoenix Eco cells were grown to ~50% confluency in 100-mm culture dishes and then transfected with 4 μg of plasmid DNA using LipofectAMINE 2000. The culture medium was replaced at 24 h after transfection, and retrovirus-containing medium was collected at 48 h after transfection and filtered through a 0.45-μm filter. For long term storage the retrovirus-containing medium was frozen in liquid nitrogen and stored at −80 °C. BALB3T3 fibroblasts were grown to ~10–20% confluency in 100-mm culture dishes and then transduced by addition of 0.5–2 ml of retrovirus-containing medium from packaging cells in the presence of 4 μg/ml Polybrene. The culture medium was replaced at 24 h after transfection and culture medium containing 750 μg/ml Geneticin or 300 μg/ml hygromycin B was added at 48 h to select stable cell populations for RevTet-On or pRevTRE, respectively.

**Cell Viability, Proliferation, and Cell Death Assays**—Cells were treated with TNF-α by changing to growth medium containing 1 or 10 ng/ml TNF-α. Growth factor withdrawal was achieved by changing to growth medium without fetal bovine serum or with 0.1% fetal bovine serum. For UV light treatment, growth medium was removed, cells were washed with PBS, and 0.5–2 ml of UV-washed cells was added to 750 μl of 250 J/m2 in a UV-cross-linker in 25-mm coverslips. After 1 h, a new growth medium was added. Cell viability in response to stress stimuli was determined with the CellTiter 96 AQueous One Solution cell proliferation assay. Quadruplicate cell samples were grown in 96-well plates, and cell viability was measured at 24 h after treatment by addition of the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium compound. Over a 2 h incubation period, this compound is converted to the colored formazan, which was detected at 490 nm with a plate reader. Background absorbance was corrected by subtraction of blanks with an equal volume of growth medium. Background caused by cellular debris was corrected by subtraction of sample absorbance at the reference wavelength of 630 nm. Cell proliferation was measured as the percentage of cells in DNA synthesis by BrdUrd incorporation. Cells treated with stress stimuli or untreated control cells were incubated for 24 h. BrdUrd at 10 μM was then added for the final 30 min. Cells were detached and collected by centrifugation. Collected cells were fixed with 70% ethanol, stained with a monoclonal anti-BrdUrd antibody and an Alexa-568-conjugated secondary antibody according to the manufacturer’s instructions, and analyzed by flow cytometry on a Becton-Dickinson FACScan. Cell death was measured by the uptake of propidium iodine, which indicates loss of membrane integrity. Stress stimulated-treated cells or control cells were detached and collected by centrifugation. Collected cells were stained with 0.2 μg/ml propidium iodine and analyzed by flow cytometry on a Becton-Dickinson FACScan.

**Immunoprecipitation and Western Blot**—Cells were lysed in M-PER Mammalian Protein Extraction Reagent containing 1 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid, 0.1% Triton X-100, and 1 μg/ml protease inhibitor cocktail, 1 μg/ml pepstatin, 1 μg/ml aprotinin, and 200 μM sodium vanadate. Protein concentrations were determined by a Bradford protein assay using bovine γ-globulin as a standard protein. Recombinant EGFP-tagged γ-PAK was immunoprecipitated with the polyclonal anti-EGFP antibody. Endogenous γ-PAK and α-PAK were immunoprecipitated with the polyclonal agaro-conjugated anti-γ-PAK (N19) and anti-α-PAK (N20) an-
Expression of EGFP-tagged γ-PAK—Wild-type γ-PAK, constitutively active γ-PAK-T402E, and kinase-deficient γ-PAK-K278R were subcloned with an N-terminal EGFP tag into the retroviral expression vector pRev-TRE. Expression of EGFP-tagged γ-PAK is controlled by the Tet-response element (TRE) upstream of a minimal CMV promoter. A reversed tetracycline-controlled transactivator is expressed from a second retroviral vector pRevTet-On. After addition of tetracycline or doxycycline to the culture medium, the reversed tetracycline-controlled transactivator binds to the TRE and induces expression of recombinant γ-PAK. Recombinant retroviruses were obtained with the 293T cell-derived ecotropic packaging cell line Phoenix Eco. The use of the retroviral system has the advantage that constructs are efficiently transduced into populations of cells rather than a few selected clones, which may show unspecific effects due to disruption or activation of genes by the integration into the host genome. BALB3T3 mouse fibroblasts were transduced with pRevTet-On and selected with Geneticin to obtain a stable BALB3T3-On cell population, which expressed the reversed tetracycline-controlled transactivator. BALB3T3-On cells where then transduced with pRev-TRE containing EGFP-tagged wild-type γ-PAK, constitutively active γ-PAK-T402E, kinase-deficient γ-PAK-K278R, or the EGFP-tag alone. Stable cell populations for the expression of EGFP-γ-PAK, EGFP-γ-PAK-T402E, EGFP-γ-PAK-K278R, or EGFP alone were obtained by selection with hygromycin B.

To determine the optimal concentration and incubation time for the induction of expression, EGFP-γ-PAK-T402E cells were treated with increasing amounts of doxycycline. Expression was monitored by fluorescence microscopy and by Western blot with an anti-EGFP antibody (Fig. 1). Without addition of doxycycline, a basal level of EGFP-γ-PAK-T402E was observed, which might be due to translation from transcripts initiated by the 5′-viral long terminal repeat. The expression was stimulated with increasing amounts of doxycycline and reached saturation at ~2 μg/ml. Doxycycline-stimulated expression increased with time and reached a steady-state level at ~48 h (data not shown). Similar results were obtained for EGFP-γ-PAK, EGFP-γ-PAK-K278R, and EGFP alone. Therefore, expression was stimulated with 2 μg/ml doxycycline for 48 h in all subsequent experiments. Because basal levels were present without addition of doxycycline, BALB3T3-On cells, which contain only the pRevTet-On vector, were used as controls rather than uninduced cells.

Protein kinase activity of recombinant wild-type and mutant EGFP-tagged γ-PAK was determined by immunoprecipitation and immunocomplex protein kinase assays (Fig. 2). BALB3T3 cells expressing EGFP-γ-PAK, EGFP-γ-PAK-T402E, EGFP-γ-PAK-K278R, or EGFP alone, which were dividing at approximately the same time intervals of 22 h, were grown to ~70% confluence. Cells were lysed, and EGFP-γ-PAK, EGFP-γ-PAK-T402E, EGFP-γ-PAK-K278R, or EGFP alone were immunoprecipitated with a polyclonal anti-EGFP antibody using equal amounts of cell lysate protein. Equal amounts of cell lysate protein and equal aliquots of immunoprecipitate for each construct were analyzed by Western blot with a monoclonal anti-EGFP antibody. Approximately equal amounts of EGFP fusion protein was detected with the different constructs. This suggests that EGFP-γ-PAK, EGFP-γ-PAK-T402E, and EGFP-γ-PAK-K278R are expressed at very similar levels in BALB3T3 fibroblasts. Equal aliquots of immunoprecipitate from cells expressing EGFP-γ-PAK, EGFP-γ-PAK-T402E, and EGFP-γ-PAK-K278R were assayed for autophosphorylation and activity toward histone 4 (Fig. 2, B and C).

Samples without immunoprecipitate were used as controls and subtracted from the activity values. Activation of γ-PAK involves autophosphorylation at Thr-402 in the activation loop (30, 41). Once γ-PAK is activated, autophosphorylation also occurs at several serine residues in the regulatory domain, and exogenous substrates such as histone 4 and myelin basic pro-
tein can be phosphorylated. No autophosphorylation and low levels of histone 4 phosphorylation were observed with the kinase-deficient EGFP-γ-PAK-K278R in the absence and presence of Cdc42(GTPγS). The low level of histone 4 phosphorylation with the EGFP-γ-PAK-K278R immunoprecipitate could be due to co-precipitating protein kinases, including endogenous γ-PAK. EGFP-γ-PAK showed a low basal level of auto-phosphorylation and no significant increase in phosphorylation of histone 4 as compared with kinase-deficient EGFP-γ-PAK-K278R. Addition of Cdc42(GTPγS) enhanced autophosphorylation and increased phosphorylation of histone 4 by 2- to 3-fold. High levels of autophosphorylation and a 5-fold increase in phosphorylation of histone 4 were observed with EGFP-γ-PAK-T402E. Addition of Cdc42(GTPγS) enhanced autophosphorylation but only slightly increased histone 4 phosphorylation. Therefore, EGFP-γ-PAK-T402E was constitutively active and expression of EGFP-γ-PAK-T402E was used to mimic endogenously activated γ-PAK.

**Effects of Activated γ-PAK on Cell Survival of BALB3T3 Fibroblasts**—BALB3T3 cells, which express EGFP-γ-PAK or EGFP-γ-PAK-T402E, were analyzed for effects on cell survival in response to TNF-α, growth factor withdrawal, and UVC light. Parental BALB3T3-On cells and transformed K-BALB cells, which express a constitutively active K-Ras oncogene, were used as controls. Cell viability was measured after 24 h with a colorimetric proliferation/viability assay. Cell viability levels of untreated K-BALB cells, BALB3T3-On cells, and cells that express EGFP-γ-PAK or EGFP-γ-PAK-T402E were very similar to each other and used as reference values at 100% (Fig. 3A). In addition, cell viability was also analyzed by phase contrast microscopy (Fig. 3B). BALB3T3-On cells were highly sensitive to TNF-α, growth factor withdrawal, and UVC light. Cell viability decreased significantly to 30–40% of that of untreated control cells. Similar results were obtained with BALB3T3 cells, indicating that transduction with the pRe- vTet-On vector does not affect cell viability (data not shown). There were no significant differences in cell viability between BALB3T3-On cells and cells expressing EGFP-γ-PAK, but in response to 1 ng/ml TNF-α, complete growth factor withdrawal (0% FBS), and UVC light, cells expressing EGFP-γ-PAK showed a trend of slightly higher cell survival than BALB3T3-On cells. In contrast, cells expressing constitutively active EGFP-γ-PAK-T402E showed increased cell survival in response to TNF-α, growth factor withdrawal, and UVC light. Cell viability decreased only to 60–85% of that of untreated control cells. Similar results were obtained with transformed K-BALB cells, which express constitutively active K-Ras. In response to TNF-α, growth factor withdrawal and UVC light cell viability decreased only to 65–90% of that of untreated control cells.

Increased cell viability in response to stress stimulants could be the result of stimulation of proliferation and/or protection from cell death. Proliferation and cell death was measured at 24 h after treatment with 1 ng/ml TNF-α or complete growth
factor withdrawal to 0% fetal bovine serum. Untreated cells were used as controls. Proliferation was measured as numbers of cells in DNA synthesis by BrdUrd incorporation, whereas cell death was measured by the uptake of propidium iodine, which enters cells that have lost membrane integrity (Fig. 4). The total numbers of K-BALB cells, BALB3T3-On cells, and cells that express EGFP-γ-PAK or EGFP-γ-PAK-T402E were very similar to each other and used as reference values at 100%. Without any treatment, parental BALB3T3-On cells and cells expressing EGFP-γ-PAK or EGFP-γ-PAK-T402E showed similar proliferation with 46–53% of BrdUrd-incorporating cells (Fig. 4A). K-BALB cells, which express an activated K-Ras oncogene, showed the fastest proliferation with 68% of cells incorporating BrdUrd. Treatment with TNF-α did not affect proliferation, whereas growth factor withdrawal resulted in a significant decrease of BrdUrd-incorporating cells, which is probably the result of arrested cell growth due to the lack of growth factor stimulation. For BALB3T3-On cells and cells expressing EGFP-γ-PAK, growth factor withdrawal decreased the number of BrdUrd-incorporating cells to 21% and 26%, respectively. For K-BALB cells and cells expressing EGFP-γ-PAK or EGFP-γ-PAK-T402E, growth factor withdrawal decreased the number of BrdUrd-incorporating cells only to ~40%. Without treatment, the rates of cell death for K-BALB cells, BALB3T3-On cells, and cells expressing EGFP-γ-PAK or EGFP-γ-PAK-T402E were similar at 7–12% (Fig. 4B). Treatment with TNF-α or growth factor withdrawal increased cell death of parental BALB3T3-On cells to 43% or 49%, respectively. Cells expressing EGFP-γ-PAK were partially protected; cell death was at 23% with TNF-α and at 38% with growth factor withdrawal. Cells expressing EGFP-γ-PAK-T402E were highly protected; cell death was at 16% with TNF-α and at 23% with growth factor withdrawal. The transformed K-BALB cells, which express an activated K-Ras oncogene, also showed protection against cell death. But in comparison with cells expressing EGFP-γ-PAK or EGFP-γ-PAK-T402E protection of K-BALB cells was more efficient in response to growth factor withdrawal than after treatment with TNF-α. Cell death of K-BALB cells was at 26% with TNF-α and at 16% with growth factor withdrawal. Therefore, stimulation of cell survival by expression of EGFP-γ-PAK-T402E, and to a lower degree by EGFP-γ-PAK, is a result of protection from cell death rather than by stimulation of the proliferation rate. However, cells expressing EGFP-γ-PAK-T402E were able to grow under growth factor-deprived conditions to a similar degree as K-BALB cells, which express an activated K-Ras oncogene.

Effects of Caspases and Reactive Oxygen Species on Cell Survival of BALB3T3 Fibroblasts—The role of caspases and reactive oxygen species (ROS) in cell death of BALB3T3 cells in response to 1 ng/ml TNF-α, growth factor withdrawal to 0% fetal bovine serum, and UVC light at 50 J/m² were determined with the general caspase inhibitor ZVAD-FMK and the ROS scavenger butylated hydroxyanisol (BHA). Parental BALB3T3-On cells and cells expressing EGFP-γ-PAK-T402E were preincubated for 1 h in ZVAD-FMK or BHA and then treated with TNF-α, growth factor withdrawal, or UVC light. After 24 h cells were assayed for cell viability with a colorimetric proliferation/viability assay. Cell viability levels of control cells in growth medium alone were very similar to each other and used as reference values at 100%. The solvent Me₂SO, BHA, and ZVAD-FMK had no significant effects on cell viability (Fig. 5A). Treatment with TNF-α, growth factor withdrawal, or UVC light resulted in a decrease of cell viability to ~50% for parental BALB3T3-On cells. In contrast, cells expressing EGFP-γ-PAK-T402E maintained cell viability at similar levels as untreated control. BHA and ZVAD-FMK had no effect on cell viability.
viability in the absence of TNF-α. In response to TNF-α, treatment with 10 μM ZVAD-FMK and 100 μM BHA partially increased cell viability of BALB3T3-On cells to 49% as compared with 33% with 10 μM ZVAD-FMK alone and completely restored the stimulation of cell survival by EGFP-γ-PAK-T402E. The protection by BHA indicates that TNF-α-induced cell death requires the generation of ROS, whereas the lack of protection by BHA indicates that ROS are not required for cell death induced by growth factor withdrawal and UVC light. A caspase or a caspase-like protease appears to protect BALB3T3 fibroblasts from TNF-α-induced cell death by counteracting the ROS-mediated stimulation of cell death.

Cell death in BALB3T3 cells does not follow a classical apoptotic pathway. A study on cell death by growth factor withdrawal reported that BALB3T3 cells exhibit some features of apoptosis such as cell rounding and nuclear chromatin condensation but not internucleosomal DNA fragmentation (42). We also did not observe internucleosomal DNA fragmentation in BALB3T3 cells treated with TNF-α, growth factor withdrawal, UVC light or cisplatin, whereas controls of human mesangial cells treated with cisplatin showed internucleosomal DNA fragmentation (data not shown). In addition, we did not observe stimulation of caspase 3 activity in BALB3T3 cells in response to stress stimuli (data not shown).

**Effects of Activated γ-PAK on the Pro-apoptotic Bcl-2 Family Protein Bad**—The role of the pro-apoptotic Bcl-2 family protein Bad as a target of γ-PAK in the protection of BALB3T3 fibroblasts from cell death was examined. Phosphorylation of Bad at Ser-112 and Ser-136 inhibits its pro-apoptotic activity by preventing heterodimerization with Bcl-2 and Bcl-XL. Both α-PAK and γ-PAK have been shown to phosphorylate Bad at Ser-112 and Ser-136 in vitro (28). The phosphorylation of endogenous Bad at Ser-112 and Ser-136 in response to TNF-α and growth factor withdrawal was analyzed by Western blot with phospho-specific antibodies. In parental BALB3T3-On cells, little or no stimulation of Ser-112 phosphorylation was observed in response to TNF-α and growth factor withdrawal (Fig. 6A). However, expression of EGFP-γ-PAK-T402E increased phosphorylation of Bad at Ser-112. Phosphorylation of endogenous Bad at Ser-136 could not be detected in BALB3T3-On cells or cells expressing EGFP-γ-PAK-T402E, probably because the anti-phospho-Ser-136 antibody is less sensitive than the anti-phospho-Ser-112 antibody. Western blots, with an antibody that detects unphosphorylated and phosphorylated Bad equally, showed that the protein levels of Bad did not change significantly.

To determine if phosphorylation of Bad by constitutively active EGFP-γ-PAK-T402E protects BALB3T3 fibroblasts from cell death, we generated the constitutive retroviral expression construct pBMNZ-mBad. Ecotropic retroviruses for pBMNZ-mBad or the pBMNZ vector alone were transduced into BALB3T3-On cells and cells expressing EGFP-γ-PAK-T402E. Cell death was analyzed at 24, 48, and 72 h after transduction by uptake of propidium iodide into cells that lost membrane integrity (Fig. 6B). BALB3T3-On cells and cells expressing EGFP-γ-PAK-T402E transduced with the pBMNZ vector alone showed basal levels of cell death. BALB3T3-On cells transduced with Bad started to show increased levels of cell death at 48 h and reached ~35% of cell death at 72 h. Cells expressing EGFP-γ-PAK-T402E transduced with Bad showed no significantly increased levels of cell death. Phosphorylation of recombinant Bad at Ser-112 and Ser-136 was greatly stimulated by expression of EGFP-γ-PAK-T402E as compared with parental BALB3T3-On cells (Fig. 6C). Under the same conditions, phosphorylation of endogenous Bad in cells transduced with pBMNZ vector alone was not detectable (data not shown). Therefore, phosphorylation of Bad appears to be one of the
mechanisms by which constitutively activated γ-PAK-T402E protects cells from cell death.

Effects of Activated γ-PAK on Activation of ERK, JNK, and p38—The stimulation of ERK, JNK, and p38 in BALB3T3-On cells and cells expressing EGFP-γ-PAK-T402E was measured in Western blots using phospho-specific antibodies, which specifically detect the active forms of these protein kinases (Fig. 7). In BALB3T3-On cells, TNF-α activated ERK, JNK, and p38 within 15 min, and activation was sustained up to 24 h. Expression of EGFP-γ-PAK-T402E changed the activation profile of ERK, JNK, and p38. In cells expressing EGFP-γ-PAK-T402E, ERK activation was more increased between 15 and 30 min as compared with parental BALB3T3-On cells, but ERK activation was significantly decreased at later time points. EGFP-γ-PAK-T402E stimulated p38 activation at 15 min as compared with parental BALB3T3-On cells, but p38 activation was greatly decreased at later time points. Western blots with antibodies that detect unphosphorylated and phosphorylated forms of ERK, JNK, and p38 equally showed that the protein levels did not change (data not shown). Growth factor withdrawal did not activate JNK and p38 as compared with basal levels and only resulted in slightly increased stimulation of ERK (data not shown).

Activation of Endogenous γ-PAK in Response to TNF-α and Growth Factor Withdrawal—To analyze if endogenous γ-PAK is activated in response to stress stimulants, we performed in-gel assays with myelin basic protein as substrate (Fig. 8). First, γ-PAK bands in the in-gel assays were identified by immunoprecipitations and by Western blots with specific anti-γ-PAK antibodies. BALB3T3-On cell lysates were immunoprecipitated with specific anti-γ-PAK and anti-α-PAK antibodies. Immunoprecipitates and BALB3T3-On cell lysate were analyzed by in-gel assay with myelin basic protein (Fig. 8A). Protein kinase bands at 62 and 58 kDa were detected with the anti-γ-PAK immunoprecipitate, whereas the anti-α-PAK immunoprecipitate did not result in detectable protein kinase bands. Rat brain and BALB3T3-On cell lysates were analyzed by Western blots with specific anti-γ-PAK and anti-α-PAK antibodies (Fig. 8B). Protein bands at 62 and 58 kDa were detected in rat brain and BALB3T3-On cells with the specific
anti-γ-PAK antibody, whereas the anti-α-PAK antibody detected a protein band at 68 kDa. The results show that the protein kinase bands at 62 and 58 kDa both represent γ-PAK. Because the predicted molecular mass of rat and rabbit γ-PAK is ~58 kDa, both activity bands may represent full-length mouse γ-PAK, which differs in post-translational modifications. α-PAK protein is present at low levels in BALB3T3 fibroblasts, but active α-PAK was not detected.

In-gel assays were carried out to examine activation of the 62- and 58-kDa γ-PAK bands in response to TNF-α and growth factor withdrawal (Fig. 8C). The 62-kDa γ-PAK band did not change significantly, but the 58-kDa γ-PAK band showed a biphasic activation. In response to TNF-α, the early activation phase of the 58-kDa γ-PAK band was between 30 min and 1 h and the second activation phase was between 3 and 12 h. In response to growth factor withdrawal, the early activation phase was between 15 and 30 min and the second activation phase was between 12 and 24 h. In cells expressing EGFP-γ-PAK-T402E, a faint protein kinase band was visible at ~87 kDa, which probably represents EGFP-γ-PAK-T402E. It appears that the EGFP tag reduces the ability of EGFP-γ-PAK-T402E to refold in the in-gel assay. However, protein levels of endogenous γ-PAK and recombinant EGFP-γ-PAK-T402E were similar (data not shown) and immunoprecipitated EGFP-γ-PAK-T402E showed a high level of constitutive activity (Fig. 2). Expression of EGFP-γ-PAK-T402E stimulated the activation of the 58-kDa band of endogenous γ-PAK in response to TNF-α or growth factor withdrawal, especially in the late activation phase (3–24 h). Therefore, activated EGFP-γ-PAK-T402E appears to, directly or indirectly, stimulate the activation of the endogenous full-length γ-PAK.

A Western blot with a C-terminal PAK antibody identified this band as caspase-cleaved γ-PAKp34 (data not shown). Therefore, stress stimulants induce activation of full-length γ-PAK (58 kDa) and at later time points activation of γ-PAKp34 by
caspase cleavage. Activation of α-PAK was not detected in BALB3T3-On cells or cells expressing EGFP-tagged γ-PAK-T402E in response to TNF-α and growth factor withdrawal. 

**DISCUSSION**

The coordination and balance between cell growth, cell survival, and cell death requires a complex signaling network, including multiple checkpoints to determine cell fate. Stress stimulants activate pathways for cell survival as well as pathways for programmed cell death, which start a racetrack for life or death (2). The final decision to live or to die depends on the integration of all activated cell survival and programmed cell death pathways. These signaling pathways include key-point regulators that act as molecular switches at checkpoints between cell survival and cell death. The ubiquitous p21-activated protein kinase γ-PAK appears to be such a key-point regulator. γ-PAK has been shown to be reversibly activated as a full-length enzyme in response to ionizing radiation in 3T3-L1 fibroblasts and U937 leukemia cells, and by UV light, DNA-damaging drugs, and hyperosmolarity in 3T3-L1 fibroblasts (43, 44). Irreversible activation of γ-PAK by caspase 3 or a caspase 3-like protease has been shown in response to Fas and ceramide in Jurkat cells, TNF-α in MCF-7 cells, heat shock in BALB3T3 and Hep 3B cells, and UVC light in A431 cells (29, 30).
Proteolytic cleavage correlates with cell death and ectopic expression of the active γ-PAKp34 fragment is sufficient to trigger apoptosis in CHO, HeLa, and Jurkat cells (33, 34). Here, we report that activated full-length γ-PAK acts as a cell survival factor and protects cells from stress-induced cell death in the contact-inhibited mouse fibroblast cell line BALB3T3.

BALB3T3 fibroblasts are highly sensitive to TNF-α and growth factor withdrawal. In these cells TNF-α induces cell death without the need to inhibit protein synthesis. This indicates that BALB3T3 fibroblasts lack the ability to induce expression of survival factors in response to TNF-α. As little as 1 ng/ml TNF-α was sufficient to reduce cell viability of BALB3T3-On cells to −50% within 24 h. Growth factor withdrawal by incubation in FBS-deprived medium also results in reduction of cell viability to −50% within 24 h. Therefore, BALB3T3 fibroblasts do not only require serum factors for proliferation but also for cell survival. Expression of constitutively active EGFP-γ-PAK-T402E results in a dramatic stimulation of cell survival in response to TNF-α, growth factor withdrawal, and UV light whereas expression of wild-type EGFP-γ-PAK has a much lower effect. Stimulation of cell survival by expression of EGFP-γ-PAK-T402E, and to a lower degree by wild-type EGFP-γ-PAK, is mainly due to protection from cell death rather than by stimulation of proliferation. However, suppression of cell death results in increased net cell growth.

Cell death in BALB3T3 cells does not follow a classical apoptotic pathway (42). We did not observe internucleosomal DNA fragmentation in BALB3T3 cells treated with TNF-α, growth factor withdrawal, UV light, or cisplatin (data not shown). The general caspase inhibitor ZVAD-FMK did not protect BALB3T3-On cells and cells expressing EGFP-γ-PAK-T402E from cell death by TNF-α, growth factor withdrawal, and UV light. In addition, we did not observe stimulation of caspase 3 activity in BALB3T3 cells in response to stress stimuli (data not shown). However, cell death is executed by intracellular signaling pathways that involve the Bcl-2 family protein Bad and therefore is programmed and not accidental. In response to TNF-α, the caspase inhibitor ZVAD-FMK sensitizes cell death in response to TNF-α and inhibits protection from cell death by EGFP-γ-PAK-T402E. Such a sensitization of TNF-α-induced cell death by caspase inhibitors also occurs in mouse L929 fibrosarcoma cells (1, 45). The mitochondrial ROS scavenger BHA protects BALB3T3-On cells from TNF-α-induced cell death, abrogates the sensitizing effect of ZVAD-FMK partially for BALB3T3-On cells, and completely abrogates the effect for cells expressing EGFP-γ-PAK-T402E. BHA also protects L929 cells from TNF-α-induced cell death by abrogating the sensitizing effect of caspase inhibitors (1, 45). It is suggested that the TNF-α-induced cell death pathway in L929 and BALB3T3 cells involves the formation of mitochondrial ROS and that a putative caspase acts as a negative regulator in this cell death pathway possibly by removing damaged mitochondria. BHA has no effect on cell death induced by growth factor withdrawal and UV light, indicating that mitochondrial ROS formation is not required in these death pathways. Our results show that expression of EGFP-γ-PAK-T402E protects BALB3T3 fibroblasts from cell death induced by several signaling pathways. Therefore, constitutively active γ-PAK appears to act at a central position where several death pathways converge.

Mitochondria play a central role in many cell death signaling pathways. The interactions of pro- and anti-apoptotic Bcl-2 family proteins at the outer mitochondrial membrane appear to control the release of cytochrome c and other pro-apoptotic factors such as apoptosis-inducing factor (2, 46). Phosphorylation of the pro-apoptotic Bcl-2 family protein Bad inhibits cell death by abrogating dimerization with anti-apoptotic Bcl-2 and Bcl-XL. Both α-PAK and γ-PAK have been shown to phosphorylate Bad at the critical Ser-112 and Ser-136 residues in vitro (28). However, we did not detect α-PAK activation in BALB3T3-On cells or cells expressing EGFP-γ-PAK-T402E in response to TNF-α or growth factor withdrawal. Therefore, phosphorylation of Bad in BALB3T3 fibroblasts appears to be due to γ-PAK and not α-PAK. We have found that expression of EGFP-γ-PAK-T402E increases phosphorylation of endogenous Bad in response to TNF-α and growth factor withdrawal. Ectopic expression of Bad by retroviral transduction induces cell death in BALB3T3-On cells whereas cells expressing constitutively active EGFP-γ-PAK-T402E are greatly protected. Protection from cell death by recombinant Bad correlates with increased phosphorylation of Bad at Ser-112 and Ser-136. Therefore, phosphorylation of Bad appears to be at least one of the mechanisms by which activated full-length γ-PAK protects BALB3T3 fibroblasts from cell death. Phosphorylation of Bad by α-PAK appears to involve a signaling pathway from Ras via phosphoinositide 3-kinase and Akt to PKA. Interestingly, we did not detect activated Akt with a phospho-specific antibody in BALB3T3 cells (data not shown). The lack of Akt activation might explain why BALB3T3 cells are highly sensitive to TNF-α and growth factor withdrawal and why expression of constitutively active γ-PAK-T402E so effectively protects BALB3T3 fibroblasts from cell death by these stimuli.

Another mechanism by which γ-PAK may affect cell survival is by acting on ERK, JNK, and p38 signaling pathways. ERK, JNK, and p38 pathways are involved in the response to growth factors, cytokines, and stress stimulants. γ-PAK phosphorylates and positively regulates c-Raf, the MEKK in the ERK pathway (19). Expression of constitutively active EGFP-γ-PAK-T402E does not affect basal activity of ERK. In response to TNF-α, EGFP-γ-PAK-T402E stimulates ERK activation within the first 30 min but reduces ERK activity after 1 h. Transient overexpression of PKA in some cell types has been shown to activate JNK and p38 (4, 16–18). Stable expression of EGFP-γ-PAK-T402E stimulates basal activity of JNK-p46 but not JNK-p54. In response to TNF-α, EGFP-γ-PAK-T402E expression also stimulates JNK-p46 activation within the first 30 min but reduces JNK-p46 activity after 1 h. Although stable expression of EGFP-γ-PAK-T402E does not affect basal p38 activity, it stimulates p38 activation within the first 15 min of treatment with TNF-α and greatly reduces p38 activity after 30 min. ERK, JNK, and p38 do not appear to be involved in pro- or anti-apoptotic signaling in response to growth factor withdrawal. Stimulation of early and/or reduction of late activation of ERK, JNK-p46, and p38 by expression of EGFP-γ-PAK-T402E might be crucial for stimulation of cell survival in response to TNF-α but not growth factor withdrawal.

Treatment with TNF-α and growth factor withdrawal induces rapid activation of endogenous full-length γ-PAK and at later time points results in proteolytic activation of γ-PAK by caspases or caspase-like proteases. The early activation of full-length γ-PAK coincides with the phosphorylation of Bad and the early activation of ERK, JNK, and p38. Caspase-activated γ-PAKp34 appears at 6–24 h after treatment and correlates with cell death in BALB3T3-On cells. Because stimulation of caspase 3 activity could not be detected in BALB3T3 fibroblasts in response to stress stimulants, the proteolytic activation of γ-PAK appears to occur by another caspase or caspase-like protease. In MCF-7 breast cancer cells, proteolytic activation of γ-PAK occurs independent of caspase 3. MCF-7 cells are deficient in caspase 3 because of a deletion in the caspase 3 gene.
Our results show that, in addition to the documented pro-apoptotic function after caspase cleavage, γ-PAK has an anti-apoptotic function if activated as a full-length enzyme. This is the first report that demonstrates such an anti-apoptotic activity for the ubiquitous γ-PAK. Activated full-length γ-PAK protects fibroblasts from cell death induced by different pathways and, therefore, appears to play a central role in the decision between cell survival and cell death. One mechanism for protection from cell death by activated full-length γ-PAK is through phosphorylation of the pro-apoptotic Bcl-2 family protein Bad, and other survival mechanisms might include regulation of the stress-induced activation of ERK, JNK, and p38 pathways.

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