The serine-threonine kinase PAK1 is activated by small GTPase-dependent and -independent mechanisms and promotes cell survival. However, the role of tyrosyl phosphorylation in the regulation of PAK1 function is poorly understood. In this study, we have shown that the prolactin-activated tyrosine kinase Jak2 phosphorylates PAK1 in vivo. Wild type, but not kinase-dead, Jak2 directly phosphorylates PAK1 in cells and in an in vitro kinase assay. PAK1 tyrosines 153, 201, and 285 were identified as sites of Jak2 tyrosyl phosphorylation by mass spectrometry and two-dimensional peptide mapping. Mutation of PAK1 tyrosines 153, 201, and 285 to phenylalanines individually or in combination implicated these PAK1 tyrosines in the regulation of PAK1 kinase activity. Tyrosyl phosphorylation by Jak2 significantly increases PAK1 kinase activity, whereas similar phosphorylation of the PAK1 Y153F, Y201F, Y285F mutant has no effect on PAK1 activity. Tyrosyl phosphorylation of wild type PAK1 decreases apoptosis induced by serum deprivation and staurosporine treatment and increases cell motility. In contrast, these parameters are unaltered in the PAK1 Y153F, Y201F, Y285F mutant. Our findings indicate that Jak2 phosphorylates PAK1 at these specific tyrosines and that this phosphorylation plays an important role in cell survival and motility.

PAK1 is a member of a conserved family of p21-activated serine-threonine kinases and is important for a variety of cellular functions, including cell morphogenesis, motility, survival, mitosis, and malignant transformation (for review see Refs. 1–3). The emerging roles of PAK1 in the regulation of multiple fundamental cellular processes have directed significant attention toward understanding how PAK1 activity is controlled. Autoinhibition of the PAK1 C-terminal catalytic domain by the N-terminal domain is a key mechanism of PAK1 regulation. Several layers of inhibition, involving dimerization and occupation of the catalytic cleft by contact between the N- and C-terminal domains, keep PAK1 kinase activity in check (4). Autoinhibition of PAK1 occurs in trans, meaning that the inhibitory domain of one PAK1 molecule interacts with the kinase domain of another PAK1 molecule (5). Association of GTP-bound forms of Cdc42 and Rac1 with the PAK1 PBD/CRIB domain induces conformational changes in the N-terminal domain that no longer support its autoinhibitory function. In addition to Cdc42 and Rac1, PAK1 is activated by the binding of small GTPases, Ras and Rac3, as well as TC10, CHP, and Wrch-1 proteins (6–11). PAK1 is a predominantly cytoplasmic protein, but is activated upon recruitment to the cell membrane. PAK1 membrane localization occurs through interaction with adaptor proteins Nck, Grb2, and PIX, all of which are activated by ligation of growth factor receptors (12–15). Membrane recruitment of PAK1 via adapter proteins and subsequent PAK1 activation may involve phosphorylation at Thr423 (a site that is also autophosphorylated when PAK1 is activated by Rac1 and Cdc42) by PDK1 (16) or interaction with lipids, such as sphingosine, that can activate PAK1 in a GTPase-independent manner (17). In addition to PDK, several other protein kinases regulate PAK1. Thus, Akt1 phosphorylates PAK1 at Ser21, decreasing Nck binding to the PAK1 N terminus and stimulating PAK1 activity (18, 19). The p35-bound form of Cdk5, a neuron-specific protein kinase, associates with and phosphorylates PAK1 at Thr212 (20) and inhibits PAK1 kinase activity (20, 21). The cyclin B-bound form of Cdc2 also phosphorylates PAK1 at Thr212 (22, 23) affecting PAK1 protein-protein interaction but not PAK1 activation (22).

The role of tyrosyl (Tyr) phosphorylation in the regulation of PAK1 function has not been well established. The non-receptor tyrosine kinase Etk/Bmx, a Tec family member, binds, phosphorylates, and activates PAK1 (24). In addition, a multiprotein complex containing Tyr-phosphorylated and highly active PAK1 was identified in constitutively activated v-ErbB receptor-transformed cells. Formation of this complex appeared to be Rho-dependent (25). A few studies also have reported Tyr phosphorylation of PAK2. PAK2 phosphorylation at Tyr130 by Src kinase strongly potentiates action of Cdc42/Rac1 on PAK2 (26). Another tyrosine kinase, Abl, associates with PAK2 in vivo and decreases PAK2 kinase activity concomitantly with PAK2 Tyr phosphorylation on multiple sites (27). These studies suggest that the tyrosine kinase-dependent modulation of PAK1
activity may provide important mechanism(s) enabling cells to respond appropriately to different external stimuli.

PAKs have been implicated in apoptosis, possessing either anti-apoptotic (PAK1, PAK4, and PAK5) or both pro-apoptotic and anti-apoptotic (PAK2) properties (for review see Refs. 1 and 3). PAK1 is activated by growth factors (epidermal growth factor and platelet-derived growth factor), and cytokines that promote cell survival. The survival signals induced by PAKs might be related to phosphorylation of the pro-apoptotic protein Bad (Bcl-2 family member), Raf-1, the forkhead transcription factor (FKHR), BimL (that interacts with and inactivates the anti-apoptotic protein Bcl-2), and dynein light chain 1 (18, 28–33). The anti-apoptotic effects of PAK1 also may be mediated by activation of NFκB, a critical transcription factor involved in cell survival (34). PAK1 mediates NFκB activation by Ras, Raf-1, and Rac1 and the expression of active PAK1 can stimulate NFκB on its own without activation of the inhibitor of κB kinases (35).

PAK1 plays a key role in coordinating dynamic reorganizations of the actin and microtubule cytoskeletons. PAK1 is localized in areas of the cortical actin cytoskeleton (36); PAK1 kinase activity participates in directional motility (37–39) and PAK1 directly phosphorylates cytoskeletal proteins, including LIM kinase (40), p41-Arc (41), and filamin (42).

JAK2 is a tyrosine kinase that is activated by approximately two-thirds of the cytokine hematopoietin superfamily of receptors, including receptors for γ-interferons, most interleukins, ciliary neurotrophic factor, leptin, growth hormone, prolactin, leukemia inhibitory factor, oncostatin M, erythropoietin, and granulocyte macrophage-colony stimulating factor (43–46). The activation of JAK2 tyrosine kinase initiates a variety of downstream signaling events that lead to diverse physiological responses to cytokines, including regulation of body growth, hematopoiesis, satiety, lactation, and various components of immune function (47). The widely expressed SH2 domain-containing protein SH2-B binds initially identified as a binding partner and substrate of JAK2 (48). SH2-B binds preferentially via its SH2 domain to the tyrosyl-phosphorylated, active form of JAK2. SH2-B binding dramatically increases JAK2 activity and enhances the tyrosyl phosphorylation of downstream targets of JAK2, such as STAT5 (49).

Both JAK2 and PAK1 play critical roles in cell survival and their dysregulation can have negative consequences in terms of human disease. However, there is a gap between upstream JAK2 events and downstream PAK1 and PAK1-dependent cell survival. Elucidation of the mechanism by which signaling events downstream of JAK2 are regulated is therefore critical for our understanding of JAK2 signaling. Here we report that prolactin-activated JAK2 phosphorylates PAK1. We mapped three sites of JAK2-dependent phosphorylation of PAK1 and showed that phosphorylation at these sites significantly increases PAK1 kinase activity. In addition, these three phosphorylated tyrosines are required for maximal anti-apoptotic and motility-promoting PAK1 activities. Together, these results indicate that PAK1 is a new member of a JAK2-dependent signaling pathway playing an important role in cell survival and cell migration.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—cDNAs encoding myc-PAK1 T423E, myc-PAK1 K299R, HA-PAK1 WT, and GST-PAK1 were provided by Dr. Chernoff (Fox Chase Cancer Center, Philadelphia, PA); cDNAs encoding JAK2, JAK2 K882E, myc-SH2-Bβ, and myc-SH2-Bβ (504–670) were provided by Dr. Carter-Su (University of Michigan, Ann Arbor, MI) (49). cDNA encoding mutant RacV12 was used with the permission of Dr. Hall (University College, London, United Kingdom). Individual tyrosines in PAK1 were mutated to phenylalanines using the QuikChange site-directed mutagenesis kit (Stratagene). The double mutant, PAK1 Y153F,Y201F was created by using PAK1 Y153F as a template and mutating Tyr201. The triple mutant PAK1 Y153F,Y201F,Y285F (PAK1 Y3F) was created by using PAK1 Y153F,Y201F,Y285F as a template and mutating Tyr285. Mutations were confirmed by sequencing by the University of Michigan DNA Sequencing Core. PAK1 pSUPER-GFP targets the PAK1 mRNA and the mutated control PAK1 pSUPER-GFP were described early (50).

**Antibody**—Monoclonal anti-phosphotyrosine (anti-Tyr(P), clone 4G10) antibody (Ab) and polyclonal anti-JAK2 (used for Fig. 2A) and rabbit anti-mouse from Upstate Biotechnology, Inc. (Millipore), monoclonal anti-HA from Covance, polyclonal anti-PAK N-20 and monoclonal anti-Myc (9E10) from Santa Cruz Biotechnology, Inc., polyclonal anti-PAK1 from Cell Signaling, and monoclonal anti-actin (Sigma) were used for immunoprecipitation and immunoblotting. Monoclonal anti-HA Ab from Roche Applied Science was used for immunocytochemistry. Anti-JAK2 antiserum was provided by Dr. Carter-Su (51) and used for immunoprecipitation, monoclonal anti-JAK2 Ab (number AHO1352, clone 691R5, BIOSOURCE) was used for immunoblotting. Anti-phospho-Thr423—PAK1 Ab was provided by Dr. Chernoff (39), anti-SH2-B was provided by Dr. Carter-Su (48), and anti-GIT1 was provided by Dr. Manser (15).

**Cells**—The stocks of prolactin-dependent Nb2 rat pre-T lymphoma cells (52) were provided by Dr. Yu-Lee (Baylor College of Medicine) and Dr. Buckley (University of Cincinnati). The cells were grown in RPMI 1640 medium (Mediatech Cellgro) supplemented with 10% fetal bovine serum (HyClone), 10% horse serum (HyClone), 10−4 M 2-mercaptoethanol (Sigma), 1 mM glutamine (HyClone), 100 units of penicillin (HyClone) per ml, and 100 mg of streptomycin (HyClone) per ml. COS-7 and 293T cells were purchased from the American Type Culture Collection (ATCC) and grown in Dulbecco’s modified Eagle’s medium (Mediatech Cellgro) supplemented with 10% fetal calf serum (for COS-7 or 10% calf serum (Mediatech Cellgro, for 293T cells), 1 mM glutamine, 100 units of penicillin/ml, and 100 mg of streptomycin/ml. Immortalized normal mammary epithelial (HME) cells were provided by Drs. Ethier (Karmanos Cancer Institute, MI) and Band (Northwestern University, IL). The cells were grown in Ham’s F-12 medium (Mediatech Cellgro) supplemented with 5 μg/ml insulin (Sigma), 1 μg/ml hydrocortisone (Sigma), 10 ng/ml epidermal growth factor (Sigma), 100 ng/ml cholaer toxin (Sigma), 2.5 μg/ml plasmocin (Amara), 2.5 μg/ml fungizone (Invitrogen), 5 μg/ml gentamicin (Invitrogen), and 5% fetal bovine serum.
Co-immunoprecipitation and Immunoblotting—For prolactin treatment, the Nb2 cells were rendered quiescent by growth in maintenance medium with 3% horse serum and no fetal bovine serum in the presence or absence of 50 μM AG 490 (Calbiochem) for 24 h at 1 × 10^7 cells/ml and treated with 10 μM ovine prolactin for 10 min (purchased from Dr. Parlov, National Hormone and Peptide Program, NIDDK, National Institutes of Health). PAK1 was immunoprecipitated with anti-PAK1 Ab and protein A-agarose, resolved by SDS-PAGE followed by immunoblotting with anti-Tyr(P), anti-PAK1, and anti-JAK2 Abs.

293T cells were transiently transfected using calcium phosphate precipitation. HA-PAK1 was overexpressed with JAK2 or JAK2 K882E, and SH2-Bβ. The cells were serum deprived overnight and PAK1 was immunoprecipitated with anti-HA, rabbit anti-mouse IgG, and protein A-agarose and resolved by SDS-PAGE followed by immunoblotting with the indicated Ab.

In Vitro Kinase Assay—HA-PAK1 was overexpressed with JAK2 or JAK2 K882E, and SH2-Bβ in 293T cells. After overnight deprivation, the cells were lysed, HA-PAK1 was immunoprecipitated with anti-HA and immune complexes were collected using protein A-agarose. The immobilized PAK1 was incubated at 30°C for 30 min in 50 μl of kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol) containing 5 μCi of [γ-32P]ATP (ICN), 10 μg/ml aprotinin, and 10 μg/ml leupeptin (39). Precipitates were washed extensively with lysis buffer and protein A-agarose-PAK1-JAK2 complexes were pelleted. Radiolabeled proteins were separated by SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography followed by immunoblotting with anti-Tyr(P), anti-JAK2, and anti-PAK1 Abs.

For pull-down assays, JAK2 or JAK2 K882E were overexpressed with SH2-Bβ (504–670) in 293T cells, and immunoprecipitated with anti-JAK2. The immobilized JAK2 was incubated with 1 μg of GST-PAK1 in the kinase buffer as described above and PAK1 phosphorylation was assessed.

To assess PAK1 in vitro kinase activity, HA-tagged wild type or mutant forms of HA-tagged PAK1 were co-expressed with JAK2 and SH2-Bβ in 293T cells. The cells were deprived of serum overnight. PAK1 were immunoprecipitated with anti-HA and incubated with 0.5 mCi of [γ-32P]ATP as described above. Proteins will be resolved by SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography. The region of the nitrocellulose containing 32P-labeled PAK1 was excised, soaked in 500 μl of 0.5% polyvinylpyrrolidone in 100 mM acetic acid at 37°C for 30 min, and digested with 5 μg of methylated trypsin (Promega) for 4 h at 37°C. Digested peptides were lyophilized, oxidized in performic acid, and re-lyophilized. Peptides were then separated first by thin layer electrophoresis and then in the second dimension by ascending chromatography (53, 54). 32P labeling peptides will be visualized by autoradiography.

Detection of Phosphorylation Sites by Tandem MS—HA- or Myc-tagged PAK1 was overexpressed in 293T cells with JAK2 and SH2-Bβ. The cells were serum deprived overnight and PAK1 was immunoprecipitated with anti-HA or anti-Myc and resolved by SDS-PAGE. Gel slices containing PAK1 were digested with 5 ng/μl sequencing grade modified trypsin (Promega) in 25 mM ammonium bicarbonate containing 0.01% n-octylglucoside for 18 h at 37°C. Peptides were eluted from the gel slices with 80% acetonitrile, 1% formic acid. Tryptic digestes were separated by capillary high pressure liquid chromatography (C18, 75 μm inner diameter PicoFrit column, New Objective) using a flow rate of 100 nl/min over a 3-h reverse phase gradient and analyzed using a LTQ linear Ion Trap LC/MS system (Thermo Electron). Resultant MS/MS spectra were matched against the PAK1 sequence using TurboSequest (BioWorks 3.2, Thermo Electron) with fragment ion tolerance <0.3 and amino acid modification variables including phosphorylation (80 Da) of Ser, Thr, and Tyr, and oxidation (16 Da) of Met.

PAK1 Gene Silencing—For synthesis of PAK1 siRNA in vivo, COS-7 cells were transiently transfected with cDNA encoding PAK1 pSUPER-GFP targeting the PAK1 mRNA or control pSUPER-GFP that produces a siRNA that is 2 base pairs different from the PAK1 siRNA using Nucleofector kit V (Amaxa Biosytems) according to the manufacturer’s protocol. After 72 h, cells were analyzed for PAK1 by immunoprecipitation and immunoblotting with anti-PAK1.

Apoptosis Assay—COS-7 cells grown on coverslips were transfected with cDNA encoding myc-PAK1 T423E, myc-PAK1 K299R, HA-PAK1 WT, HA-PAK1 Y3F, JAK2, or JAK2 K882E as indicated using FuGENE 6 (Roche) according to the manufacturer’s protocol. In 24 h the cells were serum-deprived and treated with 50 nM staurosporine. In 16 h the cells were fixed with 4% formaldehyde solution for 30 min, permeabilized with 0.1% Triton, and incubated with 2% human serum for 15 min for blocking of nonspecific staining. Next, the coverslips were incubated with anti-HA or anti-Myc Ab followed by rabbit anti-mouse/Oregon Green AB (Molecular Probes, Inc.) and 4’,6-diamidino-2-phenylindole (Molecular Probes, Inc.). After washing, the coverslips were incubated with the TUNEL reaction mixture (Roche) according to the manufacturer’s protocol. The images presented are representative of at least 3 separate experiments. In all cases staining by secondary antibody reagent alone was negligible (not shown). Apoptotic index was calculated as a ratio of amount of TUNEL- and tag-positive cells to the amount of tag-positive cells (for at least 100 cells for each experiment). Each experiment was repeated at least three times, data were pooled, the average apoptotic index for each condition was plotted and analyzed using two-tailed unpaired t test. When individual experiments were analyzed, the results
were indistinguishable from those obtained from the pooled data. Differences were considered to be statistically significant at $p < 0.05$. Results are expressed as the mean ± S.E.

**Phagokinetic Assay**—For the phagokinetic assay, HME cells were transfected with cDNA encoding myc-PAK1 T423E, HA-PAK1 WT, HA-PAK1 Y3F, JAK2, or JAK2 K882E as indicated using ExpressFect (Denville Scientific, Inc.) and plated on colloid gold-covered coverslips 48 h after transfection (55, 56). Cells remove particles while they move, thereby producing areas that are free of colloid gold. The incubation time (16 h) was experimentally determined to avoid the overlapping of the particle-free areas produced by neighbor cells. In 16 h the coverslips were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 for 15 min, and incubated with anti-HA or anti-Myc followed by goat anti-mouse fluorescein isothiocyanate. F-actin was stained with Texas Red-phalloidin. Individual transfected cells were located with a fluorescein isothiocyanate filter set using a Zeiss Axiovert 200 microscope. Differential interference contrast images were collected and particle-free areas were quantified using Image Tool software. The particle-free area was measured in three independent experiments for about 50 cells. The phagokinetic index was calculated as a ratio of particle-free to cross-sectional area of the cell. The images presented in the figure are representative of three independent experiments.

**RESULTS**

**PAK1 Associates with and Is Tyrosyl Phosphorylated by JAK2 in Vivo**—Prolactin activates JAK2 in Nb2 cells, a rat pre-T lymphoma cell line (57, 58). We used this cell model to demonstrate that endogenous prolactin-activated JAK2 tyrosyl (Tyr) phosphorylates endogenous PAK1. The Nb2 cells were rendered quiescent by growth in serum-free medium in the presence or absence of AG 490 (specific JAK2 kinase inhibitor (59) and treated with (Fig. 1A, lanes 1, 3, 5, 7, and 8) or without (Fig. 1A, lanes 2 and 6) 10 nM prolactin for 10 min. Some cells were treated with 50 μM AG 490 for 24 h (lanes 3 and 7). Whole cell lysates (lanes 5–8) were immunoprecipitated with anti-PAK1 (lanes 1–3) or nonspecific Ab (lane 4). Immunoprecipitated proteins were analyzed by Western blotting with the indicated antibodies. The migrations of proteins are indicated. B, the kinase activity of JAK2 is required for tyrosyl phosphorylation of PAK1. HA-PAK1 was immunoprecipitated with anti-HA from 293T cells overexpressed with the indicated proteins and immunoblotted (IB) with the indicated antibodies. The migrations of proteins are indicated. C, HA-PAK1 was immunoprecipitated with anti-HA from 293T cells overexpressed with the indicated proteins and immunoblotted with the indicated antibodies. The migrations of proteins are indicated.

**FIGURE 1. JAK2 tyrosine kinase phosphorylates PAK1.** A, prolactin-activated endogenous JAK2 tyrosyl phosphorylates endogenous PAK1. Serum-deprived Nb2 cells were incubated without (lanes 2 and 6) or with (lanes 1, 3, 5, 7, and 8) 10 nM PRL for 10 min. Some cells were treated with 50 μM AG 490 for 24 h (lanes 3 and 7). Whole cell lysates (lanes 5–8) were immunoprecipitated with anti-PAK1 (lanes 1–3) or nonspecific Ab (lane 4). Immunoprecipitated proteins were analyzed by Western blotting with the indicated antibodies. The migrations of proteins are indicated. B, the kinase activity of JAK2 is required for tyrosyl phosphorylation of PAK1. HA-PAK1 was immunoprecipitated with anti-HA from 293T cells overexpressed with the indicated proteins and immunoblotted (IB) with the indicated antibodies. The migrations of proteins are indicated. C, HA-PAK1 was immunoprecipitated with anti-HA from 293T cells overexpressed with the indicated proteins and immunoblotted with the indicated antibodies. The migrations of proteins are indicated.
JAK2 mutant K882E. Transient overexpression of wild type JAK2 in 293T cells produced constitutively Tyr-phosphorylated JAK2 (Fig. 1B, lane 10), as observed previously by others (48, 60). In contrast, the kinase-inactive JAK2 K882E was not phosphorylated (Fig. 1B, lanes 11 and 14). Tyr-phosphorylated PAK1 was detected in cell lysates when PAK1 was coexpressed with JAK2 (lanes 10), but not with kinase-inactive JAK2 K882E (lanes 11) or control irrelevant protein (lane 9). These findings suggest that JAK2 phosphorylates PAK1. When HA-PAK1 was immunoprecipitated from the cell lysates with anti-HA Ab and immunooblotted with anti-Tyr(P) Ab, Tyr-phosphorylated PAK1 co-immunoprecipitated with Tyr-phosphorylated WT JAK2 (lanes 3 and 6). The same blot was stripped and reprobed with anti-HA Ab. The same amount of PAK1 was immunoprecipitated with anti-HA in all lanes (lanes 2–7), indicating that differences in the amount of Tyr-phosphorylated PAK1 and co-immunoprecipitated Tyr-phosphorylated JAK2 were not due to differences in levels of PAK1 expression. As expected, Tyr phosphorylation of JAK2 (lanes 10 and 13) but not kinase-inactive JAK2 K882E (lanes 11 and 14) was enhanced in cells overexpressing SH2-BB (lane 13). SH2-BB dramatically increases JAK2 activity (49). More importantly, Tyr phosphorylation of PAK1 was enhanced by the addition of SH2-BB (lane 6 versus 3), but not kinase-inactive JAK2 K882E (lane 7 versus 4) as would be expected if JAK2 was the kinase phosphorylating PAK1. The stoichiometry of PAK1 tyrosyl phosphorylation was roughly estimated to be around 17%.

PAK1 is activated by binding the small GTPases, Cdc42 or Rac1, in their GTP-bound forms (6). To test whether Rac1 is required for JAK2 Tyr phosphorylation of PAK1, WT PAK1 was overexpressed in 293T cells in the presence and absence of a constitutively active form of Rac V12 (Fig. 1C). The activated state of Rac V12 was confirmed by PBD-GST pull down (61) and activation of PAK1 by Rac V12 in 293T cells was demonstrated (data not shown). As observed above, JAK2 Tyr phosphorylates PAK1 (Fig. 1C, lane 1). In agreement with previously published data on the activation of JAK2 by Rac1 (62, 63), the quantification of Tyr(P)-JAK2 and Tyr(P)-PAK1 bands from lane 1 versus lane 3 (Fig. 1C, upper left blot) showed that Rac V12 increased tyrosyl phosphorylation of JAK2 about 1.5-fold and PAK1 about 1.7-fold. These data demonstrate that PAK1 associates with and is Tyr phosphorylated by JAK2 in vivo and activated Rac is not required but stimulates the JAK2-dependent Tyr phosphorylation of PAK1.

**JAK2 Directly Phosphorylates PAK1 in Vitro**—To determine whether JAK2 directly phosphorylates PAK1, JAK2 was immunoprecipitated from 293T cells overexpressing JAK2 and then the ability of the immunoprecipitated JAK2 to phosphorylate GST-PAK1 directly was assayed in an in vitro kinase assay (Fig. 2A) (64). As reported previously (60, 48), JAK2 is constitutively active when overexpressed in 293T cells and SH2-BB greatly enhances JAK2 tyrosyl kinase activity. GST-PAK1 and SH2-BB migrate to a similar position after SDS-PAGE. Therefore, to visualize these two proteins on a gel, we used a truncated SH2-BB (504–670) mutant that also increases JAK2 tyrosyl kinase activity (64). Exogenous PAK1 was Tyr phosphorylated in vitro by JAK2 (Fig. 2A, lane 1). As a control kinase-inactive JAK2 K882E was overexpressed in 293T cells, and the ability of the immunoprecipitated kinase-inactive JAK2 to phosphorylate PAK1 was assayed as described above. As expected, kinase-
inactive JAK2 K882E did not Tyr phosphorylate PAK1 in vitro (lane 2). PAK1 is a Ser/Thr kinase that may be autophosphorylated, as demonstrated by the \(^{32}\text{P}\) incorporation into PAK1 in both lanes 1 and 2. However, PAK1 anti-Tyr(P) signal was detected only when PAK1 was incubated with wild type, but not kinase-dead JAK2.

To provide additional evidence that JAK2 phosphorylates PAK1, HA-tagged PAK1 was co-expressed in 293T cells with JAK2 (Fig. 2B, lanes 2 and 5) or JAK2 K882E (Fig. 2B, lane 3). These cells were serum-deprived overnight to minimize PAK1 autophosphorylation. PAK1 was immunoprecipitated with anti-HA and incubated with \(^{32}\text{P}\)ATP. The immobilized PAK1-JAK2 complex was incubated with anti-HA and immune complexes were collected using protein A-agarose. The immobilized PAK1-JAK2 complex was incubated with \[^{32}\text{P}\]ATP in an in vitro kinase assay, as described above. \(^{32}\text{P}\) incorporation was increased in bands co-migrating with PAK1 and JAK2 in the PAK1 IPs (Fig. 2B, left panel, lane 5 versus lane 1) from cells expressing or not expressing SH2-B\(\beta \) (lane 2 versus lane 5), compared with cells not expressing JAK2 (lanes 1 and 4, respectively). Incubation of this blot with anti-Tyr(P) Ab showed that PAK1 was Tyr phosphorylated in vitro only in the presence of JAK2 (lanes 2 and 5), but not JAK2 K882E (lane 3). As expected, SH2-B\(\beta \) enhanced Tyr phosphorylation of both JAK2 and PAK1 (lane 5). Interestingly, an additional double band migrating at \(-90\) kDa appeared on the autoradiogram. Immunoblotting with anti-GIT1 Ab revealed that this band corresponds to GIT1 (G-protein-coupled receptor-kinase-interacting protein 1) protein (65). These findings strongly suggest that JAK2 phosphorylates PAK1 in vitro.

**MS Identifies Tyrosine 285 as a Site of Phosphorylation**—PAK1 phosphorylation was examined by LC-MS/MS-based mass spectrometry. Briefly, HA-PAK1 was overexpressed in 293T cells with JAK2 and SH2-B\(\beta \). The cells were serum-deprived overnight and PAK1 was immunoprecipitated with anti-HA, resolved by SDS-PAGE, trypsinized, and eluted peptides were analyzed by nanospray LC and tandem mass spectroscopy. Sequest analysis of resultant spectra revealed a 2+ precursor ion (1232.48 m/z) that matched the 24-amino acid PAK1 tryptic phosphopeptide from residues 276 to 299, IGQGASGTYY(phos)TAM*DVATGQEVAIK, with an \(X_{\text{corr}} = 5.3\) (Fig. 3). This peptide assignment was confirmed using Mascot and SwissPort data base (Mowse score 98, rank 1, expectation value 4.5e-07). The localization of phosphorylation at Tyr\(^{285}\) was consistent with \(b_{10}, b_{11}, y_{14}\) and \(y_{15}\) ions. In addition to Tyr\(^{285}\) phosphorylation, we also detected the oxidation at Met\(^{288}\) on this peptide. Spectra matching this Tyr\(^{285}\) Phos and Met-Ox peptide were identified in two independent PAK-1 preparations. Oxidation of Met\(^{288}\) also was detected in the absence of phosphorylation (1192.24 m/z, charge state 2+, \(X_{\text{corr}} = 5\), data not shown).

**JAK2 Phosphorylates Tyrosines 153, 201, and 285 in PAK1**—Two-dimensional peptide mapping was used to confirm that Tyr\(^{285}\) was a site of JAK2-dependent PAK1 Tyr phosphorylation and to identify possible additional phosphorylation sites. First, the WT form of HA-tagged PAK1 was co-expressed with JAK2 and SH2-B\(\beta \) in 293T cells and the cells were serum deprived overnight. HA-PAK1 was immunoprecipitated with anti-HA and incubated with \[^{32}\text{P}\]ATP. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography. The region of the nitrocellulose containing \[^{32}\text{P}\]-labeled PAK1 was excised and digested with trypsin. Tryptic peptides were oxidized in performic acid and then separated first by thin layer electrophoresis and then by ascending chromatography. The two-dimensional phosphopeptide map of PAK1 revealed multiple phosphopeptides (Figs. 4A and 5B). To determine which peptides were phosphorylated on Tyr(s), phosphoamino acid analysis was performed. All 24 phosphorylated peptides were scraped, eluted, and subjected to acid hydrolysis. WT PAK1 contained 3 peptides phosphorylated on Tyr (Fig. 4, Spots 1, 2, and 3). In addition to phosphotyrosines, each of these spots contained \[^{32}\text{P}\]-labeled phospho-Ser. Spot 3 also contains phospho-Thr (Fig. 4B). To confirm that phosphopeptides 1, 2, and 3 contained sites of JAK2 Tyr phosphorylation, a phosphopeptide map was generated using HA-PAK1 overexpressed in the absence of JAK2 and SH2-B\(\beta \). Spots 1, 2, and 3 were not present in this map (Fig. 5A), suggesting that spots 1, 2, and 3 were sites of JAK2 phosphorylation.

To identify possible additional sites of JAK2-dependent PAK1 Tyr phosphorylation, each of the 14 Tyr in full-length HA-tagged PAK1 was individually mutated to phenylalanine. Two-dimensional phosphopeptide mapping of PAK1 in which individual Tyr(s) were mutated to phenylalanines yielded maps similar to WT PAK1 (not shown) with three exceptions. In the map of PAK1(Y153F), spot 1 (Fig. 5C) completely disappeared. Similarly, spot 2 (D) and spot 3 (E) completely disappeared when Tyr\(^{201}\) and Tyr\(^{285}\) were mutated, respectively, suggesting that Tyr\(^{153}\), Tyr\(^{201}\), and Tyr\(^{285}\) are phosphorylated by JAK2 in vitro. As predicted, spots 1 and 2 disappeared when both Tyr\(^{153}\) and Tyr\(^{201}\) were mutated and spots 1, 2, and 3 disappeared when all three Tyr\(^{153}\), Tyr\(^{201}\), and Tyr\(^{285}\) were mutated (PAK1 Y3F, Fig. 5F). These data demonstrate that JAK2 phosphorylates tyrosines 153, 201, and 285 in PAK1 in vitro.

**JAK2 Phosphorylation of PAK1 Increases Kinase Activity of PAK1**—Tyr phosphorylation is required for activation of many signaling pathways. As an initial step in determining the role of
phosphorylated Tyr(s) in PAK1 function, we set out to determine whether JAK2-dependent phosphorylation of PAK1 altered PAK1 kinase activity. HA-tagged wild type or mutated PAK1 was immunoprecipitated with anti-HA or Myc-tagged JAK2. HA-PAK1 overexpressed in 293T cells with JAK2 was immunoprecipitated with anti-HA, incubated with 0.5 mCi of [γ-32P]ATP, resolved by SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography. The PAK1 band was cut from nitrocellulose and subjected to two-dimensional phosphopeptide mapping. Phosphorylated peptides scraped from a two-dimensional map of PAK1 were eluted and subjected to acid hydrolysis. Amino acids were separated by thin layer electrophoresis and visualized by autoradiography. Migration of phosphoserine (pSer), phosphothreonine (pThr), and phosphotyrosine (pTyr) standards is indicated.

FIGURE 4. Three tyrosyl-containing PAK1 phosphopeptides are phosphorylated by JAK2 in vitro. A, wild type PAK1 overexpressed in 293T cells with JAK2 was immunoprecipitated with anti-HA, incubated with 0.5 mCi of [γ-32P]ATP, resolved by SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography. The PAK1 band was cut from nitrocellulose and subjected to two-dimensional phosphopeptide mapping. B, phosphorylated peptides scraped from a two-dimensional map of PAK1 (A) were eluted and subjected to acid hydrolysis. Amino acids were separated by thin layer electrophoresis and visualized by autoradiography. Migration of phosphoserine (pSer), phosphothreonine (pThr), and phosphotyrosine (pTyr) standards is indicated.

FIGURE 5. JAK2 phosphorylates PAK1 at Tyr153, Tyr201, and Tyr285. HA-PAK1 overexpressed in 293T cells without (A) or with JAK2 and SH2-B (B-F) was immunoprecipitated with anti-HA and subjected to in vitro kinase assay and two-dimensional phosphopeptide mapping. Spots 1, 2, and 3 were not present when HA-PAK1 was overexpressed without JAK2 and SH2-B. Spot 1 disappeared when Tyr153 was mutated to phenylalanines (C). Spots 2 (D) and 3 (E) disappeared when Tyr201 and Tyr285 were mutated to phenylalanines, respectively. Spots 1, 2, and 3 disappeared when all three Tyr153 Tyr201, and Tyr285 were mutated to phenylalanines (F).

FIGURE 6. JAK2 phosphorylation of PAK1 increases kinase activity of PAK1. HA-tagged WT PAK1 and its mutants and Myc-PAK1 K299R were immunoprecipitated with anti-HA or anti-Myc, respectively, from 293T cells overexpressed with the indicated proteins and subjected to the in vitro kinase assay using histone H4 as a substrate of PAK1. PAK1 kinase activity was then normalized by the amount of immunoprecipitated PAK1 for each lane. The leftmost bar is normalized to 100% and represents WT PAK1 kinase activity without JAK2. JAK2 doubles PAK1 kinase activity as compared with vector control or kinase-dead JAK2. JAK2 increases the kinase activity of wild type PAK1 but not the mutant forms of PAK1 lacking Tyr153, Tyr201, and/or Tyr285. Each experiment was repeated at least three times. Bars represent mean ± S.E. * p < 0.05 compared with cells expressing WT PAK1.

PAK1 K299R with anti-Myc from 293T cells overexpressing the indicated proteins and each IP was assayed in vitro for kinase activity in the presence of histone H4 (PAK1 substrate). 32P incorporation into histone H4 was estimated by autoradiography and phosphorimaging. PAK1 kinase activity was then normalized to the amount of immunoprecipitated PAK1 and plotted (Fig. 6). As expected, the activity of the kinase-dead PAK1 K299R was significantly decreased (negative control). In contrast, JAK2 more than doubled the kinase activity of wild type PAK1, as compared with WT PAK1 and the vector control. As predicted, JAK2 had no effect on the kinase activity of the PAK1 Y3F mutant. Interestingly, JAK2 had no effect on the kinase activity of single tyrosine PAK1 mutants (Y153F, Y201F, or Y285F) suggesting the redundancy of PAK1 tyrosyl phosphorylation. Similarly, kinase-dead JAK2 K882E did not alter the kinase activity of WT PAK1 or PAK1 Y3F. These data indicate that JAK2 phosphorylation increases the kinase activity of PAK1 and suggests that tyrosines 153, 201, and 285 are responsible for this activation.

PAK1 Tyrosyl Phosphorylated by JAK2 Protects Cells from Apoptosis—PAK1 protects cells from apoptosis (28, 18, 66, 67). Therefore, we sought to determine whether JAK2-dependent phosphorylation of PAK1 plays a role in this process. As the combination of serum withdrawal and treatment with apoptotic inducers causes a significant increase in cell death over apoptotic inducer alone (28), we induced apoptosis by treating COS-7 cells with staurosporine (50 nM, 16 h) in serum-free medium. Because the treatment of murine Friend cells with 500 nM staurosporine inhibits phosphorylation, but increases JAK2 expression (68), the effects of 50 nM staurosporine on JAK2 activity and expression were examined. JAK2 and HA-tagged PAK1 were overexpressed in 293T cells, treated or not with 50 nM staurosporine in serum-free medium overnight, and the level of JAK2 Tyr phosphorylation assessed with anti-Tyr(P) Ab.
and the level of JAK2 expression with anti-JAK2 Ab. No differences in either Tyr phosphorylation or JAK2 expression were detected in cell lysates and IPs with anti-HA (data not shown). Therefore, HA-tagged WT PAK1, constitutively active Myc-PAK1 Thr423, or kinase-dead Myc-PAK1 K299R were overexpressed in COS-7 cells, apoptosis was induced and cell survival examined using a TUNEL assay. Label incorporation at damaged sites in the DNA was visualized by fluorescence microscopy (red or yellow-white when three colors overlaid in Fig. 7, A–F, arrows). Cells were stained with anti-HA or anti-Myc to detect PAK1 (green) and with 4′,6-diamidino-2-phenylindole to identify nuclei (blue), and the apoptotic index calculated (Fig. 7H). As expected, overexpression of PAK1 T423E (A) protected the cells from apoptosis (16% apoptotic cells), whereas overexpression of PAK1 K299R did not (43%), as compared with the cells overexpressing vectors only (27.4%). WT PAK1 overexpressed with JAK2 protected the staurosporine-treated cells from apoptosis (8% apoptotic cells). In contrast, overexpression of WT PAK1 alone (not shown), with kinase-dead JAK2 K882E (17.8%) or with mutant PAK1 lacking Tyr153, Tyr201, and Tyr285 with either JAK2 (24.7%) or JAK2 K288E (25.2%) did not protect the cells from apoptosis. The overexpression of WT PAK1, PAK1 423T, PAK1 K299R, or PAK1 Y3F with or without JAK2 did not cause apoptosis in untreated cells (data not shown). These data were verified by analyzing DNA content using flow cytometry. Cells with a DNA content lower than the G1 peak were considered apoptotic. Tyrosyl phosphorylation of WT PAK1 decreases apoptosis induced by serum deprivation and staurosporine treatment. In contrast, apoptosis was unaltered in the PAK1 Y153F,Y201F,Y285F mutant (data not shown). Because JAK2 has also been implicated in the protection of cells from apoptosis, we studied whether JAK2 alone can promote cell survival. We down-regulated endogenous PAK1 in COS-7 cells by transiently expressing pSUPER-GFP that produces a PAK1-specific siRNA. After 72 h the level of PAK1 in cells overexpressing PAK1 pSUPER-GFP was about 35% of that in cells expressing the control plasmid (Fig. 7G). This result is consistent with the level of PAK1 knockdown observed previously using this construct (50). In this experiment, the co-expression of PAK1 siRNA and JAK2 had no effect on apoptosis when compared with cells transiently expressing JAK2 alone (Fig. 7H), suggesting that 35% of the remaining endogenous PAK1 activity is sufficient to maintain “basal” cell survival, but is not sufficient to promote cell death, as observed for the PAK K299R mutant. Interestingly, in agreement with this finding, the knockdown of PAK1 expression with PAK1-siRNA also did not change apoptotic responses to UV exposure in IMR-90 human fibroblasts (33).

Taken together, these results suggest that the expression of both PAK1 and JAK2 are necessary for maximal protection of COS-7 cells from apoptosis induced by staurosporine and serum deprivation. For example, expression of either PAK1 with kinase-dead JAK2 K882E or the expression of WT JAK2 with PAK1 siRNA does not reduce the apoptotic index.

Tyrosyl Phosphorylation of PAK1 by JAK2 Is Required for Maximal Cell Motility—To examine the effect of JAK2-dependent PAK1 tyrosyl phosphorylation on cell motility, we performed a gold particle motility (phagokinetic) assay. This assay measures the disappearance of colloid gold particles from a coverslip, a process requiring both phagocytosis and locomotion. Transfected cells were plated on coverslips coated with colloid gold particles (Fig. 8, A–D). Cells remove particles while they move, thereby producing areas that are free of colloid gold.

**FIGURE 7.** Tyrosyl phosphorylation of PAK1 by JAK2 protects COS-7 cells from apoptosis. COS-7 cells overexpressed Myc-PAK T423E (A), Myc-PAK1 K299R (B), HA-PAK1 WT (C and D), or HA-PAK1 Y3F (E and F) together with JAK2, JAK2 K882E, or vector were treated with 50 nM staurosporine for 16 h in serum-free media. The cells were fixed and stained with anti-HA or anti-Myc for PAK1 visualization (green). Apoptotic cells were visualized by TUNEL assay (arrows, red or yellow-white when three colors overlaid). Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). JAK2 protects the cells from apoptosis when overexpressed with wild type PAK1 (C) but not with mutated PAK1 lacking tyrosines 153, 201, and 285 (Y3F) (E). In contrast to JAK2, kinase-dead JAK2 K882E was not able to protect cells from apoptosis when overexpressed with the wild type PAK1 (D) or PAK1 Y3F (F). As expected, constitutively active PAK1 T423E (A) but not kinase-dead PAK1 K299R (B) protects cells from apoptosis. Bar, 20 μm. G, endogenous PAK1 was immunoprecipitated with anti-PAK1 from COS-7 cells overexpressed the GFP-tagged siRNA (lane 1) or the mutated PAK1 siRNA (lane 2) and immunoblotted with anti-PAK1. The expression levels of β-actin were used as an internal control. H, apoptotic index was calculated as a ratio of the amount of TUNEL- and tag-positive cells to the amount of tag-positive cells for at least 100 cells for each experiment. Each experiment was repeated at least three times.
The phagokinetic index was calculated and plotted in Fig. 8E. The cells overexpressing both WT JAK2 and WT PAK1 and the cells overexpressing PAK T423E exhibited increased cell motility compared with cells overexpressing either WT PAK1 with kinase-dead JAK2 K882E or PAK lacking Tyr(s) (Y3F) suggesting that the tyrosyl phosphorylation of PAK1 by JAK2 is required for maximal cell motility.

DISCUSSION

We have demonstrated that PAK1 is Tyr phosphorylated by endogenous prolactin-activated JAK2, as well as by constitutively active, overexpressed JAK2. More importantly, we have identified PAK1 Tyr

\[ \text{Tyr}^{153} \text{, Tyr}^{201} \text{, and Tyr}^{285} \]

as the major targets of JAK2 phosphorylation. This conclusion is based primarily on two-dimensional phosphopeptide mapping with the phospho-Ser and phospho-Thr. All phosphorylated peptides contained Ser, Thr, or both (data not shown), regardless of the presence or absence JAK2. More importantly, only three peptides contained Tyr(s) in the presence of JAK2. PAK1 is a Ser/Thr kinase that may be either autophosphorylated or phosphorylated by other Ser/Thr kinases (16, 18, 19, 22, 23). As predicted, in phosphopeptide maps of PAK1 mutation of Tyr

\[ \text{Tyr}^{153}, \text{Tyr}^{201}, \text{or Tyr}^{285} \]

to phenylalanine eliminated one of the spots, mutation of Tyr

\[ \text{Tyr}^{153} \text{and Tyr}^{201} \]

eliminated two spots, and mutation of Tyr

\[ \text{Tyr}^{153}, \text{Tyr}^{201}, \text{and Tyr}^{285} \]

eliminated all three spots. Mutation of any of the remaining PAK1 Tyr(s) did not alter the phosphopeptide map. The increase in \( \text{32P} \) incorporation into some of the Ser and Thr spots and the appearance of new Ser/Thr spots when PAK1 was co-expressed with JAK2 most probably reflects the increase in PAK1 kinase activity caused by JAK2-dependent Tyr phosphorylation.

Presumably, Tyr phosphorylation of PAK1 serves some function. We have shown here that JAK2 significantly increases PAK1 kinase activity. In contrast, cells overexpressing PAK1 with a kinaseinactive JAK2 K882E mutant exhibited basal levels of PAK1 kinase activity. Tyrosines 153, 201, and 285 are required for this increase in kinase activity because PAK1 with these three tyrosines mutated to phenylalanines exhibited only basal kinase activity whether expressed with JAK2, kinase-dead JAK2 K882E, or vector alone. A role for the Tyr phosphorylation of PAK1 has been suggested by others. For example, the non-receptor tyrosine kinase Etk (epithelial and endothelial tyrosine kinase, also called Bmx) from the Tec family of non-receptor protein-tyrosine kinases also directly associates with, Tyr phosphorylates, and activates PAK1 (24). In addition, a multiprotein complex containing Tyr-phosphorylated and highly active PAK1 was identified in constitutively activated v-Erb receptor-transformed cells. PAK2 was activated upon Tyr phosphorylation by Src kinases and Tyr

\[ \text{Tyr}^{131} \]

which corresponds to Tyr

\[ \text{Tyr}^{131} \]

in PAK1) was identified as the major phosphoacceptor site (25, 26). In contrast, the nonreceptor kinase c-Abl Tyr phosphorylates PAK2 and causes a 6-fold decrease in PAK2 kinase activity (27). There is no consensus on a role for small GTPases in PAK Tyr phosphorylation. We have shown here that activated Rac increases JAK2-dependent Tyr phosphorylation of PAK1. How it has been shown previously, Tyr phosphorylation of PAK2 by Src kinases was strongly dependent on prior activation of PAK2 by p21 GTPases. Wild type PAK2 could only be Tyr phosphorylated when active CDC42 or Rac1 was co-transfected (26). In contrast, Tyr phosphorylation of PAK in v-Erb-transformed fibroblasts was not Rac- or CDC42-dependent but was Rho-dependent (25). Thus, our and previously published data suggest that the tyrosine kinase-dependent regulation of PAK1 activity may provide an important mechanism for the modulation of PAK1 activity and the generation of an appropriative response to specific extracellular signals. In this respect, our data strongly suggest that PAK1 is a novel member of the JAK2-dependent signaling pathway and that JAK2 activates PAK1 by phosphorylation of three distinct PAK1 tyrosines.

Both PAK1 and JAK2 play a role in cell survival. PAK activity down-regulates several important pro-apoptotic pathways.
The survival signal induced by PAKs might be related to phosphorylation of pro-apoptotic protein BAD (Bcl-2 family member), leading to the inactivation of BAD. PAK1 phosphorylates BAD on both Ser112 and Ser139, protecting cells from apoptosis induced by interleukin-3 deprivation in a pathway distinct from the PI3K-Akt pathway (18, 28). Both PAK4 and PAK5 phosphorylate BAD on Ser112, whereas Ser139 is phosphorylated by a different mechanism (presumably by Akt) (29, 30). PAK1-mediated BAD phosphorylation was described as a critical event in survival signaling induced by the HIV viral Nef protein (69). PAK1 can also phosphorylate and activate Raf-1, resulting in Raf-1 mitochondrial translocation, where Raf-1 phosphorylates and inactivates BAD (31). In addition to BAD, PAK1 phosphorylates and inactivates the forhead transcriptional factor promoting the expression of several pro-apoptotic genes (32). PAK1 also phosphorylates dimers of the pro-apoptotic protein BimL (which interacts with and inactivates an anti-apoptotic protein Bcl-2) and dynein light chain 1 resulting in their degradation (33). The anti-apoptotic effects of PAK1 also may be mediated by activation of NFκB, a critical transcriptional factor involved in cell survival (34). PAK1 mediates NFκB activation by Ras, Raf-1, and Rac1 and expression of active PAK1 can stimulate NFκB on its own without activation of the inhibitor of κB kinases (35). During Helicobacter pylori infection of human epithelial cells, PAK1 activates NFκB via activation of upstream regulatory kinase NIK (NFκB-inducing kinase) (70).

JAK2 has also been implicated in the protection of cells from apoptosis. Indeed, the JAK2/STAT5α pathway facilitates the survival of the mammary epithelium and can impact the progression of transforming growth factor α-mandated mammary tumorigenesis (71). The inactivation of STAT3 (target of JAK2) markedly inhibits cell proliferation and induces apoptosis in human prostate cancer cells, suggesting an important role of JAK2 in cell survival (72). JAK2 has an important survival role in nucleophosmin/anaplastic lymphoma kinase fusion protein (NPM/ALK)-transformed hematopoetic cells (73) and as a downstream mediator of Bcr-Abl protein in leukemia cells (74, 75).

Although the significance of both JAK2 and PAK1 in apoptosis is widely acknowledged, the mechanism involved remains poorly understood. A gap exists between upstream cytokine-activated and JAK2-dependent events and downstream PAK1-dependent functions in our understanding of apoptosis. To fill this gap, we asked whether Tyr phosphorylation of PAK1 by JAK2 would protect cells from apoptosis. We used 50 nM staurosporine in serum-free media as an inducer of apoptosis in COS-7 cells and examined survival using a TUNEL assay and fluorescence-activated cell sorter analysis. The apoptotic index in cells overexpressing JAK2 and PAK1 was three times less than that in cells overexpressing PAK1 and kinase-dead JAK2 K822E. Additionally, the three phosphorylated tyrosines (153, 201, and 285) are required for maximal anti-apoptotic PAK1 activity, because overexpression of mutant PAK1 lacking Tyr153, Tyr201, and Tyr285 together with either JAK2 or JAK2 K288E did not protect from apoptosis.

PAK1 also serves as an important regulator of cytoskeletal dynamics and cell motility. We have demonstrated that PAK1 tyrosyl phosphorylation by JAK2 is required for maximal motility of epithelial HME cells. The overexpression of both PAK Y3F and JAK K288E did not decrease the phagokinetic index, suggesting a compensatory tyrosyl-independent mechanism of PAK1 activation. In support of a role for JAK2-PAK1 in cytoskeletal dynamics, overexpressed PAK1 enhanced cell ruffling in response to growth hormone (a physiological ligand that activates JAK2), whereas a Y3F PAK mutant inhibited ruffling. One possible explanation of the anti-apoptotic and motility-promoting actions of Tyr-phosphorylated PAK1 is based on the Tyr-dependent increase in PAK1 kinase activity. Indeed, PAK1 phosphorylates many intracellular targets, such as BAD, forkhead transcriptional factor, Raf-1, BimL, and dynein light chain 1, all leading to an inhibition of apoptosis (18, 28, 31–33). PAK1 also regulates the reorganization of the actin cytoskeleton through the phosphorylation of several cytoskeletal proteins, including LIM kinase (40), p41-Arc (41), and filamin (42). Another possible explanation of anti-apoptotic and motility-promoting effects of Tyr-phosphorylated PAK1 is that tyrosyl phosphorylation alters the ability of PAK1 to find, bind, and/or phosphorylate intracellular targets, thereby amplifying the effects of PAK1 on cell survival and motility. Because phosphorylated tyrosines create high-affinity docking sites for the binding to SH2 domain-containing proteins, candidate interacting proteins should have an SH2 domain. Further studies will be required to identify targets that may bind PAK1 phosphorylated at Tyr153, Tyr201, and or Tyr285.

In summary, prolactin-activated endogenous JAK2 Tyr phosphorylates endogenous PAK1 in Nb2 cells. Using two-dimensional phosphopeptide mapping and MS/MS analysis, we have demonstrated that Tyr153, Tyr201, and Tyr285 in PAK1 are phosphorylated by JAK2. Furthermore, these tyrosines play a significant role in PAK1 activation. Finally, we demonstrated that JAK2 Tyr phosphorylation is required for the maximal anti-apoptotic and motility-promoting effect of PAK1. Therefore, the results described here introduce PAK1 as a novel JAK2 target in cytokine signaling and provide insight into the possible mechanism by which JAK2 and PAK1 participate in cell survival and cell motility.

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