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p21-Activated Kinase 2 in Neutrophils Can Be Regulated by Phosphorylation at Multiple Sites and by a Variety of Protein Phosphatases

Qian Zhan,* Qingyuan Ge,† Taisuke Ohira,*§ Thomas Van Dyke,* and John A. Badwey*‡

The p21-activated kinase (Pak) 2 undergoes rapid autophosphorylation/activation in neutrophils stimulated with a variety of chemoattractants (e.g., fMLP). Phosphorylation within the activation loop (Thr402) and inhibitory domain (Ser141) is known to increase the activity of Pak in vitro, whereas phosphorylation within the Nck (Ser20) and Pak-interacting guanine nucleotide exchange factor (Ser192 and Ser197) binding sites blocks the interactions of Pak 2 with these proteins. A panel of phosphospecific Abs was used to investigate the phosphorylation of Pak 2 in neutrophils at these sites. Pak 2 underwent rapid (≤15 s) phosphorylation at Ser20, Ser192/197, and Thr402 in neutrophils stimulated with fMLP. Phosphorylation at Ser192/197 and Thr402 were highly transient events, whereas that at Ser20 was more persistent. In contrast, Pak 2 was constitutively phosphorylated at Ser141 in unstimulated neutrophils and phosphorylation at this site was less sensitive to cell stimulation than at other residues. Studies with selective inhibitors suggested that a variety of phosphatases might be involved in the rapid dephosphorylation of Pak 2 at Thr402 in stimulated neutrophils. This was consistent with biochemical studies which showed that the activation loop of GST-Pak 3, which is homologous to that in Pak 2, was a substrate for protein phosphatase 1, 2A, and a Mg2+/Mn2+-dependent phosphate(s) which exhibited properties different from those of the conventional isoforms of protein phosphatase 2C. The data indicate that Pak 2 undergoes a complex pattern of phosphorylation in neutrophils and that dephosphorylation at certain sites may involve multiple protein phosphatases that exhibit distinct modes of regulation.

Neutrophils stimulated with a variety of chemoattractants exhibit rapid activation of p21-activated kinase (Pak) 2 (1–4). Pak 2 is a serine/threonine protein kinase that undergoes autophosphorylation/activation upon binding the activated (GTP-bound) forms of the small GTPases Rac or Cdc42 (5). A variety of studies suggest that Paks participate in a wide range of cellular events that include rapid cytoskeletal responses, certain long-term transcriptional events and the development of malignancy (for review, see Ref. 6). For example, Pak 2 is involved in altering cell morphology/chemotaxis (e.g., Refs. 7 and 8), activation/potentiation of several distinct mitogen-activated protein kinase cascades (e.g., Ref. 9), activation of NF-κB in macrophages (10), and NFAT in T cells (11). Recent studies have also implicated Pak in Ras-mediated transformation (12), Ras-mediated apoptosis (e.g., Refs. 13 and 14), invasiveness of breast cancer cells (15), and the pathogenesis of HIV (e.g., Refs. 16–18). In addition to activated Rac/Cdc42, Pak 2 also contains binding sites for the β subunits of complex G-proteins, certain adaptor proteins (Nck), and Pak-interacting guanine nucleotide exchange factors (PIXs) (for review, see Ref. 6). Thus, Paks are capable of receiving messages from a variety of signal transduction pathways.

Considerable effort has been made to understand the regulation of Pak at the molecular level. The kinase domain (KD) of Pak is located in the C-terminal region of this enzyme, whereas the N-terminal region contains a Cdc42/Rac interactive binding motif and an autoinhibitory domain (ID; e.g., Ref. 19). Biochemical and biophysical studies have demonstrated that the ID interacts with KD to maintain Pak in an inactive/low affinity state (e.g., Refs. 20 and 21). Pak 1 and 2 can form homodimers in vivo (21). Dimerized Pak adopts a trans-inhibited conformation with the N-terminal domain of one Pak molecule inhibiting the catalytic domain of the other (21). Binding of one activated Rac or Cdc42 to this dimer disrupts these interactions and promotes autophosphorylation of the activation loop (AL) (Thr402) of both kinases (21). Phosphorylation at Thr402 results in a marked increase in the activity of Pak 2 toward certain substrates that persist even in the absence of the small GTPase (e.g., Ref. 22). In addition to the Thr402, peptide mapping studies have revealed that Pak 2 can also undergo autophosphorylation in vitro at several other residues including Ser20, Ser141, Ser192, and Ser197 (e.g., Ref. 23). Serines 20, 141, and 192/197 are located within the Nck binding site of the ID and PIX binding site, respectively. Phosphorylation within the ID (Ser141) partially activates Pak (24), whereas phosphorylation at Ser20 and Ser192 blocks binding to Nck and PIX, respectively (25). To date, few studies have focused on the phosphorylation of Pak 2 in vivo, particularly on residues outside of the AL (e.g., Ref. 26).

Pak 2 undergoes a rapid but transient activation in chemoattractant-stimulated neutrophils (1, 4) with activation/inactivation corresponding to phosphorylation and subsequent dephosphorylation at Thr402 (27). Distinct signals appear to be required for the activation and inactivation of Pak 2 in neutrophils (4). Thus, phosphatase(s) active
against Pak are likely to play major roles in regulating this enzyme. Studies on cells other than neutrophils have shown that Pak can exist in complexes with protein phosphatase (PP) 2A (28) and/or with a highly specific phosphatase termed FEM-2 (29). The phosphatases active against Pak 2 in neutrophils have not been studied until now. In this paper, we have investigated the phosphorylation of Pak 2 at Ser20, Ser141, Ser192/197, and Thr402 in stimulated neutrophils along with the complex regulation of this kinase in vivo.

Materials and Methods

Materials

A phosphospecific Ab (pAb) that only recognized Pak 1, 2, or 3 when phosphorylated in the AL (pPak(AL) Ab) (Thr402 in Pak 2) was generated in rabbits (Research Genetics, Huntsville, AL) and purified/characterized in our laboratory (27). A similar affinity purified pAb to the AL of Pak 1, 2, and 3 along with affinity purified rabbit pAbs that react with Pak 2 when phosphorylated at Ser20 (pPak2(Ser20) Ab), Paks 1 and 2 when phosphorylated at Ser144 and Ser141, respectively (pPak1/2(Ser144/141) Ab) and Paks 1 and 2 when phosphorylated at Ser199 and/or Ser197, and Ser192 and/or Ser191, respectively (pPak1/2(Ser199/197) Ab and Ser192/197 Ab) were obtained from Cell Signaling Technology (Beverly, MA). The sequences of the phosphopeptide Ags used to generate these pAbs are provided in Fig. 1. The phosphopeptide Ags used to generate the pAbs described above and a rabbit polyclonal Ab that recognized both the phosphorylated and nonphosphorylated forms of Pak 2 (pPak 2 Ab) were also obtained from Cell Signaling Technology (Beverly, MA). Goat polyclonal Abs raised to peptides derived from the N terminus of human Pak 2 (γ-Pak(N-19) Ab) and human Pak 3 (β-Pak(N-19) Ab) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant calmodulin produced in Escherichia coli, PP 2B/calcineurin isolated from bovine brain, recombinant human PP 2Ca, PP 2A purified from human RBCs, PP 1 purified from rabbit skeletal muscle, recombinant human Pak 1 Ab from rabbit muscle produced in E. coli and a sheep Ab to PP 2C generated to full-length human PP 2C were obtained from Upstate Biotechnology (Lake Placid, NY). Mouse mAbs generated to human PP 2A, human PP 1ε, and human calcineurin were obtained from BD Transduction Laboratories (San Diego, CA). Okadaic acid, calcylin A, ascomycin, FK-506, and cyclosporin A were purchased from Calbiochem (La Jolla, CA). Plasmid pGEX-mPak 3 carrying mouse tissue mPak 3 fused to GST in pGEX-KG was a gift from Dr. A. Mak (Queen’s University, Kingston, Ontario, Canada). Immobilon-P transfer membranes (0.45 μm) were obtained from Millipore (Bedford, MA). Sources of all other materials are described elsewhere (1, 3, 4, 27).

Methods

Preparation of neutrophils and the neutrophil soluble fraction. Guinea pig neutrophils were prepared as described previously (30). These preparations contained >90% neutrophils with viabilities always >95%. The studies described herein were performed in compliance with all institutional policies and federal guidelines governing the use of animals in research.

Neutrophils (1.0 × 10^6) were suspended in 1.0 ml of ice-cold phosphate buffer (50 mM HEPES, pH 7.5, 100 mM KCl, 0.20 mM EDTA) containing 0.025 mM of protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO) and freeze-thawed three times with a mixture of dry ice and ethanol. The homogenate was centrifuged at 100,000 × g for 45 min and the resulting supernatant was used immediately as the source of Mg^{2+}/Mn^{2+}-dependent PP(s) that has certain properties that are different from those of conventional PP 2Cs.

Immunoblotting/detection of phosphorylated Pak 2 in neutrophils. Neutrophils (1.9 × 10^6/ml) were stimulated in disposable 1-cm plastic cuvettes at 37°C. The standard assay mixture consisted of a modified Dulbecco’s PBS medium (135 mM NaCl, 2.7 mM KCl, 16.2 mM Na_2PO_4, 1.47 mM KH.PO_4, 0.90 mM CaCl_2, and 0.50 mM MgCl_2, pH 7.35) containing 7.5 mM d-glucose. Cells were generally incubated in this medium for 10.0–30.0 min with or without inhibitors before stimulation. Concentrations of inhibitors and stimulation times are presented in the figure legends. At the appropriate time, the cells were rapidly lysed by adding 0.25 ml of 5 mM DTT to 1.0 ml of the reaction mixture and the samples were boiled for 4.0 min. The final composition of the resulting supernatant was used immediately as the source of Mg^{2+}/Mn^{2+}-dependent PP(s) that has certain properties that are different from those of conventional PP 2Cs.

Amino acid sequences of the phosphopeptide Ags used to generate the pAbs used in this study and the corresponding sequences in Paks 1, 2, and 3.

Human Pak1 & 2 / mouse Pak3

pPak (AL) Ab

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<tr>
<td>pPak3 (414-426) antigen</td>
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pPak (ser-20) Ab

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pPak1/2 (ser-144/141) Ab

|--------------------------|----------------------------------------------------------|

pPak1/2 (ser-199/204 & 192/197) Ab

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<th>pPak1 (192-211) antigen</th>
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<tr>
<td>pPak2 (185-204) antigen</td>
<td>P - R - P - D - H - Y - K - S - I - Y - T - R - S - V - I - E - P - L - P - V</td>
</tr>
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electrophoretically to Immobilon P-membranes (1). Specific phosphorylation of Pak was assayed by Western blotting with the appropriate pAb (e.g., Ref. 27). Membranes were incubated with the pAb (~1/1,000 dilution) overnight at 4°C. Phosphorylated Pak 2 was visualized with a luminoECL detection system (Pierce, Rockford, IL). Conditions for Western blotting are detailed elsewhere (4). At the end of these experiments, both the immunodetection system and the bound Ab were removed from the blot by incubating the membrane with ImmunoPure elution buffer (Pierce) for 30–60 min at room temperature followed by two washes in TBST (20 mM Tris-HCl, 150 mM NaCl, and 0.01% (v/v) Tween 20, pH 7.4). The blots were then stained with an Ab that recognized both the phosphorylated and nonphosphorylated forms of Pak 2 or 3.

The concentrations of inhibitors and conditions used in these studies did not affect cell viability, as measured by the exclusion of trypan blue or by the release of lactate dehydrogenase (data not shown; Ref. 31).

Preparation of phosphorylated GST-Pak 3 and in vitro dephosphorylation of this protein catalyzed by PP 1, PP 2A, PP 2B, and PP 2C. The GST-Pak 3 (3-Pak) fusion protein was expressed in E. coli, purified on a glutathione-agarose affinity column and eluted with reduced glutathione (GSH) (e.g., Refs. 32 and 33).

Dephosphorylation of GST-Pak 3 by PP 1 or PP 2A was carried out at 30°C for 15 min in a 0.10-ml reaction mixture containing 30 μg/ml GST-Pak 3 in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2.0 mM MnCl2, 1.0 mM EDTA, 0.50 mM DTT and 0.10 mg/ml BSA. The assay conditions for PP 2B (calcineurin) were identical to those described above but also included 0.20 mM CaCl2 and 70 μg/ml calmodulin (34). The assay conditions for PP 2C were identical to those for PP 1 and PP 2A but in this case MnCl2 was replaced by 5.0 mM MgCl2 (e.g., Ref. 35). The assay mixtures were allowed to equilibrate for 10 min at 30°C before the reactions were initiated by the addition of phosphatase. The units of phosphatase activity used are defined in the legend to Fig. 3. The concentrations of inhibitors used in these studies did not affect cell viability, as measured by the exclusion of trypan blue or by the release of lactate dehydrogenase.

Dephosphorylation of GST-Pak 3 by Mg2+/Mn2+-dependent phosphatases in neutrophils. These assay conditions were modified from those used to initiate phosphorylation of Pak 2 at other sites (e.g., Thr402; see below) because both of these sites are currently capable of distinguishing between these possibilities. In contrast to Ser92/197, phosphorylation at Ser20 was sustained with most of the phosphorylation at this site still evident at 3.0 min. The double band observed for Ser20 at certain time points is likely to be the result of a mobility shift due to phosphorylation/dephosphorylation of Pak 2 at other sites (e.g., Thr402, see below) because both of these bands were specifically blocked by the Ser20 phosphopeptide Ag (see below). The pAb 2(Ser20) (Ser20) Ab did not detect Ser21 in Pak 1. Only three of the nine amino acids in the ~5 to +4 positions are identical when the sequences surrounding Ser21 in Pak 1 and Ser25 in Pak 2 are compared (Fig. 1). In contrast to other sites, Ser21 in Pak 2 was constitutively phosphorylated in unstimulated neutrophils. A similar situation was observed for Ser144 in Pak 1.

A variety of data indicate that the pAbs used in these experiments were specific for the designated residues. For example, these experiments reacted with only one or two major proteins when blotted against a lysate of neutrophils with the major bands corresponding to the masses of Pak 2 and/or 1. When the blots shown in Fig. 2A were washed and reblotted with a regular Ab to Pak 2, the resulting bands overlapped the bands observed with the pAbs (data not shown). Most importantly, the bands observed with each pAb were specifically blocked by the phosphopeptide Ag (5.0 μg/ml) used to generate the pAb but not by phosphopeptides corresponding to the other phosphorylation sites in Pak 1 or 2 (Fig. 2B). Finally, the unique kinetics of phosphorylation observed with three of four of these pAbs along with the different sensitivities of the various sites to phosphatases and inhibitors monitored with these reagents (see below) strongly suggest that each pAb reacts with a distinct site in Pak.

It is noteworthy that the pPak(AL) Ab recognized only one form of Pak 2 in stimulated neutrophils, whereas the pPak 2(Ser20) Ab recognized both slow and fast migrating forms of Pak 2. One explanation for these data is that the slow migrating band results from phosphorylation at Thr402. Thus, the pPak(AL) Ab would only recognize this form of Pak 2. Under these circumstances, the mobility shift observed with the pPak 2(Ser20) Ab would be due to phosphorylation at Ser20 occurring both during and after phosphorylation at Thr402 such that this pAb would bind to both the slow and fast migrating forms of Pak 2. In contrast, the rapid dephosphorylation of Pak 2 at Ser192/197 would preclude detection of the lower band with the pPak 2(Ser192/197) Ab. Consistent with this interpretation, a proteolytic fragment of Pak 1 (residues 201–491) which contains only the AL phosphorylation site (Thr125) exhibits a mobility shift when phosphorylated in vitro (20).

Interestingly, evidence of a possible mobility shift and/or dephosphorylation of Pak 2 with the pPak 1/2(Ser144/141) Ab was only observed in stimulated cells at 15 s (Fig. 2A). These data may indicate that any Pak 2 which undergoes phosphorylation at both Ser141 and Thr402 is particularly sensitive to dephosphorylation, and/or that Pak 2 phosphorylated at Ser141 does not undergo significant phosphorylation at Thr402.

Effects of inhibitors of PPs on the phosphorylation of Pak 2 at Thr402 in stimulated neutrophils

The effects of inhibitors of PP 2A (100 nM okadaic acid) (e.g., Refs. 35 and 37) and/or PP 2B (200 nM ascomycin) (e.g., Ref. 38)
on the phosphorylation of Pak 2 at Thr402 were evaluated to obtain information on the PPs that were active at or controlled phosphorylation at this residue (Fig. 3). Neutrophils were treated with these inhibitors for 30 min at 37°C and then stimulated with 1.0 μM fMLP for 15 s. Phosphorylation at Thr402 was monitored by Western blotting with the pPak(AL) Ab. Equal loading of protein per lane was monitored with an Ab that recognized both the phosphorylated and nonphosphorylated forms of Pak 2 (Pak 2 Ab). B, Summarizes data from three to five separate experiments (mean ± SD). The amount of phosphorylation at Thr402 was estimated by densitometry with the 100% value being that exhibited by cells not treated with inhibitors but stimulated with 1.0 μM fMLP for 15 s. Conditions for cell stimulation and Western blotting are provided in Materials and Methods.

FIGURE 2. Progress curves for phosphorylation of Pak 2 on different residues in stimulated neutrophils. Specificity of the pAbs. A, Neutrophils were stimulated with 1.0 μM fMLP for different time periods and phosphorylation of Pak 2 at Thr402, Ser20, Ser141, and Ser192/197 was monitored by Western blotting with pAbs generated to these sites. Data on the phosphorylation of Pak 1 on Ser144 and Ser199/204 are also presented. A Western blot with a Pak 2 Ab that reacts with both the phosphorylated and nonphosphorylated forms of Pak 2 (Pak 2 Ab) is also shown. B, Lysates of neutrophils were blotted with the pAbs listed above in the absence (−) or presence of the Ser20, Ser141, Ser199/204, or Thr423 phosphopeptide Ags used to generate these pAbs (5.0 μg/ml). Unstimulated neutrophils (pPak1/2(Ser144/141) Ab) and neutrophils stimulated with 1.0 μM fMLP for 15 s (pPak(AL) Ab and pPak1/2(Ser199/204 and Ser192/197) Ab) or 3.0 min (pPakSer20) Ab were used in these competition experiments. Conditions for cell stimulation and Western blotting are provided in Materials and Methods.
Okadaic acid inhibits dilute solutions of PP 1 and PP 2A in vitro at concentrations of 1.0–2.0 μM and 1.0–2.0 nM, respectively (35, 37). However, the effective concentrations of this inhibitor towards PP 1 and PP 2A in vivo are considerably higher due to the high concentrations of these phosphatases in cells (e.g., Refs. 35 and 37). Thus, the ability of 100 nM okadaic acid to enhance phosphorylation at Thr^{402} in stimulated neutrophils is consistent with the involvement of PP 2A in this reaction. Inhibitors of PP 1 (e.g., okadaic acid >1.0 μM, calyculin A) (37) could not be used in these studies because these compounds are known to reduce phosphorylation at Thr^{402} and block activation of Pak 2 in neutrophils (1, 3, 27). Selective inhibitors of PP 2C are not yet available.

Fig. 4 presents the progress curves for phosphorylation of Pak 2 at Thr^{402} in fMLP-stimulated neutrophils with and without okadaic acid (100 nM) plus ascomycin (200 nM). Interestingly, while these inhibitors were effective at increasing phosphorylation at all time points examined, they did not prevent the pronounced loss of phosphate at 1 and 3 min. Restaining this blot with an Ab that recognized both the phosphorylated and nonphosphorylated forms of Pak 2 (γ-Pak(N-19) Ab) demonstrated that this loss of phosphate was not due to a proteolytic event (Fig. 4). These data indicate that neutrophils are likely to contain phosphatase(s) in addition to PP 2A and/or PP 2B that are active against Thr^{402}.

In contrast to the pPak(AL) Ab, the combination of okadaic acid plus ascomycin produced only a small increase in the reactivity of the pPak 1/2(Ser^{199/204} and Ser^{192/197}) Ab at 15 and 30 s with no increases at 1.0 and 3.0 min (Fig. 4A). Thus, it is unlikely that the

![FIGURE 4](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org)
effects of these inhibitors on Thr^{402} were the result of a general/overall enhancing effect of okadaic acid plus ascomycin on protein phosphorylation in neutrophils.

**Dephosphorylation of GST-Pak 3 by PPs**

Many serine/threonine PPs can be broadly divided into different categories (e.g., PP 1, PP 2A, PP 2B, PP 2C) on the basis of their substrate specificity, requirement for divalent cations, and sensitivity to various tumor promoters (e.g., okadaic acid, calyculin A) (e.g., Ref. 35). The presence of PP 1, PP 2A, PP 2B, and PP 2C in neutrophils was demonstrated with a panel of Abs that specifically reacted with the catalytic subunits of these enzymes (Fig. 5). Each of these Abs reacted with a major protein when blotted against a lysate of unfractionated neutrophils with the resulting bands corresponding to the expected molecular masses of these phosphatases.

The ability of these phosphatases to catalyze dephosphorylation of the AL and ID of Pak in vitro was investigated with a GST-Pak 3 fusion protein serving as the substrate. The GST-Pak 3 fusion protein was expressed in *E. coli* containing mouse Pak 3 fused to GST in the pGEX vector by incubation with isopropyl-β-D-thiogalactopyranoside (IPTG) and purified with GSH-sepharose beads as referenced in Materials and Methods (Fig. 6). As reported previously, GST-Pak 3 is susceptible to degradation (33). The resulting fusion protein reacted with the pPak(AL) Ab and the pPak 1/2(Ser^{144/141}) Ab (Figs. 6 and 7) which indicated that a portion of this protein was phosphorylated at Thr^{421} and Ser^{139}. Perturbation of the N-terminal region of Pak by fusion to GST results in activation of a portion of this kinase in the absence of activated Rac or Cdc42 (32). GST-Pak 3 was purified as shown in Fig. 6 and treated for 15 min at 37°C with various amounts of recombinant PP 1α (rabbit muscle) (A), PP 2A purified from human RBCs (B), PP 2B purified from rabbit brain (C), and recombinant PP 2Cα (human) (D). Each phosphatase was assayed under optimal conditions as described in Materials and Methods. Dephosphorylation at Thr^{421} and Ser^{139} in Pak 3 (analogous to Thr^{402} and Ser^{141} in Pak 2, respectively) was monitored with the pPak(AL) Ab and pPak 1/2(Ser^{144/141}) Ab, respectively. The arrow indicates the position of the GST-Pak3 fusion protein before treatment with the phosphatase.

GST-Pak 3 was treated with purified PP 1α, PP 2A, PP 2B, and PP 2C for 15 min at 37°C under conditions known to be effective for each of these phosphatases (34) and phosphorylation at Thr^{421} and Ser^{139} was monitored with the pAbs described above (Fig. 7). PP 1 and PP 2A, but not PP 2B or PP 2C, were effective against Thr^{421} under the assay conditions. In contrast, all four phosphatases were capable of catalyzing dephosphorylation at Ser^{139} which established that these phosphatases were active under the assay conditions used. In Fig. 7, the PP 2B catalyzed dephosphorylation at Ser^{139} exhibited a strong activation by calmodulin (Fig. 7C). The insensitivity of the AL phosphorylation site to PP 2B suggests that the ability of ascomycin to enhance phosphorylation at Thr^{402} in stimulated neutrophils (Figs. 3 and 4) may occur indirectly provided that the in vitro experiments summarized in Fig. 7 adequately reflect the situation for native Pak 2 in vivo (see Discussion).
Effects of neutrophil lysates on the phosphorylation of GST-Pak 3: dephosphorylation of Pak by novel phosphatase(s)

Studies were undertaken to determine if neutrophils contain novel phosphatase(s) active against the AL of Pak (Fig. 8). Incubation of GST-Pak 3 with the undialyzed, soluble fraction of neutrophils resulted in an increased detection of phosphate at Thr\(^{211}\) and Ser\(^{139}\) but less so at Ser\(^{200/205}\) when monitored with the pAbs described above (Fig. 8, lanes a and b). However, addition of 10.0 mM Mg\(^{2+}\) to the reaction mixture 30 min after the addition of the soluble fraction resulted in a pronounced, time-dependent loss of phosphate from Thr\(^{211}\) but not from the Ser\(^{139}\) or Ser\(^{200/205}\) sites. A similar loss of phosphate from Thr\(^{211}\) was also observed when 10.0 mM MnCl\(_2\) was added to the assay mixture. Because the Thr\(^{211}\) site was highly phosphorylated at the time the divergent pattern was added, these data are consistent with Mg\(^{2+}/\)Mn\(^{2+}\)-stimulating the activity of highly specific phosphatase(s) that recognize the AL of Pak. It is unlikely that this activity is due to PP 1, PP 2A, or a typical isoform of PP 2C because inhibitors of these phosphatases were present in the assay system at optimal concentrations (100 nM calyculin A, 1.0 \(\mu\)M orthovanadate) (e.g., Refs. 36 and 37). Moreover, a typical isoform of PP 2C from humans was not active against the AL in GST-Pak 3 but did recognize the Ser\(^{139}\) site (Fig. 7). Finally, sodium fluoride (100 mM) partially inhibited the Mg\(^{2+}/\)Mn\(^{2+}\)-dependent phosphatase(s) from neutrophils that were active against the AL of GST-Pak 3 (Fig. 8, compare lanes f and h). Sodium fluoride (100 mM) does not inhibit the typical isoforms of PP 2C (39). The effects of Mg\(^{2+}\) and F\(^{-}\) were estimated by densitometry by comparing the heights of bands b, f, and h in Fig. 8. Treatment of the fusion protein for 30 min with 10.0 mM MgCl\(_2\) alone or 10.0 mM MgCl\(_2\) plus 100 mM NaF reduced the signal with the pPak(AL) Ab by 70 \(\pm\) 7% and 36 \(\pm\) 15% (SD, \(n = 3\)–4), respectively. Thus, a variety of data suggest that this specific Mg\(^{2+}/\)Mn\(^{2+}\)-AL phosphatase present in neutrophils is not a typical form of PP 2C. This Mg\(^{2+}/\)Mn\(^{2+}\)-dependent activity may be responsible for the dephosphorylation of the AL of Pak 2 that occurred in stimulated neutrophils in the presence of okadaic acid and ascomycin (Fig. 4).

Discussion

Although autophosphorylation of Pak 2 in vitro has been investigated for nearly a decade, relatively few studies have focused on these reactions in intact cells, particularly on residues outside of the AL. We have now investigated the multisite phosphorylation of Pak 2 in stimulated neutrophils along with the PPs that are active against the AL and ID of this kinase. As noted above, phosphatases active against Pak are likely to play major roles in regulating this kinase. The significance of these studies is developed below.

Incubation of baculovirus expressed Pak 2 or Pak 2 purified from rabbit reticulocytes with Mg \(\cdot\) ATP alone results in autophosphorylation at Ser\(^{139}\), Ser\(^{200}\), Ser\(^{192}\), and Ser\(^{197}\). Addition of activated Cdc42 to the reaction mixture results in additional phosphorylation of Pak 2 at Ser\(^{141}\), Ser\(^{165}\), and Thr\(^{402}\) (23). In contrast, Pak 2 was not phosphorylated on Ser\(^{200}\) and Ser\(^{192/197}\) in unstimulated neutrophils but was phosphorylated on Ser\(^{141}\). Stimulation of neutrophils with fMLP resulted in rapid phosphorylation of Pak 2 at Ser\(^{20}\), Ser\(^{192/197}\), and Thr\(^{402}\) (Fig. 2A). Elicited guinea pig neutrophils and human blood neutrophils stimulated with fMLP exhibit rapid activation of Cdc42 and Rac with kinetics consistent with these GTPases functioning upstream of Pak (e.g., Refs. 31 and 40). This rapid accumulation of activated Cdc42/Rac in stimulated neutrophils would promote autophosphorylation of Pak 2 on Thr\(^{402}\) as predicted by the in vitro studies. Why phosphorylation of Pak 2 on Ser\(^{20}\), Ser\(^{192/197}\), and Thr\(^{402}\) in neutrophils does not conform to the in vitro pattern is not known, but may reflect the activities of phosphatases and kinases that are present in neutrophils and active at these sites. The peritoneal cells used here are likely to be primed to exhibit enhanced responses upon stimulation (e.g., Ref. 41). Whether this priming also affects the phosphorylation of Pak is not known. Thus, the phosphorylation/regulation of Pak 2 in stimulated neutrophils is considerably different from that predicted by in vitro studies.

We have previously reported that the rapid activation/inactivation of Pak 2 in fMLP-stimulated neutrophils is closely associated with phosphorylation/dephosphorylation of this kinase at Thr\(^{402}\) (27). A variety of data implicate PP 2A in the dephosphorylation/inactivation of Pak. PP 2A catalyzes dephosphorylation of the AL of Pak 2 immunoprecipitated from neutrophils (27) and an inhibitor of PP 2A (100 nM okadaic acid) increased phosphorylation at this site in stimulated cells (Fig. 3). PP 2A is present in neutrophils (Fig. 5) and can exist in complexes with Pak in certain cell types (28). However, it is likely that phosphatases in addition to PP 2A are also active against the AL of Pak 2 because 100 nM okadaic acid did not prevent the pronounced dephosphorylation at Thr\(^{402}\) that occurred in neutrophils at 1.0 and 3.0 min after stimulation (Fig. 4). Other potential phosphatases that can catalyze this reaction are PP 1 and FEM-2 (Fig. 7; Ref. 29). We have detected PP 1 in neutrophils (Fig. 5) along with a specific Mg\(^{2+}/\)Mn\(^{2+}\)-dependent AL phosphatase. This Mg\(^{2+}/\)Mn\(^{2+}\)-dependent phosphatase differs from conventional PP 2Cs in that it is active in the presence of 1.0 \(\mu\)M orthovanadate and is partially inhibited by 100 mM NaF (Fig. 8; Refs. 36 and 39). Moreover, a typical isoform of PP 2C was not found to be active against the AL of GST-Pak 3 (Fig. 7). FEM-2 is a possible candidate for this Mg\(^{2+}/\)Mn\(^{2+}\)-dependent phosphatase because it is activated by Mg\(^{2+}\), catalyzes dephosphorylation of the AL of Pak, is partially inhibited by F\(^{-}\) and

![FIGURE 8. In vitro dephosphorylation of the activation loop of GST-Pak 3 catalyzed by Mg\(^{2+}/\)Mn\(^{2+}\)-dependent phosphatase(s) present in neutrophils. GST-Pak 3 was treated with the 100,000 \(\times\) g soluble fraction from unstimulated neutrophils for 30 min at 37°C (lane b) and then treated with 10.0 mM MgCl\(_2\) (lanes d-f) or 10.0 mM MnCl\(_2\) (lane g) for different time periods. Lane c shows the effect of increasing the incubation time of the GST-Pak 3 fusion protein with the soluble fraction from 30 to 60 min. Lane h shows the effect of NaF (100 mM) on the dephosphorylation of GST-Pak 3 when added to the reaction mixture with MgCl\(_2\). The amount of phosphorylation at Thr\(^{211}\), Ser\(^{139}\), and Ser\(^{200/205}\) was monitored with the pPak(AL) Ab, pPak 1/2/Ser\(^{144/141}\) Ab, and the pPak 1/2/Ser\(^{200/204}\) and Ser\(^{192/197}\) Ab, respectively.](http://www.jimmunol.org/Downloadedfrom.html)
insensitive to orthovanadate and calyculin A (29, 36, 39). Chronic activation of Pak can promote viral replication, metastasis, and cellular transformation (e.g., Refs. 6, 12, 18). Perhaps the presence of multiple phosphatases active at the Thr422 site are a safeguard against these possibilities. Interestingly, the Mg$^2+$/Mn$^2+$-dependent AL phosphatase in neutrophils was not active against phosphorylation sites in the ID and PIX-binding region of Pak 3 (Fig. 8) which suggests that different sites in Pak can be recognized by distinct phosphatases that are susceptible to different modes of regulation.

PIX can function as a scaffold protein because it binds Pak and FEM-2 simultaneously (29). Thus, PIX may participate in the activation and subsequent inactivation of Pak 2 through its GTP/GDP exchange activity and ability to serve as a targeting subunit that colocalizes Pak and FEM-2 (29). As noted earlier, Pak 2 in IMLP-stimulated neutrophils undergoes a rapid and transient phosphorylation on serines 192/197 (Fig. 2A), and these modifications may promote dissociation of the PIX-FEM-2 complex from Pak (25). The physiological significance of these reactions is not yet known because isoforms of PIX have been described that have activating, inhibiting, and permissive effects on Pak (42). The isoforms of PIX in neutrophils remain to be characterized. PIX itself can undergo phosphorylation which does not affect its GTP/GDP exchange activity (43). It is interesting to note that while inhibitors of PP 2B enhanced phosphorylation of Pak 2 at Thr422 in stimulated neutrophils, isolated PP 2B did not catalyze dephosphorylation of the AL in the GST-Pak 3 fusion protein (Fig. 7). If these data with the GST-Pak 3 fusion protein adequately reflect the situation in vivo, they indicate that PP 2B regulates Pak indirectly, perhaps by catalyzing the dephosphorylation of PIX or some unknown protein that is also involved in the regulation of Pak.

As noted above, Pak 1 or 2 can form dimers with the ID of one Pak molecule inhibiting the catalytic domain of the other (21). Monomeric Pak possesses considerable intrinsic kinase activity even in the absence of activated Rac or Cdc42 (cf. Ref. 21). Dimerization severely restricts this intrinsic/basal activity of Pak (21). Serine 141 resides within the ID and phosphorylation at this site partially activates Pak 2 by preventing the ID from packing against the kinase domain (21, 24). The constitutive phosphorylation of Pak 2 at Ser$^{141}$ (and Pak 1 at Ser$^{144}$) may provide for a pool of the kinase domain (21, 24). The constitutive phosphorylation of Pak 2 at Ser$^{141}$ may provide for a pool of the kinase domain (21, 24). The constitutive phosphorylation of Pak 2 at Ser$^{141}$ may provide for a pool of the kinase domain (21, 24).

In summary, we report that Pak 2 undergoes phosphorylation in neutrophils on multiple sites in a manner that exhibits significant differences from that observed during activation of this kinase in vitro. Dephosphorylation at some sites may involve multiple protein phosphatases that are susceptible to different modes of regulation. These data highlight the complex regulation that is necessary for coordinating the many cellular functions of Pak 2.

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References

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