Caspase-Activated PAK-2 is Regulated by Subcellular Targeting and Proteasomal Degradation.

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Running title: Targeting and degradation of caspase-activated PAK-2

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ABSTRACT

p21-activated protein kinases (PAK) are a family of serine/threonine protein kinases which are activated by binding of the p21 G-proteins Cdc42 or Rac. The ubiquitous PAK-2 (γ-PAK) is unique among the PAK isoforms, because it is also activated through proteolytic cleavage by caspases or caspase-like proteases. In response to stress stimulants such as tumor necrosis factor α (TNF-α) or growth factor withdrawal, PAK-2 is activated as a full-length enzyme and as a proteolytic PAK-2p34 fragment. Activation of full-length PAK-2 stimulates cell survival, while proteolytic activation of PAK-2p34 is involved in programmed cell death. Here we provide evidence that the pro-apoptotic effect of PAK-2p34 is regulated by subcellular targeting and degradation by the proteasome. Full-length PAK-2 is localized in the cytoplasm while the proteolytic PAK-2p34 fragment translocates to the nucleus. Subcellular localization of PAK-2 is regulated by nuclear localization and nuclear export signal motifs. A nuclear export signal motif within the regulatory domain prevents nuclear localization of full-length PAK-2. Proteolytic activation removes most of the regulatory domain and disrupts the nuclear export signal. The activated PAK-2p34 fragment contains a nuclear localization signal and translocates to the nucleus. However, levels of activated PAK-2p34 are tightly regulated through ubiquitination and degradation by the proteasome. Inhibition of degradation by blocking poly-ubiquitination results in significantly increased levels of PAK-2p34, and as a consequence in stimulation of programmed cell death. Therefore, nuclear targeting and inhibition of degradation appear to be critical for stimulation of the cell death response by PAK-2p34.

INTRODUCTION

In a multicellular organism tissue homeostasis is maintained by the coordinated regulation of proliferation, cell survival and programmed cell death. Proliferation, cell survival
and programmed cell death are regulated by extracellular signals and intracellular signal transduction pathways. Defects in any component of these signal transduction pathways can trigger abnormalities in cell growth, including transformation of cells and the development of tumors. Intracellular signaling pathways depend on controlled spatial and temporal localization of signaling molecules. Signaling molecules have to be targeted to their proper subcellular compartments and in many cases undergo signal-induced translocations. Regulation of proliferation, cell survival and cell death also involves controlled degradation of signaling molecules. The stability and lifetime of many signaling proteins is regulated by ubiquitination and subsequent degradation by the 26S proteasome complex.

Among the many different signaling molecules which regulate cell survival and cell death are the p21-activated protein kinases (PAK). PAKs are activated in response to extracellular signals and regulate cell shape and motility as well as cell survival and programmed cell death. The mammalian PAK family consists of six members which can be divided into two subfamilies according to sequence homology. The first subfamily consists of PAK-1 (α-PAK), PAK-2 (γ-PAK) and PAK-3 (β-PAK). PAK-1 and PAK-3 are tissue-specific with the highest levels in brain, while PAK-2 is ubiquitous (1-7). The second subfamily consists of the more recently identified PAK-4, PAK-5, and PAK-6 (8-10). PAKs have been named according to their activation by the monomeric, p21 G-proteins Cdc42 and Rac (10-13). These p21 G-proteins in the GTP-bound state bind to a conserved region within the N-terminal regulatory domain of PAKs and release block of the catalytic site by an overlapping auto-inhibitory domain (AID) (14). The ubiquitous PAK-2 is unique among the PAK family; it is also activated by proteolytic cleavage by caspases or caspase-like proteases (15,16). Proteolytic cleavage removes most of the N-terminal regulatory domain including the AID and generates constitutively active PAK-2p34, a 34 kDa C-terminal fragment which contains the entire catalytic domain. Caspase-activation has not been demonstrated for any other PAK isoform and among the PAK family appears to be a unique characteristic for PAK-2. Both, activation by Cdc42(GTP) and caspases require autophosphorylation at Thr-402 of PAK-2 (15,17).
Activated full-length PAK-2 stimulates cell survival and cell growth. Expression of constitutively active PAK-2T402E, used to mimic the activation of full-length PAK-2 by activators such Cdc42 and Rac, promotes cell survival and suppresses cell death of BALB3T3 fibroblasts in response to stress stimulants such as TNF-α, growth factor withdrawal, and UVC light (18). The stimulation of cell survival by activated full-length PAK-2 is, at least in part, mediated by phosphorylation and inhibition of the pro-apoptotic Bcl-2 family protein Bad. In addition to PAK-2, PAK-1 and PAK-4 also suppress cell death and promote survival pathways that result in the phosphorylation of Bad (19-21). In contrast to activation of PAK-2 by Rac or Cdc42, cleavage and activation of PAK-2 by caspases or caspase-like proteases correlates with programmed cell death. Caspase-activated PAK-2p34 was observed during Fas- and ceramide-induced apoptotic 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 UV-light-induced apoptosis of A431 cells (16,22,23). Several reports suggest that PAK-2p34 is involved in the execution of programmed cell death. Recombinant expression of PAK-2p34 induced morphological changes characteristic of apoptotic cell death in a variety of cell lines and increased cell death in HeLa and CHO cells (24). In the same report, expression of dominant negative PAK mutants delayed apoptosis of CHO cells in response to Fas receptor ligation. In another report, recombinant expression of PAK-2p34 induced apoptotic cell death of Jurkat cells (25). In Jurkat cells expression of a dominant negative PAK mutant inhibited the formation of apoptotic bodies but did not delay cell death in response to Fas ligation (16). Since dominant negative mutants affect both proteolytically cleaved PAK-2p34 and full-length PAK-2 or other PAKs, the differences in the effect of dominant negative mutants in CHO cells and Jurkat cells could be due to different ratios of anti-apoptotic full-length PAKs and proteolytically cleaved PAK-2.

In this study we show that protein levels of proteolytically activated PAK-2p34 are tightly regulated through degradation. PAK-2p34 but not full-length PAK-2 is rapidly degraded by the 26S proteasome. Stabilization of PAK-2p34 by inhibition of its degradation results in dramatic stimulation of cell rounding and apoptotic chromatin condensation indicating increased
levels of programmed cell death. Furthermore, this stimulation of programmed cell death appears to require the generation and stabilization of PAK-2p34. Therefore, PAK-2 appears to be unique among the PAK isoforms; full-length PAK-2 stimulates cell survival whereas proteolytically activated PAK-2p34 is involved in the cell death response. This dual function could, at least in part, be regulated by subcellular targeting. We show that subcellular localization of full-length PAK-2 and proteolytically activated PAK-2p34 is regulated by signal sequences for nuclear localization and nuclear export. Full-length PAK-2 contains both nuclear localization (NLS) and nuclear export (NES) sequences, but the NES dominates over the NLS and prevents nuclear accumulation of full-length PAK-2. Proteolytic activation disrupts the NES and allows nuclear localization of the PAK-2p34 fragment.

EXPERIMENTAL PROCEDURES

Materials – The inducible retroviral expression system pRevTet-On, the pEGFP-N3 vector, and the monoclonal anti-EGFP antibody were obtained from CLONTECH. The bicistronic retroviral expression vector pRetroIRES-GFP was generated from RetroTet RTRg(-)gfp (Dr. Helen M Blau, Stanford University). The cloning vector pKoz/EGFP and the expression vector pExpress/EGFP were generated as described previously (26,27). pMT107 and pMT123 expression vectors for His-ubiquitin (His-Ub) and HA-ubiquitin (HA-Ub), respectively were gifts from Dr. D. Bohmann, Rochester University. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. Cloned pfu Turbo DNA polymerase was purchased from Stratagene. Ni²⁺-NTA-agarose and kits for plasmid DNA isolation were obtained from QIAGEN. TransIT-LT1 and TransIT-HeLaMONSTER transfection reagents were from Mirus. Super Signal Pico chemiluminescence reagent, M-PER Mammalian Protein Extraction Reagent and Gelcode Blue Staining Reagent were from Pierce. Dulbecco's Modified Eagle Medium (DMEM) and customized primers were obtained from Invitrogen/Life Technologies. Fetal
bovine serum (FBS) was from Hyclone. Tumor necrosis factor (TNF)-α, ATP, MG-132, lactacystin and E64 were purchased from Calbiochem. Monoclonal anti-FLAG antibody, polyclonal anti-glucose-6-phosphate-dehydrogenase antibody, Hoechst 33342 and myelin basic protein were from Sigma. The antibody specific for full-length PAK-2 (γPAK-V19), a C-terminal antibody that detects PAK-1, PAK-2 and PAK-3 (αPAK-C19), and the anti-Oct-1 (C-21) antibody were purchased from Santa Cruz. The polyclonal anti-ubiquitin antibody was a gift from Dr. A. Haas, Medical College of Wisconsin. Leptomycin B was purified by Dr. B. Wolff, Novartis, Austria (28), and provided to us by Dr. P. Ferreira, Medical College of Wisconsin. [γ-32P]ATP was purchased from Perkin Elmer Lifesciences.

**Subcloning and Site-directed Mutagenesis** - cDNAs encoding the protein coding region of PAK-2p34 and PAK-2p34K278R were amplified by PCR with *pfu* Turbo DNA polymerase using p34-5’ (5’-ATCTCGAGCATATGAGTGGTGCCAAGTCTTCAG) and PAK-2-3’ (5’-CGGCAGGATCTTAGCGGTTACTCTTCATTGC) oligonucleotide primers and wild-type PAK-2 or the kinase-deficient mutant PAK-2K278R as templates, respectively. The cDNA encoding the regulatory domain (RD) of PAK-2 was amplified by PCR using PAK-2-5’ (5’-TCTGACATATGCTCTGATAACGGAGAAGCTGG) and RD-3’ (5’-TGAATTCTAGAGGATCTTAGGGTCACCTATGCT) oligonucleotide primers and wild-type PAK-2 as template. The fragment 197-246 was amplified with 197-5’ (5’-TCTGACATATGCTCTGATAACGGAGAAGCTGG) and RD-3’ (5’-TGAATTCTAGAGGATCTTAGGGTCACCTATGCT) oligonucleotide primers and wild-type PAK-2 as template. The fragment 197-246 was amplified with 197-5’ (5’-TCTGACATATGCTCTGATAACGGAGAAGCTGG) and RD-3’ (5’-TGAATTCTAGAGGATCTTAGGGTCACCTATGCT) oligonucleotide primers, the fragment 213-246 with p34-5’ and RD-3’ oligonucleotide primers, and the fragment 197-240 with 197-5’ and 240-3’ (5’-TGTATGGATCCTACACAAATAGTTATGTAATTTCCTCCAT) oligonucleotide primers. PAK-2RDmuNES was amplified with PAK-2-5’ and muNES-3’ (5’-TAATGGATCCTACTTACACAAATAGTTATGTAATTTCCTCCAT) oligonucleotide primers. PAK-2(197-524)muNLS was generated by site-directed mutagenesis according to the megaprimer PCR method (29,30). The megaprimer was amplified with muNLS-5’ (5’-ACCTATGCATGAATATACAAAGATATGAAAAATTGG) and PAK-2-
3’ oligonucleotide primers and wild-type PAK-2 as template. Then, the megaprimer was used together with the 197-5’ oligonucleotide primer and wild-type PAK-2 as template to amplify PAK-2(197-524)μuNLS. Amplified cDNAs were subcloned into pKoz/EGFP, pExpress/EGFP and/or pKoz/FLAG to form fusions with enhanced green fluorescent protein (EGFP) or the FLAG tag using NdeI and BamHI sites incorporated into the oligonucleotide primers. The previously described EGFP-PAK-2T402E was subcloned into pKoz/FLAG. Sense (5’-TATGCCTAAGAAAAATATACACAGATAAG) and antisense (5’-GATCCTTATCTTTGTATTTTTTCTTAGGCA) oligonucleotides encoding the putative NLS were annealed and subcloned into pExpress/EGFP using NdeI and BamHI sites. New cDNA constructs were sequenced to confirm the clones and to ensure the absence of accidental mutations due to misincorporation during PCR and cloning. EGFP-PAK-2p34 was subcloned into the inducible retroviral expression vector pRevTRE. FLAG-PAK-2p34 FLAG-PAK-2p34K278R and FLAG-PAK-2T402E were subcloned into the bicistronic retroviral expression vector pRetroIRES-GFP.

**Cell Culture, Transfection and Retroviral Transduction** - The human embryonic kidney cell line 293T, the ecotropic 293T-cell derived packaging cell line Phoenix Eco (31), the mouse fibroblast cell line BALB3T3 (32), the human cervix carcinoma cell line HeLa (33), and the human breast cancer cell line Hs578T (34) were obtained from American Tissue Culture Collection (ATCC). Cells were maintained in DMEM containing 10% heat-inactivated FBS, 2 mM glutamine 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 overnight at -80°C and stored in liquid nitrogen. For transient expression 293T, BALB3T3 and Hs578T cells were transfected with TransIT-LT1 transfection reagent, and HeLa cells with TransIT-HeLaMONSTER transfection reagent. Ecotropic retroviruses for the transduction of murine cells were obtained by transient transfection of the retroviral vectors into the packaging cell line Phoenix Eco. 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 approximately 10 to 20% confluency in 100-mm culture dishes and then transduced by addition of 0.5 to 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 mg/ml geneticin or 300 mg/ml hygromycin B was added at 48 h to select stable cell populations for pRevTet-On or pRevTRE, respectively. BALB3T3-On cells, a stable cell line for the expression of the reversed tetracycline/doxycycline-controlled transactivator were obtained as described previously (18).

**Analysis of Programmed Cell Death** - Cells transfected with an EGFP expression vector alone, co-transfected with an EGFP expression vector and the His-Ub expression vector, or co-transfected with the bicistronic vector for EGFP and FLAG-PAK-2p34 expression and the His-Ub expression vector were stained with 10 µg/ml Hoechst 33342 for 10 min and analyzed by fluorescence microscopy. To determine levels of programmed cell death 500 transfected (EGFP-positive) cells were counted and analyzed for apoptotic chromatin condensation.

**Purification of Ubiquitinated Protein** – Cells transfected with constructs for PAK-2p34, His-Ub, or both were lysed in 1 ml of 6M guanidin-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄, pH 8.0 plus 5mM imidazole per 100 mm dish at 40 h after transfection (35). The lysate (1 ml) was sonicated to reduce viscosity and centrifuged at 12,000 g to remove insoluble material. The cleared lysate was incubated with 0.1 ml bed volume of Ni²⁺-NTA-agarose for 4 h at room temperature. The mixture was applied to a spin column and centrifuged at 500 g. The spin column was washed successively with 0.5 ml of 6M guanidin-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄, pH 8.0; 1 ml of 6M guanidin-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄, pH 5.8, 0.5 ml of 6M guanidin-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄, pH 8.0; 1 ml of 6M guanidin-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄, pH 8.0/protein buffer (50 mM Na₂HPO₄/NaH₂PO₄, pH 8.0, 0.1 M KCl, 20 % glycerol, and 0.2 %
NP-40) 1:1; 1 ml of 6 M guanidin-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄, pH 8.0/protein buffer 1:3; 1 ml protein buffer; and 1 ml protein buffer plus 10 mM imidazole. Elution was in 0.5 ml protein buffer plus 0.2 M imidazole. The eluate was precipitated with 12% TCA, washed with ice-cold ethanol and dissolved in 0.1 ml of SDS sample buffer.

**Western Blot** - Cells were lysed in M-PER Mammalian Protein Extraction Reagent containing 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 25 mM NaF, 25 mM β-glycerophosphate and 200 µM sodium vanadate. Protein concentrations were determined by a Bradford protein assay using bovine γ-globulin as a standard protein. Western blots were performed with cell lysates (30 µg) or 20 µl aliquots of the Ni²⁺-NTA purification by SDS-polyacrylamide gel electrophoresis, semidy transfers onto polyvinylidene membranes and detection by chemiluminescence using horseradish peroxidase-conjugated secondary antibodies and Supersignal reagent.

**In-Gel Protein Kinase Assays** - In-gel assays were performed with 0.1 mg/ml of myelin basic protein co-polymerized in the separating gel of 11% SDS-polyacrylamide gels (36). Cell lysates were prepared as described above. Cell lysates (30 µg) were separated by electrophoresis in the substrate-containing gels. After electrophoresis, gels were washed twice for 1 h in 50 mM Tris-HCl, pH 8.0, 20% 2-propanol, once for 1 h in 50 mM Tris-HCl, pH 8.0, 2 mM 2-mercaptoethanol (buffer A), and denatured twice for 1 h in buffer A containing 6 M guanidine-HCl at room temperature. Renaturation was performed with 5 changes of buffer A containing 0.04% Tween 40 for 16 to 24 h at 4°C. Phosphorylation was carried out in 20 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 0.4 mM EGTA, 30 mM 2-mercaptoethanol and 50 µM [γ⁻³²P]ATP (500 cpm/pmol) for 1 h at room temperature. Excess of [γ⁻³²P]ATP was removed by washing in 5% trichloroacetic acid, 1% sodium pyrophosphate. Gels are stained with Gelcode Blue, dried and subjected to autoradiography.
Separation of Nuclei and Cytoplasm – BALB2T3 fibroblasts were resuspended in 0.5 ml/10 cm culture dish of lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM MgCl₂, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 200 µM sodium vanadate) and incubated on ice for 15 min. An aliquot of 200 µl of lysate was saved for Western blot analysis. Nuclei were collected by centrifugation at 500 g for 5 min at 4°C. The supernatant was transferred to a new tube and re-centrifuged at 12,000 g for 15 min at 4°C. The resulting supernatant is the cytoplasm. The nuclear pellet was washed twice with 1 ml lysis buffer and re-centrifuged each time at 500 g for 5 min at 4°C. The final nuclear pellet was resuspended in 200 µl lysis buffer. Lysate, cytoplasm and nuclei were adjusted to 1x SDS sample buffer and analyzed by Western blot.

RESULTS

Recombinant Expression of PAK-2p34 – To study the role of proteolytically activated PAK-p34, we generated a construct for the expression of the proteolytic fragment from amino acid residues 213 to 524 of PAK-2. The cDNA segment corresponding to amino acid residues 213 to 524 of PAK-2 was subcloned with an N-terminal EGFP tag (EGFP-PAK-2p34) into the retroviral expression vector pRev-TRE. We observed that transfection of a vector that contains EGFP-PAK-2p34 only results in 1% to 2% of 293T cells showing green fluorescence, whereas transfection of a vector that contains EGFP alone or EGFP fusion proteins with full-length PAK-2 or the regulatory domain of PAK-2 results in 25% to 50% of 293T cells that show green fluorescence. Expression of PAK-2p34 has been shown to stimulate apoptosis in HeLa, CHO, and Jurkat cells (24,25). Therefore, the low apparent transfection rate of EGFP-PAK-2p34 could be due to stimulation of cell death of 293T cells by EGFP-PAK-2p34. However, we have not observed cell death at a sufficient rate to account for the low apparent transfection rate. To determine the reason for the low apparent transfection rate of EGFP-PAK-2p34, we used a
bicistronic expression vector with an internal ribosome entry site (IRES) to express FLAG-PAK-2p34 and EGFP as independent proteins. 293T cells were transfected with either a bicistronic construct for expression of EGFP and FLAG-PAK-2p34 or a construct for expression of an EGFP-PAK-2p34 fusion protein (Fig. 1A). Cells transfected with the bicistronic construct for expression of EGFP and FLAG-PAK-2p34 showed a much higher apparent transfection rate as indicated by the number of green fluorescent cells than cells transfected with the construct for expression of EGFP-PAK-2p34. Therefore, it appears that the low apparent transfection rate with EGFP-PAK-2p34 is caused by degradation of the EGFP-PAK-2p34 protein rather than by cell death. If cell death was the cause, we would also have expected a low apparent transfection rate for the bicistronic construct for expression of EGFP and FLAG-PAK-2p34.

**PAK-2p34 is Degraded by the 26S Proteasome Complex** - To determine if degradation of EGFP-PAK-2p34 is mediated by the 26S proteasome, we treated 293T cells transfected with either the bicistronic construct for expression of EGFP and FLAG-PAK-2p34, or the construct for expression of an EGFP-PAK-2p34 fusion, with the proteasome inhibitor MG-132. Treatment with the proteasome inhibitor significantly increased the number of green fluorescent cells in cultures that were transfected with the construct for expression of EGFP-PAK-2p34, but not in cultures transfected with the bicistronic construct for expression of EGFP and FLAG-PAK-2p34 (Fig. 1A). Western blot analyses of 293T cell lysates with monoclonal anti-EGFP and anti-FLAG antibodies confirmed these results. Treatment with the proteasome inhibitor significantly increased the amount of EGFP-PAK-2p34 and FLAG-PAK-2p34, but not of EGFP (Fig. 1B). Western blot analyses of an EGFP fusion protein of full-length PAK-2 (EGFP-PAK-2) showed similar amounts in the absence and presence of the proteasome inhibitor. EGFP-PAK-2p34 or EGFP alone were also stably transduced into BALB3T3 mouse fibroblasts using the retroviral inducible expression system RevTet-On. Expression was induced with doxycycline and cells were incubated in absence and presence of MG-132. Western blot analysis with the anti-EGFP antibody showed that levels of EGFP-PAK-2p34 but not EGFP were increased in presence of the
proteasome inhibitor (Fig. 1C). Similar results were obtained with another proteasome inhibitor, lactacystin whereas the lysosomal cysteine protease inhibitor E64 had no effect on EGFP-PAK-2p34 levels (data not shown). The findings indicate that proteolytically activated PAK-2p34, but not full-length PAK-2 is rapidly degraded by the proteasome.

Endogenous PAK-2p34 is generated by caspase or caspase-like protease mediated cleavage in response to apoptotic stimulants. We have shown previously that incubation of BALB3T3 cells with 1 ng/ml TNF-α or incubation in serum-free medium induces cell death and results in the generation of PAK-2p34 (18). Degradation by the proteasome could be a mechanism to regulate the levels of PAK-2p34 and prevent programmed cell death. To determine if endogenous PAK-2p34 is also degraded by the proteasome, we treated BALB3T3 mouse fibroblasts with TNF-α or by growth factor withdrawal (Fig. 2). Activity bands of full-length PAK-2 were detected at 62 and 58 kDa by in-gel assays in lysates of untreated BALB3T3 fibroblasts as well as cells treated with pro-apoptotic stimulants such as TNF-a, or growth factor withdrawal, whereas caspase-activated PAK-2p34 was only detected after treatment with the pro-apoptotic stimulants (18). Therefore, we determined effects of the proteasome inhibitor MG-132 on accumulation of endogenous PAK-2p34 at 6 h and 24 h of incubation with 1 ng/ml TNF-α or in serum-free medium by in-gel assays with myelin basic protein as a substrate. Untreated control cells, cells with TNF-α or cells in serum-free medium were incubated for 6 h in the absence or presence of 50 μM MG-132 (Fig. 2A). Incubation of BALB3T3 cells with proteasome inhibitors for more than 6 h results in stimulation of cell death. For the 24 h time point, untreated control cells, cells with TNF-α or cells in serum-free medium were pre-incubated for 18 h and then incubated for an additional 6 h in the absence or presence of 50 μM MG-132 (Fig. 2B). In the absence of proteasome inhibitor, untreated control cells showed no PAK-2p34 at 6 or 24 h, while cells treated with TNF-α or by growth factor withdrawal showed increasing amounts of PAK-2p34 from 6 h to 24 h. Presence of the proteasome inhibitor dramatically increased the levels of PAK-2p34 at 6 and 24 h in cells treated with TNF-α or by growth factor withdrawal. Incubation with the proteasome inhibitor also resulted in increased
levels of PAK-2p34 in control cells. It appears that inhibition of proteasomal degradation results in accumulation of PAK-2p34 that is generated by basal activities of caspases or caspase-like proteases. The results suggest that levels of endogenous PAK-2p34 are regulated through cleavage by caspases or caspase-like proteases and through degradation by the 26S proteasome complex.

Proteins are targeted for degradation by the 26S proteasome by covalent conjugation of ubiquitin chains. To determine if degradation of PAK-2p34 is accompanied by ubiquitination, FLAG-PAK-2p34 was co-transfected with a construct for the expression of ubiquitin with a N-terminal His-tag (His-Ub). This approach allows the isolation of ubiquitinated proteins by affinity chromatography on a Ni\(^{2+}\)-NTA resin under denaturing conditions to prevent degradation and de-ubiquitination (35). Surprisingly, co-expression of epitope-tagged ubiquitin stabilized the levels of PAK-2p34 even more than the proteasome inhibitor (Fig. 3). This stabilization was specific for PAK-3p34, since FLAG-PAK-2p34 and kinase-deficient FLAG-PAK-2p34K278R levels were increased, whereas constitutively active full-length FLAG-PAK-2T402E or EGFP were not affected (Fig. 3A). Co-transfection with an empty vector or increasing amounts of His-Ub showed that the effect is specific and dose-dependent (Fig. 3B). Similar results were obtained when HA-Ub was co-transfected indicating that the effect is not due to the His tag. Co-expression of His or HA-tagged ubiquitin appears to inhibit degradation of PAK-2p34. Conjugation of epitope-tagged ubiquitin has been shown previously to inhibit degradation of certain proteins (37). However, the degree of this inhibition varies from virtually quantitative to not measurable. For example, co-expression of His or HA-tagged ubiquitin did not affect degradation of c-Jun (35).

To determine if PAK-2p34 undergoes ubiquitination, cells which express FLAG-PAK-2p34 and His-Ub were lysed under denaturing conditions and His-Ub conjugates were isolated using Ni\(^{2+}\)-NTA agarose (Fig. 3C). Cells which express FLAG-PAK-2p34 or His-Ub alone were used as controls. Lysates and eluates from the Ni\(^{2+}\)-NTA-agarose were analyzed by Western blot with a C-terminal anti-PAK antibody (αPAK-C19) to detect proteolytically activated PAK-2p34.
This antibody cross-reacts with PAK-1, PAK-2 and PAK-3, but since PAK-1 and PAK-3 are not proteolytically activated, it is specific for caspase-cleaved PAK-2p34. In the lysates from cells which express FLAG-PAK-2p34 alone a weak band was detected at approximately 34 kDa which corresponds to unmodified FLAG-PAK-2p34. In the lysates from cells which express both FLAG-PAK-2p34 and His-Ub, the levels of unmodified FLAG-PAK-2p34 were significantly increased and a weak band was detected at approximately 42 kDa, which would correspond to mono-ubiquitinated FLAG-PAK-2p34. As expected FLAG-PAK-2p34 was not detected in lysates of cells which express His-Ub alone. Endogenous full-length PAK-2 and PAK-1 were detected in all lysates. After Ni\textsuperscript{2+}-NTA-agarose affinity purification the anti-FLAG antibody detected a band at approximately 42 kDa and a weaker band at approximately 50 kDa, which would correspond to mono- and bi-ubiquitinated FLAG-PAK-2p34, in samples from cells that co-express FLAG-PAK-2p34 and His-Ub. The weak band corresponding to bi-ubiquitinated FLAG-PAK-2p34 suggests that some longer ubiquitin chains can be formed if endogenous untagged ubiquitin is conjugated to FLAG-PAK-2p34 first. A weak band corresponding to unmodified FLAG-PAK-2p34 suggests that a small amount of unmodified FLAG-PAK-2p34 co-purifies with His-Ub conjugated FLAG-PAK-2p34. No PAK-2 immunoreactive bands were detected in Ni\textsuperscript{2+}-NTA-agarose purified samples from cells that express FLAG-PAK-2p34 or His-Ub alone. Similar results were obtained when the samples were analyzed with an anti-FLAG antibody. As a control lysates and eluates from the Ni\textsuperscript{2+}-NTA-agarose were also analyzed with an anti-ubiquitin antibody. Ubiquitin conjugates were detected in all lysates. After Ni\textsuperscript{2+}-NTA-agarose affinity purification ubiquitin conjugates were detected in samples from cells which express FLAG-PAK-2p34 and His-Ub or His-Ub alone. These represent proteins which underwent ubiquitination with His-Ub. As expected purified samples from cells which express FLAG-PAK-2p34 alone showed no ubiquitin conjugates. Similar results were obtained when the samples were analyzed with an anti-His antibody. The results suggest that the incorporation of a His-tagged ubiquitin prevents further addition of ubiquitin and locks most of PAK-2p34 in the
mono-ubiquitinated state. Since degradation by the proteasome requires poly-ubiquitination this might explain the stabilization of PAK-2p34 by co-expression of His- or HA-tagged ubiquitin.

**Stabilization of PAK-2p34 Causes Programmed Cell Death** – Since co-expression of epitope-tagged ubiquitin inhibited degradation of recombinant PAK-2p34, we examined if stabilization of PAK-2p34 results in induction of programmed cell death. 293T cells were transfected with the bicistronic construct for expression of EGFP and FLAG-PAK-2p34 alone or co-transfected with the bicistronic construct and the construct for expression of His-Ub. Transfected cells were incubated for 48 h and analyzed for apoptotic morphological changes (Fig 4). Cellular morphology was visualized by fluorescence of expressed EGFP, nuclear morphology was visualized by staining with Hoechst 33342. Cells co-transfected with the bicistronic construct for EGFP and FLAG-PAK-2p34 and a construct for His-Ub showed cell rounding, whereas cells transfected with the bicistronic construct for EGFP and FLAG-PAK-2p34 alone displayed no significant changes in cell morphology (Fig. 4A). Nuclear staining showed that the rounded cells also displayed apoptotic chromatin condensation (Fig. 4B). Co-expression of His-Ub and FLAG-PAK-2p34 resulted in cell rounding and apoptotic chromatin condensation. Eventually, at 48 h to 72 h after transfection, cells detached and/or formed apoptotic bodies (data not shown). Control cells co-transfected with a construct for EGFP and a construct for His-Ub showed no cell rounding and no apoptotic chromatin condensation. The data suggest that stabilization of PAK-2p34 induces programmed cell death. To determine if the induction of programmed cell death requires protein kinase activity, 293T cells were co-transfected with a bicistronic construct for EGFP and FLAG-PAK-2p34K278R or the bicistronic construct alone. Co-expression of kinase-deficient FLAG-PAK-2p34K278R did not result in cell rounding or apoptotic chromatin condensation. To determine if the induction of programmed cell death is specific for caspase-activated PAK-2p34, 293T cells were co-transfected with a bicistronic construct for EGFP and FLAG-PAK-2T402E or the bicistronic construct alone. Co-expression of constitutively active FLAG-PAK-2T402E did not result in cell rounding or apoptotic chromatin condensation.
condensation. These results indicate that protein kinase activity of caspase-activated but not full-length PAK-2 induces programmed cell death. Cell rounding and apoptotic chromatin condensation were also observed when EGFP-PAK-2p34 was co-expressed with His-Ub indicating that the epitope tag does not affect the death response (data not shown). Therefore, cell rounding and apoptotic chromatin condensation are specific effects of PAK-2p34 and correlate with its stabilization by overexpression of epitope-tagged ubiquitin.

Incubation with proteasome inhibitor increased levels of endogenous PAK-2p34 in BALB3T3 cells suggesting that PAK-2p34 is generated by basal activities of caspases or similar proteases. Therefore, we examined whether expression of epitope-tagged ubiquitin increases levels of endogenous PAK-2p34 and programmed cell death. BALB3T3, HeLa, Hs578T, and 293T cells were transfected with an EGFP expression vector alone, or co-transfected with EGFP and His-Ub expression vectors and incubated for 48 h (Fig. 5). Expression of His-Ub stimulated levels of endogenous PAK-2p34 in BALB3T3, HeLa and Hs578T cells but not in 293T cells, whereas full-length PAK-2 was detected in all cell lines independent of the expression of His-Ub (Fig. 5A). However, His-Ub stimulated levels of FLAG-PAK-2p34 in 293T cells co-transfected with the bicistronic construct for expression of EGFP and FLAG-PAK-2p34. Therefore, endogenous PAK-2p34 generated by basal activities of caspases or similar proteases appears to be stabilized by His-Ub in BALB3T3, HeLa, and Hs578T cells. Since His-Ub did not increase levels of endogenous PAK-2p34 but increased levels of recombinant FLAG-PAK-2p34, it appears that there is no basal cleavage of endogenous PAK-2p34 in 293T cells. In addition, 293T, unlike BALB3T3, HeLa, and Hs578T cells, do not generate endogenous PAK-2p34 in response to apoptotic stimuli (data not shown). Expression of His-Ub also stimulated programmed cell death in BALB3T3, HeLa, and Hs578T cells but not in 293T cells. His-Ub increased the percentage of transfected cells showing apoptotic chromatin condensation from 9% to 37% in BALB3T3 cells, from 10% to 53% in HeLa cells and from 11% to 54% in Hs578T cells. In 293T cells His-Ub increased the percentage of transfected cells showing apoptotic chromatin condensation only slightly from 3% to 10% whereas in 293T which express
recombinant FLAG-PAK-2p34 His-Ub the percentage increased from 4% to 80%. Therefore, levels of programmed cell death correlate with the stabilization of endogenous or recombinant PAK-2p34.

Subcellular Localization of PAK-2 – Full-length PAK-2 and PAK-2p34 contain the same protein kinase domain but have opposing effects on cell viability. Activated full-length PAK-2 stimulates cell survival whereas caspase-activated PAK-2p34 induces a cell death response. Therefore, we examined if full-length PAK-2 and caspase-activated PAK-2 differ in their subcellular localization. The localization of recombinant PAK-2 was examined using EGFP fusion constructs (18,26). The EGFP fusion proteins and EGFP alone were transiently expressed in 293T cells and subcellular localization was monitored by fluorescence microscopy. EGFP alone was evenly distributed throughout the cell with no particular subcellular localization. An EGFP fusion protein of full-length PAK-2 (EGFP-PAK-2) was localized in the cytoplasm, while an EGFP fusion protein of PAK-2p34 (EGFP-PAK-2p34) was localized in the cytoplasm and the nucleus (Fig. 6). This suggests that full-length PAK-2 is actively excluded or exported from the nucleus. Since an EGFP fusion protein of the regulatory domain of PAK-2 (EGFP-PAK-2RD) was also localized in the cytoplasm, the nuclear exclusion or nuclear export signal appears to exist within the regulatory domain. To distinguish between nuclear exclusion and nuclear export, 293T cells which express EGFP-PAK-2RD were treated with the nuclear export inhibitor leptomycin B. Subcellular localization of EGFP-PAK-2RD was monitored by fluorescence microscopy after 3 h of treatment. Cells treated with 10 or 30 ng/ml of leptomycin B showed nuclear localization of EGFP-PAK-2RD (Fig. 6). Since leptomycin B is an inhibitor of nuclear exportin-1 (CRM1), it appears that EGFP-PAK-2RD is exported from nuclei by an exportin-1-dependent mechanism.

In order to determine the subcellular localization of endogenous PAK-2 we carried out cell fractionation of BALB3T3 fibroblasts. To stimulate proteolytic activation of PAK-2 by caspases or caspase-like proteases cells were treated by growth factor withdrawal for 24 h.
Treated cells and control cells were homogenized and separated into nuclei and cytoplasm. Western blots were performed to examine the localization of full-length PAK-2 and proteolytically activated PAK-2p34 (Fig 7). A N-terminal antibody that is specific for PAK-2 (γPAK-V19) was used to detect full-length PAK-2. A C-terminal antibody that detects PAK-1, PAK-2 and PAK-3 (αPAK-C19) was used to detect proteolytically activated PAK-2p34. Since PAK-1 and PAK-3 are not proteolytically activated, this antibody is specific for caspase-cleaved PAK-2p34. Full-length PAK-2 was detected in total cell lysates and the cytoplasm but not in the nuclei of untreated control cells and cell treated by growth factor withdrawal. Caspase-activated PAK-2p34 was not detected in control cells. In cells treated by growth factor withdrawal caspase-activated PAK-2p34 was present in the total cell lysate and in the nuclei. The purity of the cytoplasmic and nuclear fractions was analyzed with antibodies for glucose-6-phosphate-dehydrogenase as a cytosolic marker and the transcription factor Oct-1 as a nuclear marker. The cytosolic fractions showed no significant contamination of the nuclear marker, and the nuclear fractions showed no significant contamination of the cytosolic marker. Since proteolytically activated PAK-2p34 was detected in the nuclear fraction whereas full-length PAK-2 was detected in the cytoplasmic fraction, it appears that PAK-2p34 generated in response to growth factor withdrawal translocates to the nucleus.

Subcellular Localization of PAK-2 is Regulated by Nuclear Localization and Nuclear Export Signals - In order to identify the motifs which regulate the subcellular localization of PAK-2 we generated EGFP fusion constructs of PAK-2 fragments. The EGFP fusion proteins were transiently expressed in 293T cells and subcellular localization was determined by fluorescence microscopy (Fig. 8). The minimal sequence which was sufficient for nuclear export was the 50 amino acid segment of residues 197 to 246. The segment containing residues 213 to 246, the N-terminal 34 amino acid residues of PAK-2p34, showed cytoplasmic and nuclear localization in most cells. However, partial nuclear export was observed in a small subset of cells. This indicates that residues within 197 to 212 are required for efficient nuclear export and
that PAK-2p34 can accumulate in the nucleus because part of a nuclear export signal (NES) is removed by proteolytic cleavage. The segment containing residues 197 to 240 was detected in both the cytoplasm and the nuclei. Therefore, the C-terminal part of the regulatory domain appears to be strictly required for nuclear export. Concomitantly, a mutant regulatory domain, in which the motif IVSIG from amino acid residues 239 to 243 was changed to REGRS (PAK-2RDmuNES), showed no nuclear export and accumulated in the nucleus.

Computer analysis with PSORT II prediction software identifies the motif PKKKYTR from amino acid residues 245 to 251 as a potential nuclear import signal (NLS). To determine if this motif serves as a NLS, we cloned complementary synthetic oligonucleotides encoding the motif C-terminal of the EGFP open reading frame, and expressed an EGFP-NLS fusion protein in 293T cells (Fig. 8). In addition, we generated a mutant PAK-2p34 which contains a functional NES and a disrupted NLS motif (PAK-2(197-524)muNLS). To restore the NES motif we added residues 197 to 212 to the N-terminus of PAK-2p34 which generates a PAK-2 fragment from residues 197 to 524. To disrupt the NLS motif we changed the three lysine residues in the NLS motif PKKKYTR to PMHEYTR. The EGFP-NLS showed no exclusive nuclear localization but enhanced nuclear accumulation, suggesting that the PKKKYTR motif may not be sufficient to promote exclusive nuclear localization. The EGFP-PAK-2(197-524)muNLS, which contains a functional NES but a mutant NLS motif, showed only little nuclear localization, indicating that the NLS motif PKKKYTR is required for nuclear localization. In a protein kinase assay with immunoprecipitated EGFP-PAK-2 fusion proteins PAK-2(197-524)muNLS showed similar activity towards myelin basic protein as PAK-2p34, indicating that the mutations do not affect protein kinase activity (data not shown). Thus, we have identified signals for nuclear localization and nuclear export that regulate the subcellular localization of PAK-2.
DISCUSSION

Activation of full-length PAKs by stimulators such as Cdc42 or Rac stimulates cell survival and protects cells from programmed cell death. Expression of constitutively active mutants of PAK-1, PAK-2 and PAK-4 have been shown to suppress programmed cell death through stimulated phosphorylation of the pro-apoptotic Bcl-2 family protein Bad. In contrast, proteolytic activation by caspases or caspase-like proteases, which among the PAK family is a unique feature of PAK-2, appears to be involved in cell death. Proteolytic activation which results in the constitutively active PAK-2p34 fragment is observed in response to different pro-apoptotic stimuli in a variety of cell lines (16,22,23). However, only limited evidence exists for a direct involvement of PAK-2p34 in the cell death response. Recombinant expression of PAK-2p34 has so far only been shown to stimulate cell death in Jurkat, HeLa and CHO cells (24,25). Here, we show that PAK-2p34 is rapidly degraded in HEK 293T cells and mouse BALB3T3 fibroblasts and that significant levels of cell death are only observed if degradation of PAK-2p34 is inhibited.

Surprisingly, degradation of PAK-2p34 can be inhibited by co-expression of epitope-tagged ubiquitin. A similar effect has been observed previously with L-β-galactosidase which was stabilized by co-expression of Myc-tagged ubiquitin (37). However, this is not a general phenomena since most proteins are still degraded when epitope-tagged ubiquitin is co-expressed. For example, co-transfection of the same His- or HA-tagged ubiquitin plasmids as used in our study, resulted in normal ubiquitination and degradation of c-Jun (35). In the case of PAK-2p34, conjugation of a His-tagged ubiquitin appears to block further ubiquitin conjugation. Since poly-ubiquitination is required for degradation by the proteasome this would be sufficient to prevent degradation. Inhibition of PAK-2p34 degradation with proteasome inhibitors results in increased levels of unmodified PAK-2p34, but not as expected in accumulation of mono- or poly-ubiquitinated PAK-2p34. Similarly inhibition of PAK-2p34 degradation by conjugation of His-tagged ubiquitin does only result in small amounts of mono-ubiquitinated PAK-2p34, while
most of the PAK-2p34 accumulates in the unmodified form. This suggests that ubiquitination is in balance with de-ubiquitination (37-39), and if poly-ubiquitination and/or degradation is blocked the balance shifts in favor of de-ubiquitination.

Endogenous PAK-2p34 also appears to be degraded by the proteasome. PAK-2p34 generated in response to TNF-a or growth factor withdrawal is increased in the presence of proteasome inhibitor. This suggests that PAK-2p34 that is generated through proteolytic cleavage by caspases or similar proteases is also ubiquitinated and degraded by the 26S proteasome. In the presence of proteasome inhibitor or expression of epitope-tagged ubiquitin PAK-2p34 is also generated in the absence of an apoptotic stimulus. This suggests that PAK-2p34 is always generated by a basal activity of a caspase or a similar protease, but is normally degraded by the proteasome to prevent cell death. Therefore, levels of PAK-2p34 appear to be regulated by cleavage through caspases or similar proteases and by ubiquitination and degradation by the 26S proteasome. Apoptotic stimuli could increase generation of PAK-2p34 through activation of caspases or similar proteases, and reduce degradation of PAK-2p34 through several potential mechanisms that decrease the level of ubiquitinated PAK-2p34. First, the conjugation of ubiquitin onto proteins is a reversible process, and ubiquitin chains on PAK-2p34 could be removed by de-ubiquitinating enzymes such as ubiquitin C-terminal hydrolases or ubiquitin-specific processing proteases (38). For example, the tumor suppressor protein p53 is stabilized through de-ubiquitination by the ubiquitin-specific processing protease HAUSP (39,40). Since inhibition of PAK-2p34 degradation does not result in accumulation of ubiquitinated PAK-2p34 but in increased levels of the unmodified form, it appears that degradation of PAK-2p34 can be regulated by competing ubiquitinating and de-ubiquitinating activities. Second, ubiquitination (or de-ubiquitination) of PAK-2p34 could be regulated by phosphorylation. For example, phosphorylation of IκBα, the inhibitor of NFκB triggers subsequent ubiquitination and degradation of IκBα (41,42). In addition, the activity of the ubiquitin ligase APC is regulated by phosphorylation (43). Therefore, phosphorylation of PAK-2p34 or of an ubiquitinating or de-ubiquitinating enzyme could regulate degradation of PAK-
Third, ubiquitination of PAK-2p34 could be prevented by sumoylation. For example, covalent modification of the oncogene product Mdm2 by the ubiquitin-related protein SUMO1 protects it from ubiquitination and subsequent degradation (44). In a similar manner competing sumoylating and ubiquitinating enzymes could regulate degradation of PAK-2p34.

Stabilization of endogenous or recombinant PAK-2p34 by expression of epitope-tagged ubiquitin resulted in a dramatic increase of cells undergoing programmed cell death. This effect was specific for active PAK-2p34 since co-expression of His-tagged ubiquitin with PAK-2p34K278R, a kinase-deficient PAK-34 mutant, and with PAK-2T402E, a constitutively active full-length PAK-2 mutant, did not result in stimulation of cell death. In BALB3T3, HeLa and Hs578T cells stimulation of programmed cell death by expression of His-Ub correlated with increased levels of endogenous PAK-2p34. In 293T cells expression of His-Ub only increased programmed cell death slightly and did not increase levels of endogenous PAK-2p34. However, expression of recombinant PAK-2p34 in 293T cells restored the stimulation of programmed cell death by expression of His-Ub indicating that PAK-2p34 is involved in programmed cell death. The results suggest that degradation of caspase-activated PAK-2p34 by the proteasome protects cells from cell death and that stimulation of cell death by PAK-2p34 requires its stabilization by inhibiting its ubiquitination and degradation by the proteasome.

It is unclear how the anti- and pro-apoptotic functions of PAK-2 and PAK-2p34 are regulated, since both forms contain the same protein kinase domain. Their active sites will likely recognize the same consensus sequences for phosphotransfer. Therefore, substrate specificity must be determined by other mechanisms such as specific protein-protein interactions outside the active site and/or subcellular localization. We observed that recombinant and endogenous full-length PAK-2 is localized in the cytoplasm while recombinant and endogenous PAK-2p34 translocates to the nucleus. MEKK1 is another protein kinase that induces both anti- and pro-apoptotic signals. Full-length MEKK1 is located at the particulate fraction and protects from cell death. Caspase cleavage of MEKK1 causes translocation of the 91 kDa kinase fragment from the particulate fraction to a soluble cytoplasmic fraction. Translocation of MEKK1 catalytic activity
is required for the pro-apoptotic function (45). Mst1 is another protein kinase that translocates upon caspase-activation. Full-length Mst1 is excluded from the nucleus and localized in the cytoplasm. Caspase cleavage removes two C-terminal nuclear export signals and results in nuclear localization of the N-terminal catalytic domain of Mst1. Inhibition of nuclear localization of Mst1 reduces chromatin condensation (46).

Therefore, subcellular localization of signaling molecules appears to be important in regulation of cell survival and cell death. Many signaling proteins undergo rapid redistribution between the cytosol and the nucleus by traveling in and out of the nucleus through the nuclear pore complex (47). Small proteins (typically less than 50 kDa) diffuse freely through the nuclear pores. Larger proteins require a nuclear localization signal (NLS) or a nuclear export signal (NES) to enter or exit the nucleus. An EGFP-fusion protein of the regulatory domain of PAK-2 is localized in the cytoplasm, but accumulates in the nucleus in presence of the nuclear export inhibitor leptomycin B. Therefore, it appears that cytoplasmic localization of full-length PAK-2 is regulated by nuclear export rather than by cytoplasmic retention. We have identified sequence motifs which are involved in nuclear localization and nuclear export. The NLS motif PKKKYTR is required for efficient nuclear localization but by itself not sufficient for nuclear localization suggesting that further motifs or additional mechanisms are involved in nuclear localization of PAK-2p34. The minimal sequence required for nuclear export is rather large and does not contain a cluster of leucine residues characteristic for typical NES motifs. Therefore, nuclear export of PAK-2 may occur by binding of residues 197 to 246 to another protein that contains a NES rather than by direct binding to exportin-1. Alternatively, the 50 amino acid segment of residues 197 to 246 could contain several NES motifs which do not contain the typical leucine residues.

Our data suggest that the anti- and pro-apoptotic functions of full-length PAK-2 and proteolytically activated PAK-2p34 could be regulated by differential subcellular targeting. Cytoplasmic localization of PAK-2 appears to promote cell survival through phosphorylation of “survival substrates” whereas nuclear localization of PAK-2p34 appears to promote cell death.
through phosphorylation of “death substrates”. In addition, the apoptotic response induced by proteolytic activation of PAK-2p34 is regulated by ubiquitination and degradation by the proteasome. PAK-2p34 but not full-length PAK-2 undergoes rapid degradation. Degradation of PAK-2p34 could be part of a safety mechanism to protect cells from cell death by untimely activation of caspases or similar proteases.

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**FOOTNOTES**

1 The abbreviations use are: PAK, p21-activated protein kinase; AID, auto-inhibitory domain; NLS, nuclear localization signal; NES, nuclear export signal; TNF, tumor necrosis factor; CHO, Chinese hamster ovary; EGFP, enhanced green fluorescent protein; PCR, polymerase chain reaction; UV, ultraviolet; LPB, leptomycin B; Ub, ubiquitin.

2 Jakobi et al., unpublished data

**FIGURE LEGENDS**

Fig. 1. Recombinant PAK-2p34 is degraded by the 26S proteasome. Degradation and ubiquitination of recombinant PAK-2p34 was analyzed using transiently transfected 293T cells and stable BALB3T3 cell lines. A, 293T cells were transfected with either a bicistronic construct for expression of EGFP and FLAG-PAKp34 or a construct for expression of an EGFP-PAK-2p34 fusion protein. At 40 h after transfection the medium was changed and cells were incubated for 8 h in the absence or presence of 50 µM MG-132. Cells were analyzed by fluorescence
microscopy. B, 293T cells were transfected with the bicistronic construct for EGFP and FLAG-PAKp34 or the construct for EGFP-PAK-2p34 as well as EGFP-PAK-2 and treated with MG-132 or left untreated. The blot with lysates of the bicistronic construct for EGFP and FLAG-PAKp34 was reprobed with a mouse anti-FLAG antibody. The positions of EGFP-PAK-2, EGFP-PAK-2p34, EGFP and FLAG-PAK-2p34 are indicated at the right. C, stable BALB3T3 cell lines for EGFP or EGFP-PAK-2p34 were induced for expression with 2 µg/ml doxycycline for 42 h and then incubated for 6 h in the absence and presence of 50 µM MG-132. Lysates were analyzed by Western blot with a mouse anti-EGFP antibody. The positions of EGFP-PAK-2p34 and EGFP are indicated at the right.

Fig. 2. Levels of endogenous PAK-2p34 are stimulated by the proteasome inhibitor MG-132. BALB3T3 mouse fibroblasts were left untreated as a control, treated with 1 ng/ml TNF-α or treated by growth factor withdrawal in serum-free medium in the absence or presence of 50 µM MG-132. Levels of proteolytically activated PAK-2p34 were analyzed by in-gel assays with myelin basic protein. Positions of PAK-2p62, PAK-2p58 and PAK-2p34 are indicated at the right A, untreated control cells, cells with TNF-α, or cells in serum-free medium were incubated for 6 h in the absence or presence of MG-132. B, untreated control cells, cells with TNF-α, or cells in serum-free medium were pre-incubated for 18 h and then incubated for 6 h in the absence or presence of MG-132.

Fig. 3. Degradation of recombinant PAK-2p34 is inhibited by conjugation of His-tagged ubiquitin. The effect of co-expression of His-tagged ubiquitin (His-Ub) on levels of recombinant PAK-2 was measured by Western blot analyses. A, 293T cells were transfected with bicistronic constructs for EGFP and FLAG-PAK-2p34, FLAG-PAK-2p34K278R or FLAG-PAK-2T402E and incubated for 40 h. Cells were left untreated (Con) or were treated with MG-132 (MG). In addition cells were co-transfected with the bicistronic constructs and a construct for His-Ub. Levels of FLAG-tagged PAK-2 were examined by Western blot with an anti-FLAG...
antibody. Blots were re-probed with an anti-EGFP antibody to verify even transfection efficiencies. Positions of FLAG-PAK-2, FLAG-PAK-2p34 and EGFP are indicated at the right. 

B, 293T cells were transfected with bicistronic constructs for EGFP and FLAG-PAK-2p34 and indicated amounts of the construct for His-Ub or an empty vector. Cells were analyzed by Western blot with anti-Flag and anti-EGFP antibodies at 48 h after transfection. Positions of FLAG-PAK-2p34 and EGFP are indicated at the right. 

C, 293T cells expressing FLAG-PAK-2p34 alone, FLAG-PAK-2p34 plus His-Ub, or His-Ub alone. Aliquots of lysates were examined for ubiquitination either directly or after affinity purification on Ni\textsuperscript{2+}-NTA agarose under denaturing conditions. Lysates and purified samples were analyzed by Western blot with anti-PAK (\(\alpha\)PAK-C19) and anti-ubiquitin antibodies. Positions of molecular weight standard proteins are indicated at the right in kilodalton, positions of unmodified FLAG-PAK-2p34 and mono- and bi-ubiquitinated FLAG-PAK-2p34 or ubiquitin-conjugates (Ub-conjugates) are indicated at the right. Immuno-reactive bands corresponding to endogenous full-length PAK-2 and PAK-1 are indicated by closed and open arrowheads, respectively. The arrow marks an unspecifically cross-reacting protein that is purified on Ni\textsuperscript{2+}-NTA agarose independent of transfection with FLAG-PAK-2p34 or His-Ub.

Fig.4. **Inhibition of degradation of recombinant PAK-2p34 results in apoptotic morphological changes.** 293T cells were co-transfected with a construct for EGFP or bicistronic constructs for EGFP and FLAG-PAK-2p34, FLAG-PAK-2p34K278R or FLAG-PAK-2T402E and a construct for His-tagged ubiquitin (His-Ub). A, cell morphology was analyzed by fluorescence microscopy. B, apoptotic chromatin condensation was analyzed by staining with Hoechst 33342 and fluorescence microscopy. Transfected cells are visualized by EGFP fluorescence, nuclear morphology is analyzed by Hoechst fluorescence. Overlay images of EGFP and Hoechst fluorescence are also shown. Nuclei with condensed chromatin are indicated by arrows.
Fig. 5. Levels of endogenous PAK-2p34 and programmed cell death are stimulated by expression of epitope-tagged ubiquitin. BALB3T3, HeLa, and Hs578T cells were transfected with a construct for EGFP alone or co-transfected with a construct for EGFP and His-Ub. 293T cells were co-transfected with a construct for EGFP or bicistronic a construct for EGFP and FLAG-PAK-2p34. Transfected cells were incubated for 48 h. A, One set of samples were lysed and analyzed by Western blot with a C-terminal anti-PAK antibody. Positions of PAK-1, PAK-2, FLAG-PAK-2p34 and PAK-2p34 are shown at the right. The co-expression of His-Ub is indicated at the bottom. B, A second set of samples was stained with Hoechst 33342 and analyzed for programmed cell death. For each sample transfected (EGFP-positive) cells were analyzed for apoptotic chromatin condensation. Levels of apoptotic cells in control samples and samples co-transfected with His-Ub are shown as percentages of transfected (EGFP-positive) cells.

Fig. 6. Subcellular localization of recombinant PAK-2. 293T cells were transfected with EGFP or EGFP fusion constructs containing full-length PAK (EGFP-PAK-2), PAK-2p34 (EGFP-PAK-2p34) or the regulatory domain of PAK-2 (EGFP-PAK-2RD). Cells transfected with EGFP-PAK-2RD were also treated for 3 h with the nuclear export inhibitor leptomycin B (LPB) at the indicated concentrations. A schematic representation of the EGFP fusion constructs is shown on the top and their subcellular localization as monitored by fluorescence microscopy at the bottom.

Fig. 7. Subcellular localization of endogenous PAK-2. BALB3T3 cells treated by growth factor withdrawal (0% FBS) for 24 h or untreated control cells were lysed and separated into nuclei and cytoplasm. Endogenous full-length PAK-2 and caspase-activated PAK-2p34 were identified by Western blots using a N-terminal anti-PAK-2 antibody or a C-terminal anti-PAK antibody. Protein loaded was 30 µg for lysate (Lys), 20 µg for cytoplasm (Cyto) and 10 µg for nuclei (Nuc). The purity of the fractions was analyzed with antibodies for glucose-6-phosphate-
dehydrogenase (anti-G6PD) as a cytosolic marker and the transcription factor Oct-1 (anti-Oct-1) as a nuclear marker. Positions of PAK-1, PAK-2 and PAK-2p34 as well as G6PD and Oct-1 are shown at the right.

Fig. 8. Subcellular localization of PAK-2 is regulated by signal sequences for nuclear localization and nuclear export. 293T cells were transfected with EGFP fusion constructs containing fragments of the regulatory domain of PAK-2 (197-246, 213-246, and 197-240), a regulatory domain with a mutant NES (PAK-2RDmuNES), the putative NLS of PAK-2 (NLS) and a PAK-2p34 mutant with a restored NES and a disrupted NLS (PAK-2(197-524)muNLS). A schematic representation of the EGFP fusion constructs is shown on the top and their subcellular localization as monitored by fluorescence microscopy at the bottom. Arrows indicate enhanced nuclear accumulation of EGFP-NLS.
Figure 1

A

EGFP + PAK-2p34

EGFP-PAK-2p34

B

EGFP + FLAG-PAK-2p34

EGFP-PAK-2p34

EGFP-PAK-2

EGFP

anti-EGFP

anti-FLAG

FLAG-PAK-2p34

C

EGFP

EGFP-PAK-2p34

anti-EGFP

EGFP

EGFP-PAK-2p34

MG-132
Figure 2

A  6 hours

|          | Control | TNF-α | Growth factor withdrawal |
|----------|---------|-------|-------------------------|
|          | -       | +     |                         |

B  24 hours

|          | Control | TNF-α | Growth factor withdrawal |
|----------|---------|-------|-------------------------|
|          | -       | +     |                         |

- PAK-2 (62 kDa)
- PAK-2 (58 kDa)
- PAK-2p34 (34 kDa)
- MG-132
Figure 3

A

EGFP + FLAG-PAK-2p34

EGFP + FLAG-PAK-2p34K278R

EGFP + FLAG-PAK-2T402E

Con  MG  His-Ub  Con  MG  His-Ub  Con  MG  His-Ub

FLAG-PAK-2

FLAG-PAK-2p34

EGFP

B

EGFP + FLAG-PAK-2p34 (1000 ng)

0  1  10  100  1000  10000  ng

His-Ub  Con

FLAG-PAK-2p34

EGFP

C

MW  Lysates  Ni\(^{2+}\)-NTA-purified

anti-PAK

anti-Ub

217  112  80  47  32

FLAG-PAK-2p34

FLAG-PAK-2p34

mono/bi-Ub

Hi5-Ub

Ub-conjugates
Figure 4

A

EGFP

EGFP+ FLAG-PAK-2p34

EGFP+ FLAG-PAK-2 p34K278R

EGFP+ FLAG-PAK-2T402E

Control

+ His-Ub

B

EGFP/ His-Ub

EGFP+ FLAG-PAK-2p34/ His-Ub

EGFP+ FLAG-PAK-2 p34K278R/ His-Ub

EGFP+ FLAG-PAK-2T402E/ His-Ub

EGFP

Hoechst

Overlay
Figure 5

A

B

Apoptotic Cells (%)

| BALB3T3 | HeLa | Hs578T | 293T | 293T + p34 |
|---------|------|--------|------|-----------|
| Control | -    | -      | -    | -         |
| His-Ub  | +    | +      | +    | +         |
Figure 6

| 1 | 100 | 200 | 300 | 400 | 500 | 524 aa |
|---|-----|-----|-----|-----|-----|--------|
|   | **Regulatory Domain** | **Catalytic Domain** |       |     |     |        |
|   | PAK-2 (1-524) |                |       |     |     |        |
|   | PAK-2p34 (213-524) |              |       |     |     |        |
|   | PAK-2RD (1-246) |               |       |     |     |        |

- EGFP
- PAK-2
- PAK-2p34
- PAK-2RD
- PAK-2RD 10ng/ml LPB
- PAK-2RD 30ng/ml LPB
Figure 7

|                   | Control          | Growth factor withdrawal |
|-------------------|------------------|--------------------------|
|                   | Lys   | Cyto  | Nuc   | Lys   | Cyto  | Nuc   |
| anti-PAK-2 (N-terminal) |       |       |       |       |       |       |
| anti-PAK (C-terminal)  |       |       |       |       |       |       |
| anti-G6PD         |       |       |       |       |       |       |
| anti-Oct-1       |       |       |       |       |       |       |
Figure 8

![Diagram showing the regulatory and catalytic domains of PAK-2, with various modifications highlighted.]

- Regulatory Domain
- Catalytic Domain

- PAK-2RDmuNES (239IVSIG to 239REGRS)
- NLS (245PKKYYTR)
- PAK-2(197–524)muNLS (245PKKYYTR to 245PMHEYTR)

197-246, 213-246, 197-240, PAK-2RDmuNES, NLS, PAK-2(197–524)muNLS
Caspase-activated PAK-2 is regulated by subcellular targeting and proteasomal degradation
Rolf Jakobi, Corine C. McCarthy, Mark A. Koeppel and Daniel K. Stringer

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