Activation of the p21-activated protein kinases from neutrophils with an antibody that reacts with the N-terminal region of Pak 1

Jian P. Lian[a], John A. Badwey[a,b,*]

[a]*Boston Biomedical Research Institute, 20 Staniford St., Boston, MA 02114, USA*
[b]*Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA*

Received 13 January 1997; revised version received 27 January 1997

Abstract Neutrophils contain two renaturable p21-activated protein kinases (Paks) with molecular masses of ca. 69 and 63 kDa that undergo rapid activation upon stimulation of these cells with the chemoattractant fMet-Leu-Phe. We now report that these kinases undergo a massive, ATP-dependent activation in lysates of unstimulated neutrophils during immunoprecipitation with an antibody generated to residues 2–21 of the N-terminal region of Pak1. This activation was specific as it was completely blocked by a peptide that corresponds to residues 2–21 of Pak1 and was not observed with an antibody generated to the C-terminal region of Pak 1. The properties of the Paks activated with the antibody were virtually identical to those observed for these kinases from stimulated neutrophils, or activated in vitro with Rac-GTPγS plus ATP. These data indicate that perturbation of the N-terminal region of Pak can trigger activation of this enzyme, and that both the 69 and 63 kDa kinases may represent forms of Pak 1 that differ in their content of phosphate.

Key words: Protein kinase; Antipeptide antibody; Structure-function relationship

1. Introduction

Neutrophils stimulated with the chemoattractant fMet-Leu-Phe (fMLP) exhibit a number of phenomena that are critical to the antimicrobial responses of these cells. These events include chemotaxis, phagocytosis and the release of large quantities of superoxide and hydrogen peroxide [1]. Cells triggered with this agonist also exhibit rapid activation of four protein kinases with molecular masses of 69, 63, 49 and 40 kDa [2–4]. These kinases can be detected by their ability to undergo renaturation and autophosphorylation, or to catalyze the phosphorylation of a substrate fixed within a gel [2–4]. Activation of all four of these kinases is blocked by antagonists of phosphatidylinositol 3-kinase (e.g. wortmannin, LY294002) [5,6]. These inhibitors are known to abrogate phagocytosis, degranulation and superoxide/peroxide release by stimulated neutrophils [7,8]. Recent studies have established that the 69 and 63 kDa enzymes are p21-activated kinases (Paks) [9,10]. Paks and their substrates (e.g. myosin VI isoforms) are thought to participate in the polarization and turnover of the actin cytoskeleton [11–13].

*Corresponding author. Fax: (1) (617) 523-6649.

Abbreviations: fMLP, fMet-Leu-Phe; Pak, p21-activated kinase; p47-*phox*, the 47 kDa component of the phagocyte oxidase; Pak(N-20) ab, antipeptide antibody raised against residues 2–21 of rat Pak1; Pak(C-19) ab, antipeptide antibody raised against residues 525–544 of rat Pak1; ip-B, immunoprecipitation buffer; SDS-B, solubilization buffer

Paks undergo autophosphorylation/activation upon binding the active (GTP-bound) forms of the small GTP-binding proteins (p21) Rac or Cdc42 [14]. The mechanism of this activation remains to be completely elucidated, but evidence suggests that it may involve the removal of an inhibitory domain from the MgATP binding site and the displacement of a pseudosubstrate sequence from the active site [15]. A number of studies have established that antipeptide antibodies can be used as valuable probes to evaluate possible regulatory regions within various protein kinases along with the structural events that modulate catalysis (e.g. [16,17]). In this communication, we describe the effects of two antipeptide antibodies on the 69 and 63 kDa Paks from neutrophils.

2. Materials and methods

2.1. Materials

Affinity purified, rabbit polyclonal antibodies raised against peptides corresponding to amino acid residues 2–21 (Pak(N-20) ab) and 525–544 (Pak(C-19) ab) of rat Pak1 and the peptide antigens were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Myosin light chain from chicken gizzard was a gift from Dr. Renee Lu, Boston Biomedical Research Institute. Alkaline phosphatase from bovine intestinal mucosa (4900 U/mg) and apyrase from potato (>200 U/mg) were purchased from Sigma Chemical Co., St. Louis, MO. Sources of all other materials are described elsewhere [2,3,10].

2.2. Preparation of neutrophils

Guinea pig peritoneal neutrophils were prepared as described previously [18].

2.3. Preparation of samples, immunoprecipitation of kinases, and SDS-PAGE

Neutrophils ($3.0–5.0\times10^7$/ml) were stimulated with 1.0 μM fMLP as described [2]. At the appropriate time, 0.50 ml of the reaction mixture was transferred to a microcentrifuge tube containing 0.50 ml of 2× concentrated 'solubilization buffer' (SDS-B) or 2× concentrated 'immunoprecipitation buffer' (ip-B) and rapidly mixed. The final composition of SDS-B after mixing was 2.3% (w/v) SDS, 62.5 mM Tris-HCl (pH 6.8), 5.0 mM EDTA, 10.0% (w/v) glycerol, 5.0% (w/v) 2-mercaptoethanol, and 0.002% (w/v) bromophenol blue. The final composition of ip-B was 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5.0 mM $MgCl_2$, 1.0 mM EGTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1.0% (v/v) Nonidet P-40, 2.5% (v/v) glycerol, 1.0 mM Na_3VO_4, and 1.0 mM phenylmethylsulfonyl fluoride.

Immunoprecipitation reactions for neutrophils lysed with the ip-B ($1.5–2.5\times10^7$ cell equivalents/1.0 ml) were performed as described [9,10]. After the final wash, the protein A coated beads containing the bound kinases were resuspended in 0.10 ml of SDS-B. All samples were immersed in a boiling water bath for 3 min and then subjected to SDS-PAGE (0.07 ml/lane) on 9.0% (w/v) polyacrylamide slab gels as described [3].

2.4. Detection of renaturable protein kinases in polyacrylamide gels

Protein kinases were detected directly in gels by their ability to undergo renaturation and catalyze the phosphorylation of a peptide substrate 'fixed' within the gel that corresponds to residues 297–331 of p47-*phox*. These techniques have been presented in detail [3].

PII S0014-5793(97)00134-8

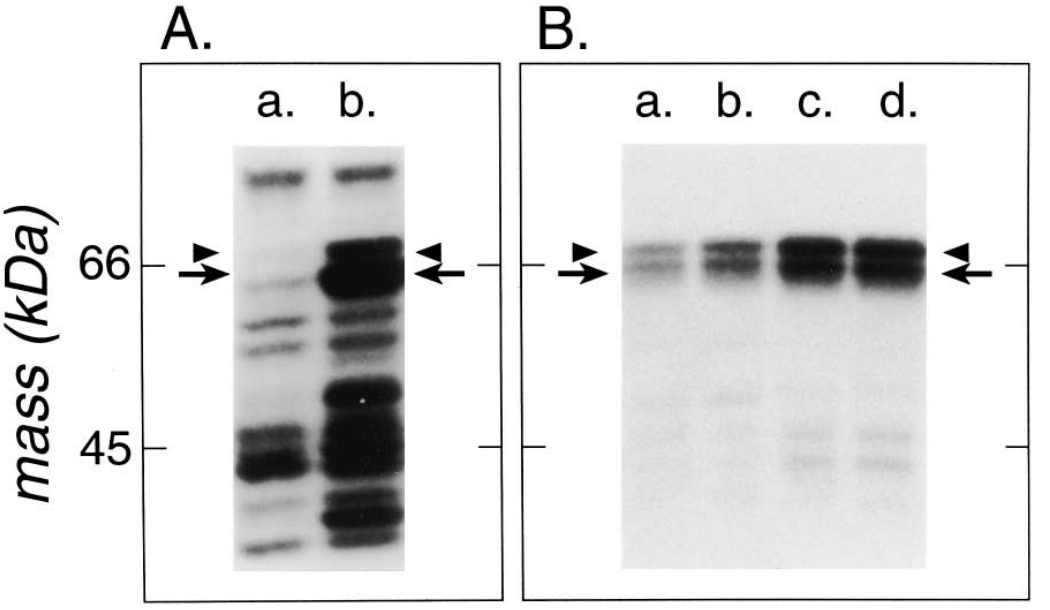

Fig. 1. In vitro activation of the 69 and 63 kDa protein kinases (Paks) during immunoprecipitation with an antipeptide antibody generated to the N-terminal region of Pak 1. Activities of the 69 and 63 kDa Paks were monitored by the ability of these enzymes to undergo renaturation and catalyze the phosphorylation of the p47-*phox* peptide fixed within a gel. The lysates utilized contained ca. 18–30 μM endogenous ATP. Equal amounts of the denatured antibody-Pak complexes were applied to each lane. Neutrophils were stimulated with 1.0 μM fMLP for 15 s. A: Comparison of the renaturable kinases in whole cell lysates from: (a) unstimulated and (b) stimulated neutrophils. B: Comparison of the kinases immunoprecipitated from lysed neutrophils by antibodies generated to the C-terminal (Pak(C-19) ab) and N-terminal (Pak(N-20) ab) regions of Pak1. The autoradiograms shown were derived from: (a) unstimulated cells treated with the Pak(C-19) ab; (b) stimulated cells treated with the Pak(C-19) ab; (c) unstimulated cells treated with the Pak(N-20) ab and (d) stimulated cells treated with the Pak(N-20) ab. The 69 and 63 kDa Paks are indicated by the arrowhead and arrow, respectively.

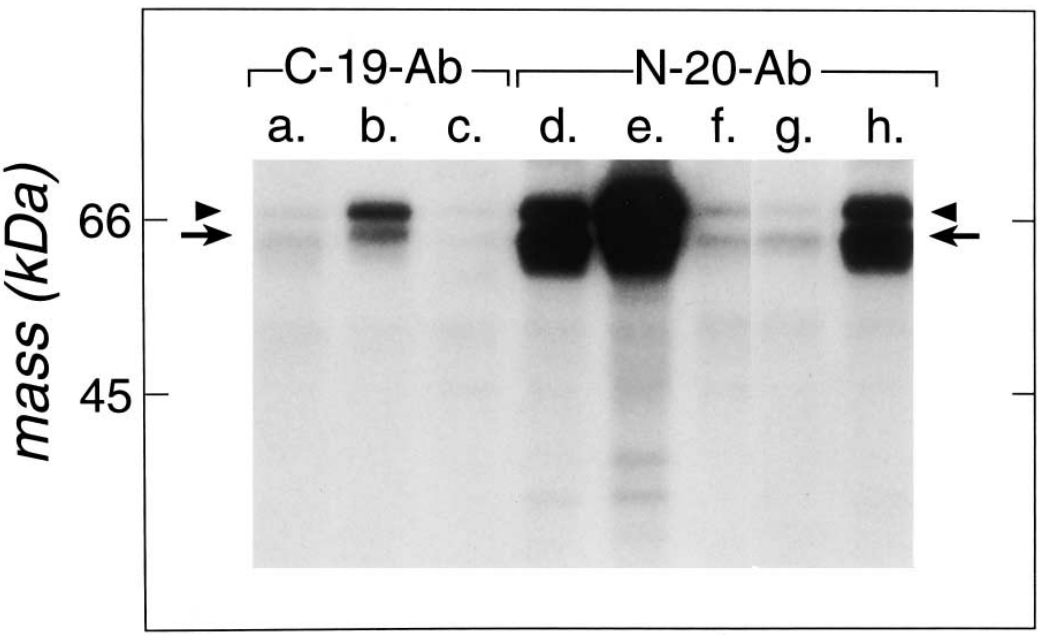

Fig. 2. In vitro activation of the 69 and 63 kDa protein kinases by the Pak(N-20) ab monitored by autophosphorylation. The 69 and 63 kDa Paks were monitored by their abilities to undergo autophosphorylation with [γ-^{32}P]ATP. Pak was precipitated from lysates of neutrophils that contained ca. 18–30 μM endogenous ATP, the complexes were washed and incubated with 0.7 μM [γ-^{32}P]ATP (10 μCi) as referenced in Section 2. Equal amounts of antibody-Pak complexes were applied to each lane. Neutrophils were treated for 15 s with 0.25% (v/v) Me_2SO (unstimulated cells) or 1.0 μM fMLP (stimulated cells). When present, the peptide antigens were utilized at a concentration of 5.0 μg/ml. The autoradiograms shown were from lysates of: (a) unstimulated cells treated with the Pak(C-19) ab; (b) stimulated cells treated with the Pak(C-19) Ab; (c) stimulated cells treated with the Pak(C-19) ab and the C-peptide antigen; (d) unstimulated cells treated with the Pak(N-20) ab; (e) stimulated cells treated with the Pak(N-20) ab; (f) unstimulated cells treated with the Pak(N-20) ab and the N-peptide antigen; (g) stimulated cells treated with the Pak(N-20) ab and the N-peptide antigen; and (h) unstimulated cells treated with the Pak(N-20) ab and the C-peptide antigen. The 69 and 63 kDa Paks are indicated by the arrowhead and arrow, respectively.

2.5. Autophosphorylation and phosphorylation of exogenous proteins by Pak

These reactions were monitored as described [9,10].

2.6. Analysis of data

Unless otherwise noted, all of the autoradiographic observations were confirmed in at least three separate experiments performed on different preparations of cells. The number of observations (n) is also based on different cell preparations.

3. Results

Stimulation of neutrophils with the chemoattractant fMLP is known to result in rapid activation of four renaturable protein kinases with molecular masses of ca. 69, 63, 49 and 49 kDa. These kinases can catalyze the phosphorylation of a peptide substrate fixed within a gel that corresponds to residues 297–331 of p47-*phox* (Fig. 1A [2–4]). The 69 and 63 kDa kinases are Paks [10]. Little or no activity was observed for these enzymes in unstimulated neutrophils (Fig. 1A,a [2–6]). Interestingly, the 69 and 63 kDa Paks exhibited substantial amounts of activity when immunoprecipitated from lysates of unstimulated cells with an antipeptide antibody (Pak(N-20) ab) generated to residues 2–21 of rat Pak1 (Fig. 1B,c). These lysates contained ca. 18–30 μM ATP which was released from the disrupted cells (calculated from the observation that 1×10^9 packed cells occupy a volume of ca. 1.0 ml and contain ca. 1.2 mM ATP [19]). In contrast, little activity was obtained from lysates of unstimulated neutrophils with an antipeptide antibody generated to the C-terminal region of Pak1 (Fig. 1B,a) or with an antibody generated to a fusion protein that corresponds to residues 176–306 of rat Pak1 [10]. However, both of these antibodies were capable of immunoprecipitating the active kinases from stimulated neutrophils (e.g. Fig. 1B,b [10]). It is unlikely that the Pak(N-20) ab preserved or protected a high level of Pak activity that was present in unstimulated neutrophils because little activity was observed for these kinases in resting cells disrupted under denaturing conditions that rapidly inactivate protein phosphatase and proteases (e.g. with SDS-B; Fig. 1A,a [2–6]). Rather, the results strongly indicate that perturbation of the N-terminal region of Pak results in the activation of these kinases in a reaction that is dependent upon ATP (see below). Immunoprecipitation experiments followed by Western blotting of the samples with the Pak(N-20) ab and/or the Pak(C-19) ab indicated that these antibodies exhibited similar affinities for the 69 and 63 kDa kinases (data not shown).

Activation of Paks can also be monitored by the ability of these enzymes to undergo enhanced autophosphorylation

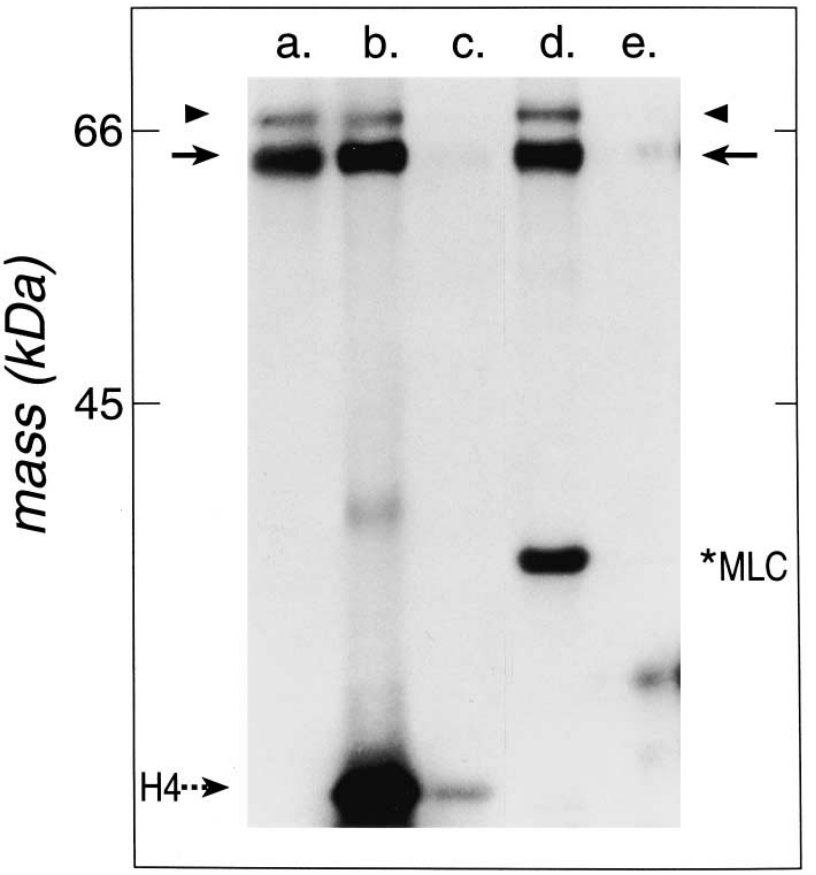

Fig. 3. Substrate specificity of the Paks activated with the Pak(N-20) ab. The 69 and 63 kDa protein kinases were immunoprecipitated from lysates of unstimulated neutrophils with the Pak(N-20) ab and their ability to catalyze the phosphorylation of various exogenous substrates were examined as referenced in Section 2. The lysates contained ca. 18–30 μM endogenous ATP. Equal amounts of material were applied to each lane. The autoradiograms shown were derived from reaction mixtures that contained: (a) no substrate; (b) histone H4 (8.0 μg/50 μl); (c) histone H4; but the N-peptide antigen (5 μg/1.0 ml) was present during the immunoprecipitation step; (d) gizzard myosin light chain (20 μg/50 μl) and (e) gizzard myosin light chain, but the N-peptide antigen was present during the immunoprecipitation step. The 69 and 63 kDa Paks are designated by the arrowhead and arrow, respectively. Histone H4 (lanes b and c) and myosin light chain (lanes d and e) are indicated by the broken arrow and asterisk, respectively.

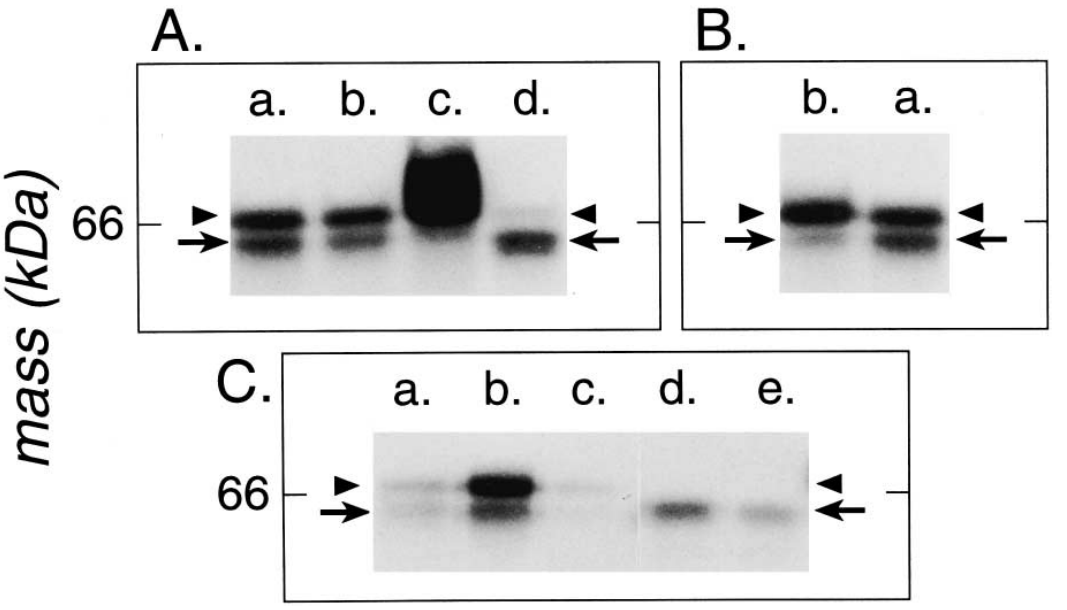

Fig. 4. Effects of ATP, alkaline phosphatase and apyrase on the activation of the 69 and 63 kDa Paks. The 69 and 63 kDa Paks were immunoprecipitated from lysates of unstimulated neutrophils and monitored by their ability to undergo renaturation and catalyze the phosphorylation of the p47-*phox* peptide. The lysates employed contained ca. 18–30 μM ATP. A: Activity of the kinases after immunoprecipitation with the Pak(N-20) ab (a), and after a subsequent incubation of the immunoprecipitates in 'kinase buffer' (50 mM HEPES, pH 7.8, 10 mM $MgCl_2$, 10 mM $MnCl_2$ and 0.20 mM dithiothreitol) for 40 min at 37°C with: (b) no additional reagents; (c) 1.0 mM ATP and (d) alkaline phosphatase (330 U/50 μl). B: Activities of the kinases after immunoprecipitation with: (a) normal ip-B and (b) ip-B plus an additional 1.0 mM ATP. C: Activities of the kinases immunoprecipitated with: (a) Pak(C-19) ab; (b) Pak(N-20) ab; (c) Pak(N-20) ab plus the N-peptide antigen (5 μg/50 μl); (d) Pak(N-20) ab but with all phosphatase inhibitors omitted from ip-B and (e) Pak(N-20) ab with all phosphatase inhibitors omitted and apyrase (30 U/50 μl) added to ip-B. Equal amounts of material were applied to each lane. The 69 and 63 kDa protein kinases are indicated by the arrowhead and arrow, respectively.

after activation [2,14]. For example, the 69 and 63 kDa kinases from fMLP-stimulated neutrophils underwent significant autophosphorylation after immunoprecipitation with the Pak(C-19) ab, whereas the enzymes from unstimulated cells did not (Fig. 2,ab). In contrast, dramatic autophosphorylation of the 69 and 63 kDa kinases was observed in immunoprecipitates obtained from unstimulated neutrophils with the Pak(N-20) ab (Fig. 2,d). The specificity of the Pak(N-20) ab was verified by demonstrating that the addition of the N-peptide antigen to the immunoprecipitation step prevented this autophosphorylation reaction (Fig. 2,fg) and the phosphorylation of the p47-*phox* peptide (Fig. 4C,c), whereas the C-peptide antigen did not (Fig. 2,h). However, the C-peptide antigen was effective in blocking immunoprecipitation of the kinases when the Pak(C-19) ab was employed (Fig. 2,c).

Histone H4 is a selective substrate for activated Paks [2,15]. The 69 and 63 kDa kinases activated with the Pak(N-20) ab exhibited substantial activity with this substrate and with myosin light chain from gizzard (Fig. 3). These reactions were not observed when the N-peptide antigen was present during immunoprecipitation of the kinases (Fig. 3). The 69 and 63 kDa kinases activated with the Pak(N-20) ab also exhibited substantial activity with myelin basic protein, histone HIII and histone 2B (data not shown). This specificity is similar to that reported for purified H4 kinase/Pak [20,21].

The mechanism by which the Pak(N-20) ab activated the kinases and the role of ATP in this process were investigated (Fig. 4). Incubation of the isolated antibody-kinase complex after immunoprecipitation with 1.0 mM ATP for 40 min at 37°C resulted in the disappearance of the 63 kDa kinase band and the appearance of a broad band of activity that originated in the 69 kDa region of the renaturation gel (Fig. 4A,c). This profile is identical to that observed when Pak 1 was activated by the addition of either Rac1-GTPγS or Cdc42-GTPγS plus ATP [10] and may reflect the significant size heterogeneity of Pak that occurs after hyperphosphorylation [11,22]. The highly basic nature of the peptide substrate fixed within the gel may exacerbate this situation (10 of the 35 residues are positively charged). Addition of ATP (1.0 mM) to the ip-B during the immunoprecipitation procedure also resulted in an increase in the 69 kDa kinase and a diminution of the 63 kDa band (Fig. 4B). The sharp band of activity observed for the 69 kDa kinase under the conditions described in Fig. 4 B,b may reflect a more limited phosphorylation of Pak at 4°C than occurred at 37°C (i.e. Fig. 4A,c). In contrast, treatment of the antibody-kinase complex with alkaline phosphatase (Fig. 4A,d) or omission of phosphatase inhibitors from ip-B during the immunoprecipitation reaction (Fig. 4C,d) resulted in a marked reduction of the 69 kDa kinase band. The activity of the 63 kDa kinase that persisted under these circumstances may reflect phosphorylation of this enzyme by endogenous ATP at a site that is inaccessible to phosphatases. This possibility was supported by the fact that apyrase was very effective in reducing the activity of the 63 kDa kinase under these conditions (Fig. 4C,e). Apyrase can catalyze the hydrolysis of the endogenous ATP present in the ip-B which was released from the disrupted neutrophils. These effects of alkaline phosphatase and apyrase strongly suggest that the 69 and 63 kDa Paks undergo phosphorylation with endogenous ATP during immunoprecipitation with the Pak(N-20) ab, and that this phosphorylation was necessary for the activation of these enzymes.

4. Discussion

In this communication, we report that an antipeptide antibody that reacts with the N-terminal region of Pak1 (residues 2–21) activates both the 69 and 63 kDa Paks from unstimulated neutrophils. The N-terminal region of Pak1 contains the binding motif for the small GTPases Rac and Cdc42 (ISXPXXXXFXHXXHVG) (residues 75–90) [23] and an SH3 binding site that can interact with the small adapter protein Nck (PPAPP) (residues 12–16) [24,25]. Activation of Pak is thought to involve the removal of an inhibitory region from the MgATP substrate binding site followed by autophosphorylation/displacement of a pseudosubstrate sequence from the active site [15]. A second phosphorylation reaction may facilitate the disassociation of the phosphorylated pseudosubstrate sequence from the peptide/substrate binding grove for low affinity substrates [15]. The autophosphorylation site/putative pseudosubstrate sequence (-KRST-) (residues 419–422) conforms to the preferred consensus sequence for H4 kinase/Pak (KRXS) [26] and lies within the 'activation segment' found in many protein kinases that are boarded by the sequences DFG (residues 406–408) and APE (residues 431–433) [27]. Activation segments are known to play major roles in the regulation of a variety of protein kinases [27]. A constitutively active Pak mutant can be generated by substituting threonine 422 with a glutamic acid residue [11].

It is likely that the Pak(N-20) ab and Rac/Cdc42 induce similar conformational changes in the N-terminal region of Pak since the properties exhibited by the kinases activated with these reagents were virtually identical. These similarities include activation of two forms of Pak (i.e. the 69 and 63 kDa species) (Figs. 1 and 2), their substrate specificities (Fig. 3), ability to undergo autophosphorylation (Fig. 2), and the necessity for ATP/autophosphorylation for activation (Fig. 4) [10]. Similar properties have also been reported for these kinases from stimulated neutrophils [2–4]. Previous studies have reported that Pak can be constitutively activated either through a variety of amino acid substitutions in the N-terminal region of the kinase (e.g. [11,28]) or by fusing the protein to glutathione *S*-transferase [12]. Finally, it is noteworthy that antipeptide antibodies generated to both the N-terminal and C-terminal regions of Pak1 immunoprecipitated both the 69 and 63 kDa kinases from neutrophils (Figs. 1 and 2). Moreover, treatment of the immunoprecipitated kinases with ATP reduced the activity of the 63 kDa enzyme and increased the activity of the 69 kDa species (Fig. 4). These data indicate that the 69 and 63 kDa Paks may represent different forms of Pak1 which vary in their content of phosphate. This possibility and the question as to whether these different forms of Pak vary in their kinetic properties towards certain substrates remain to be addressed.

Acknowledgements: These studies were supported by Grants DK-50015 and AI-23323 from the National Institutes of Health. We are grateful to Ms. Angela J. DiPerri for typing the manuscript.

References

[1] Baggiolini, M., Boulay, F., Badwey, J.A. and Curnutte, J.T. (1993) FASEB J. 7, 1004–1010.
[2] Ding, J. and Badwey, J.A. (1993) J. Biol. Chem. 268, 5234–5240.
[3] Ding, J. and J.A. Badwey (1993) J. Biol. Chem. 268, 17326–17333.
[4] Grinstein, S., Furuya, W., Butler, J.R. and Tseng, J. (1993) J. Biol. Chem. 268, 20223–20231.
[5] Ding, J. and Badwey, J.A. (1994) FEBS Lett. 348, 149–152.
[6] Ding, J., Vlahos, C.J., Liu, R., Brown, R.F. and Badwey, J.A. (1995) J. Biol. Chem. 270, 11684–11691.
[7] Baggiolini, M., Dewald, B., Schnyder, J., Ruch, W., Cooper, P.H. and Payne, T.G. (1987) Exp. Cell Res. 169, 408–418.
[8] Dewald, B., Thelen, M. and Baggiolini, M. (1988) J. Biol. Chem. 263, 16179–16184.
[9] Knaus, U.G., Morris, S., Dong, H.-J., Chernoff, J. and Bokoch, G.M. (1995) Science 269, 221–223.
[10] Ding, J., Knaus, U.G., Lian, J.P., Bokoch, G.M. and Badwey, J.A. (1996) J. Biol. Chem. 271, 24869–24873.
[11] Lim, L., Manser, E., Leung, T. and Hall, C. (1996) Eur. J. Biochem. 242, 171–185.
[12] Wu, C., Lee, S.-F., Furmaniak-Kazmierczak, E., Cote, G.P., Thomas, D.Y. and Leberer, E. (1996) J. Biol. Chem. 271, 31787–31790.
[13] Brzeska, H. and Korn, E. (1996) J. Biol. Chem. 271, 16983–16986.
[14] Manser, E., Leung, T., Salihuddin, H., Zhao, Z.-S. and Lim, L. (1994) Nature 367, 40–46.
[15] Benner, G.E., Dennis P.B. and Masaracchia, R.A. (1995) J. Biol. Chem. 270, 21121–21128.
[16] Makowske, M. and Rosen, O.M. (1989) J. Biol. Chem. 264, 16155–16159.
[17] Wangsgard, W.P., Meixell, G.E., Dasgupta, M. and Blumenthal, D.K. (1996) J. Biol. Chem. 271, 21126–21133.
[18] Badwey, J.A. and Karnovsky, M.L. (1986) Methods Enzymol. 132, 365–368.
[19] DePierre, J.W. and Karnovsky, M.L. (1974) J. Biol. Chem. 249, 7111–7120.
[20] Tahara, S.M. and Traugh, J.A. (1992) J. Biol. Chem. 256, 11558–11564.
[21] Tuazon, P.T. and Traugh, J.A. (1984) J. Biol. Chem. 259, 541–546.
[22] Manser, E., Chong, C., Zhao, Z.-S., Leung, T., Michael, G., Hall, C. and Lim, L. (1995) J. Biol. Chem. 270, 25070–25078.
[23] Burbelo, P.D., Drechsel, D. and Hall, A. (1995) J. Biol. Chem. 270, 29071–29074.
[24] Galisteo, M.L., Chernoff, J., Su, Y.-C., Skolnik, E.Y. and Schlessinger, J. (1996) J. Biol. Chem. 271, 20997–21000.
[25] Bokoch, G.M., Wang, Y., Bohl, B.P., Sells, M.A., Quilliam, L.A. and Knaus, U.G. (1996) J. Biol. Chem. 271, 25746–25749.
[26] Eckols, T.K., Thompson, R.E. and Masaracchia, R.A. (1983) Eur. J. Biochem. 134, 249–254.
[27] Johnson, L.N., Noble, M.E.N. and Owen, D.J. (1996) Cell 85, 149–158.
[28] Bagrodia, S., Derijard, B., Davis, R.J. and Cerione, R.A. (1995) J. Biol. Chem. 270, 27995–27998.