Autophosphorylation and Protein Kinase Activity of p21-Activated Protein Kinase γ-PAK Are Differentially Affected by Magnesium and Manganese†

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ABSTRACT: To examine the requirements for activation of the p21-activated protein kinase γ-PAK (Pak2, PAK I) from rabbit reticulocytes by Cdc42(GTPγS), autophosphorylation with ATP(Mg) or ATP(Mn) and its effects on protein kinase activity were examined. Autophosphorylation with ATP(Mg) alone was minimal with negligible protein kinase activity; the rate of autophosphorylation was increased 3−4-fold upon binding of Cdc42(GTPγS), resulting in a 3-fold stimulation of protein kinase activity with peptide and protein substrates. The rate of autophosphorylation with ATP(Mn) was 4.7-fold faster than with ATP(Mg) alone and was stimulated 2-fold by Cdc42(GTPγS). However, γ-PAK autophosphorylated with ATP(Mn) in the presence or absence of Cdc42(GTPγS) did not phosphorylate peptide or protein substrates in the presence of ATP(Mn), indicating that γ-PAK can utilize ATP(Mn) for autophosphorylation but not for phosphorylation of exogenous substrates. Tryptic phosphopeptide maps of γ-PAK autophosphorylated with ATP(Mg) alone showed 3 phosphopeptides, while with Cdc42(GTPγS) a total of 9 major phosphopeptides was observed. When γ-PAK was autophosphorylated with ATP(Mn) in the presence or absence of Cdc42(GTPγS), 7 major phosphopeptides were observed, which were identical to peptides obtained with Cdc42(GTPγS) and ATP(Mg). Utilizing a recombinant mutant of γ-PAK with alanine replacing threonine 402 in the catalytic region (T402A), it was determined that the two additional phosphopeptides observed in active PAK (peptides 7 and 8) were due to phosphorylation of threonine 402. These results show that Mn sustains autophosphorylation on serine but does not support autophosphorylation of threonine 402, which is required for activity toward exogenous substrates, or phosphorylation of these substrates.

p21-activated protein kinases (PAKs)[1] are activated by autophosphorylation upon binding of small G proteins such as Rac and Cdc42 in the presence of GTP (*1*−*9*). PAK enzymes contain an amino-terminal regulatory domain with a binding site for G proteins and a carboxyl-terminal catalytic domain. γ-PAK (PAK I, Pak2) has a molecular weight of 58 000 Da and is present in numerous tissues and species (*1*, *4*, *8*−*13*). In addition to showing activation via autophosphorylation in response to Cdc42(GTPγS), Walter et al. (*14*) have determined that autophosphorylation is required for activation of γ-PAK following cleavage into 2 fragments by CPP32 (caspase 3). Autophosphorylation sites on serine are present in the p27 regulatory domain as shown by phosphopeptide mapping. Only one autophosphorylation site is identified in the catalytic domain, threonine 402. In other studies, the substrates histone 4 and 2B have been shown to stimulate autophosphorylation resulting in activation of γ-PAK without addition of Cdc42(GTPγS) or cleavage by caspase (15).

Autophosphorylation is a property common to a number of protein kinases and is now recognized in many cases to have an important role in the regulation of the function of these enzymes. With some protein kinases, such as, cyclic AMP-dependent protein kinase, cyclic GMP-dependent protein kinase, tyrosine receptor kinases, dsRNA-activated protein kinase, protein kinase C, and calmodulin-dependent protein kinase I, it has been established that autophosphorylation is required to fully activate the enzyme (*16*−*24*). With other protein kinases such as calmodulin kinases II and IV, autophosphorylation has been shown to inactivate the enzyme (*25*−*28*).

In these studies, we examine the requirement for activation of the native γ-PAK holoenzyme (p58) from rabbit reticulocytes by Cdc42(GTPγS). γ-PAK is present primarily as an inactive holoenzyme (*10*). By utilizing ATP(Mg) and ATP(Mn), it is possible to distinguish between autophosphorylation, which does not result in activation of γ-PAK, from autophosphorylation, which is essential for γ-PAK activity. Autophosphorylation with ATP(Mn) occurs at a faster rate than with ATP(Mg) in the presence or absence of Cdc42(GTPγS). However, autophosphorylation with ATP(Mn) does not support protein kinase activity toward exogenous substrates when assayed in the presence of ATP(Mn). Thus, γ-PAK can utilize ATP(Mn) for autophosphorylation but not phosphorylation of exogenous substrates. This is consistent with data showing that ATP(Mg) is absolutely required for the phosphorylation of threonine 402 in the presence of Cdc42(GTPγS) and for the full expression of protein kinase activity toward exogenous substrates.

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[1] Abbreviations: PAK, p21-activated protein kinase; GST, glutathione S-transferase.

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EXPERIMENTAL PROCEDURES

Materials. Histone I (IIIS), mixed histone (IIAS), trypsin (diphenylcarbamyl chloride-treated), soybean trypsin inhibitor, bovine serum albumin (fatty acid-free), myelin basic protein, and magnesium chloride were purchased from Sigma. Histones 2B and 4, ATP, GTPγS, GDP, aprotinin, antipain, leupeptin, and pepstatin were obtained from Boehringer Mannheim Chemicals. (γ-^{32}P)ATP was purchased from NEN. Manganese chloride was from Mallinckrodt. Peptides were synthesized and purified as described previously (*29*). P81 phosphocellulose paper was purchased from Whatman, and cellulose thin-layer chromatography sheets were from Kodak. The clone for GST-Cdc42 was expressed and purified as described by Jakobi et al. (*8*).

Purification of γ-PAK. γ-PAK was purified from rabbit reticulocytes to apparent homogeneity by chromatography on DEAE-cellulose, SP-Sepharose, protamine agarose, and FPLC on Mono Q and Mono S; phosphatase inhibitors (1 mM sodium pyrophosphate and 1 mM sodium vanadate) were added to the postribosomal supernatant and to all buffers. An enzyme unit is the amount of enzyme that incorporates one picomole of phosphate into histone IIAS per minute at 30 °C.

Recombinant γ-PAK from rabbit spleen and the T402A mutant were expressed in insect cells as GST-γ-PAK, purified on glutathione-Sepharose and cleaved with thrombin to produce p58 as described elsewhere (*14*).

Autophosphorylation and Activation of γ-PAK. γ-PAK was activated by autophosphorylation in the presence of Cdc42(GTPγS) as described elsewhere (*8*). Autophosphorylation was carried out in 50 μL reactions containing 20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$ or 6 mM $MnCl_2$, 30 mM 2-mercaptoethanol, 0.2 mM (γ-^{32}P)ATP (specific activity 3000−5000 cpm/pmol), and γ-PAK (10−20 units) with Cdc42 (0.8−1.0 μg) preloaded with GTPγS (0.18 mM). Incubation was at 30 °C for 10−15 min; reactions were terminated by the addition of 10 μL of 100 mM ATP and sample buffer and analyzed by SDS−PAGE in 10% polyacrylamide gels (*8*). Phosphorylated γ-PAK was analyzed by autoradiography, and the radioactivity was quantified by excising the bands from the gel and counting the ^{32}P incorporated by Cerenkov or liquid scintillation counting.

To measure changes in activity following autophosphorylation and activation, 2 μg of protein substrate were added to 10−20 units of enzyme prephosphorylated with ATP(Mg) or ATP(Mn) in 50 μL reaction mixtures. Incubation was for 15 min, and the reactions was analyzed in 15% polyacrylamide gels as described above. These conditions are kinetically valid since the reaction was linear with time and less than 10% of the substrate was phosphorylated.

Reactions with peptide (1 mM) as substrate were carried out in 25 μL reaction mixtures containing 5−6 units of prephosphorylated γ-PAK. Following incubation for 30 min, the reactions were terminated with 5 μL of 100 mM nonlabeled ATP and the peptide was precipitated on P81 phosphocellulose paper with 75 mM H_3PO_4 as described previously (*29*). Reactions were linear with time, and less than 10% of substrate was phosphorylated under these conditions.

K_m and V_{max} values were determined from Lineweaver−Burk plots of the rate of phosphorylation at 4−6 different concentrations of histone IIAS (0−1.0 mg/mL) and at a fixed concentration of (γ-^{32}P)ATP (0.2 mM).

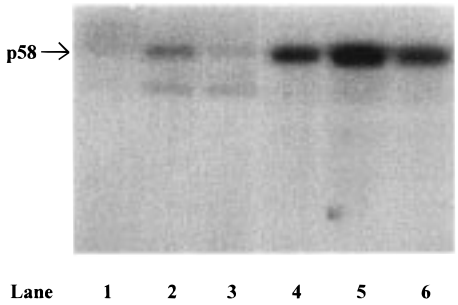

FIGURE 1: Effects of magnesium and manganese on autophosphorylation of γ-PAK. Autophosphorylation was carried out with (γ-^{32}P)ATP and 10 mM $MgCl_2$ (lanes 1−3) or 6 mM $MnCl_2$ (lanes 4−6): lanes 1 and 4, no addition; lanes 2 and 5, with Cdc42(GTPγS); lanes 3 and 6, with Cdc42(GDP). The autoradiogram is shown.

Tryptic Phosphopeptide Mapping and Phosphoamino Acid Analysis. The protein corresponding to autophosphorylated γ-PAK was excised from the polyacrylamide gel and extensively digested with trypsin, and peptide mapping was carried out as previously described (*30*). Peptides were separated by electrophoresis at 600 v for 2 h at 4 °C in the first dimension in pyridine/acetic acid/water (100:10:890, pH 3.5). The second dimension was thin-layer chromatography in butanol/pyridine/acetic acid/water (60:40:12:48). ^{32}P-labeled peptides were detected by autoradiography with a Phosphor Imager SI (Molecular Dynamics). Phosphoamino acid analysis was carried out on tryptic digests or phosphopeptides hydrolyzed with 6 N HCl for 2 h as previously described (*31*). Individual phosphopeptides were scraped from the phosphopeptide maps and eluted sequentially with 0.5 mL each of 0.5 M acetic acid, water, and 0.5 M pyridine. Eluates were combined, dried in a Speed Vac, and then subjected to acid hydrolysis and phosphoamino acid analysis.

RESULTS

Autophosphorylation and Activation of γ-PAK in the Presence of ATP(Mg) and ATP(Mn). γ-PAK has been shown to be activated by binding of Cdc42(GTPγS) followed by autophosphorylation (*8*). To examine the requirements for activation via autophosphorylation, γ-PAK p58 purified from rabbit reticulocytes was incubated with (γ-^{32}P)ATP and 10 mM $MgCl_2$ or 6 mM $MnCl_2$ and analyzed by SDS−PAGE followed by autoradiography. With magnesium, autophosphorylation of p58 was low in the absence of Cdc42, was increased slightly in the presence of Cdc42(GDP), and was significantly increased in the presence of Cdc42(GTPγS) (Figure 1). Quantification of autophosphorylation with magnesium showed a 1.2-fold stimulation with Cdc42(GDP) and a 3.4-fold stimulation with (Cdc42GTPγS) (Table 1). With manganese, autophosphorylation in the absence of Cdc42(GTPγS) was higher than with magnesium in the presence of Cdc42(GTPγS) (Figure 1). Autophosphorylation was stimulated 4.7-fold with ATP(Mn) alone and 9.0-fold with Cdc42(GTPγS) as compared to ATP(Mg) alone (Table 1).

The optimum metal ion concentration for autophosphorylation of γ-PAK p58 in the absence of Cdc42 was determined from rates of reaction at concentrations of magnesium

Table 1: Stimulation of Protein Kinase Activity by Autophosphorylation of γ-PAK[a]

		^{32}P incorporated			
		p58		H4	
γ-PAK	Me^{2+}	(cpm)	(-fold)	(cpm)	(-fold)
p58	Mg	108	1.0	15 031	1.0
p58 + Cdc42(GTPγS)	Mg	370	3.4	49 355	3.3
p58 + Cdc42(GDP)	Mg	132	1.2	34 447	2.3
p58	Mn	504	4.7	1 300	0.1
p58 + Cdc42(GTPγS)	Mn	970	9.0	2 568	0.2
p58 + Cdc42(GDP)	Mn	816	7.6	1 676	0.1

[a] γ-PAK (9 units) was autophosphorylated under kinetically valid conditions with (γ-^{32}P)ATP and 10 mM $MgCl_2$ or 6 mM $MnCl_2$ and analyzed by SDS−PAGE as described under Experimental Procedures. Protein kinase activity of autophosphorylated γ-PAK was measured with histone 4 (2 μg). ^{32}P incorporated was determined by Cerenkov counting gel slices in a scintillation counter.

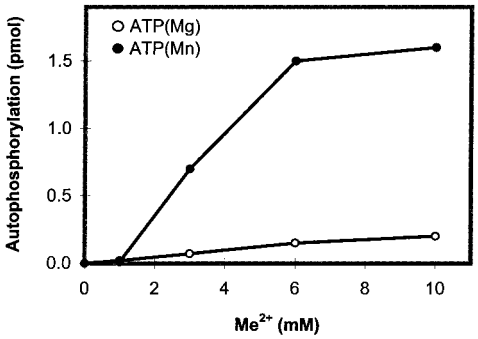

FIGURE 2: Effects of increasing concentrations of magnesium and manganese on autophosphorylation of γ-PAK. Autophosphorylation of γ-PAK p58 with (γ-^{32}P)ATP was carried out at increasing concentrations of Mg and Mn as described under Experimental Procedures, and an aliquot (25 μL) was analyzed by SDS−PAGE. The autophosphorylated protein was excised from the gel and quantified by Cerenkov counting to determine the extent of autophosphorylation.

or manganese up to 10 mM. Autophosphorylation of p58 increased as the concentration of metal ion increased and was found to be maximal at 6−10 mM Mg^{2+} or Mn^{2+} (Figure 2). The rate of autophosphorylation of p58 with manganese was 5−7-fold greater than with magnesium at the optimal concentration.

To determine the effects of autophosphorylation on protein kinase activity, we examined autophosphorylated γ-PAK with the substrate histone 4. Some phosphorylation of histone 4 was observed when γ-PAK was autophosphorylated with ATP(Mg) alone. When γ-PAK was autophosphorylated in the presence of Cdc42(GTPγS), the rate of phosphorylation of histone 4 was stimulated 3.1-fold (Table 1). Some stimulation of histone phosphorylation was also observed with Cdc42(GDP) due to the fact that histone can activate native γ-PAK to some extent. The protein kinase activity of γ-PAK autophosphorylated with ATP(Mn) was quite low, 10-fold less than the activity observed when γ-PAK was autophosphorylated with ATP(Mg). The activity was essentially unchanged by the addition of Cdc42(GTPγS). The rate of phosphorylation of histone 4 was 19-fold faster with magnesium than with manganese in the presence of Cdc42-(GTPγS).

Kinetic parameters were determined with increasing concentrations of histone using γ-PAK autophosphorylated with ATP(Mg) or ATP(Mn) in the presence of Cdc42-(GTPγS) (Table 2). The K_m value with magnesium was 0.2 mg/mL; with manganese the K_m was 6.5-fold higher than with magnesium and the V_{max} was reduced by 30%. This gives a V_{max}/K_m of 134 with ATP(Mg) and 15 for ATP(Mn).

Table 2: Kinetic Parameters for Phosphorylation of Histone with γ-PAK[a]

γ-PAK	Me^{+2}	K_m (mg/mL)	V_{max} (pmol/(min μg))	V_{max}/K_m
p58	Mg	0.2	27	134
p58	Mn	1.3	19	15

[a] γ-PAK (p58) activated in the presence of Cdc42(GTPγS) was autophosphorylated by incubation with (γ-^{32}P)ATP and 10 mM $MgCl_2$ or 6 mM $MnCl_2$ for 10 min, and the rate of phosphorylation of histone IIAS (0−1.0 mg/mL) was measured. K_m and V_{max} values were determined from Lineweaver−Burk reciprocal plots of rates and concentrations of substrates.

Table 3: Comparison of the Effects of Mg^{2+} and Mn^{2+} on the Rate of Phosphorylation of Protein and Peptide Substrates by γ-PAK[a]

	^{32}P incorporated		
substrate	Mg (pmol)	Mn (pmol)	Mg/Mn (ratio)
experiment 1			
histone 4	3.8	0.7	5
histone 2B	3.3	0.4	8
myelin basic protein	1.4	0.2	7
histone 1	0	0.2	0
experiment 2			
AKRESAA	18.6	1.0	19
AKRASAA	11.9	0.7	17
AKKASAA	0.1	0.1	1
AKAASAA	3.5	0	0

[a] Proteins (2 μg) or synthetic peptides (1 mM) were incubated with γ-PAK (2.5 units), activated by autophosphorylation in the presence of Cdc42(GTPγS) and 10 mM $MgCl_2$ or 6 mM $MnCl_2$ as described under Experimental Procedures. Phosphorylation of proteins and peptides was analyzed as described under Experimental Procedures.

Analysis of Phosphorylation of Different Substrates with ATP(Mg) and ATP(Mn). The effects of magnesium and manganese on phosphorylation of protein substrates by γ-PAK following activation by Cdc42(GTPγS) were examined (Table 3). Histones 4 and 2B were good substrates with ATP(Mg), myelin basic protein was phosphorylated to a lesser extent, and histone 1 was not phosphorylated. The rate of substrate phosphorylation was 5−8-fold higher with all three protein substrates when autophosphorylation was carried out with ATP(Mg) was compared to ATP(Mn).

Synthetic heptapeptides based on the phosphorylation site for γ-PAK in Rous sarcoma virus protein NC (KKRKSGL), identified both in vivo and in vitro, were used to examine the activity of γ-PAK with manganese and magnesium (*29*). AKKASAA and AKAASAA were not phosphorylated by γ-PAK using ATP(Mg) or ATP(Mn), which coincided with previous studies with ATP(Mg) (*29*). Peptides AKRESAA and AKRASAA were good substrates for Cdc42-activated γ-PAK. The rate of phosphorylation of the peptides with magnesium was significantly higher than with manganese, with the Mg/Mn ratios around 18. These results suggest that autophosphorylation with ATP(Mn) did not result in activation of γ-PAK or that manganese was not a suitable cofactor for phosphorylation of exogenous substrates.

GST-γ-PAK purified on glutathione-Sepharose beads was used to examine the effects of ATP(Mg) and ATP(Mn) with

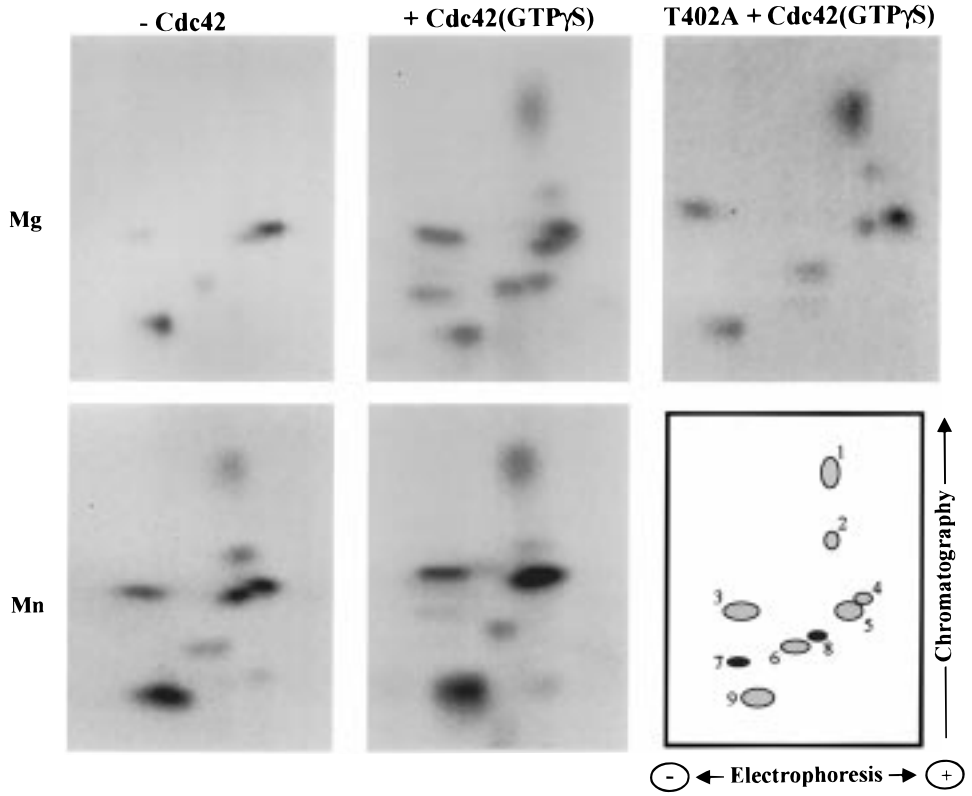

FIGURE 3: Tryptic phosphopeptide maps of γ-PAK autophosphorylated in the presence of ATP(Mg) or ATP(Mn). Autophosphorylation was carried out with (γ-^{32}P)ATP and the indicated metal ion in the presence and absence of Cdc42(GTPγS). The T402A mutant was autophosphorylated with ATP(Mg). Two-dimensional tryptic phosphopeptide mapping was carried out as described in Experimental Procedures. The autoradiograms are shown along with a cartoon identifying the phosphopeptides.

Table 4: Phosphorylation of Substrate Peptide By Autophosphorylated γ-PAK[a]

metal ion		^{32}P incorporated	
autophosphorylation	peptide phosphorylation	(cpm)	(-fold)
Mn	Mn	2 302	1.0
Mn	Mg	35 621	15.5
Mg	Mg	46 000	20.0
Mg	Mn	3 466	1.5

[a] GST-γ-PAK was autophosphorylated with (γ-^{32}P)ATP and 6 mM $MnCl_2$ or 10 mM $MgCl_2$ in the presence of Cdc42(GTPγS) for 15 min as described under Experimental Procedures, and the metal ion and Cdc42 were removed by washing on glutathione-Sepharose. Peptide AKRESAA (1 mM) and Cdc42(GTPγS) were added to 25 μL reaction mixtures with 6 mM $MnCl_2$ or 10 mM $MgCl_2$ as indicated and incubated for 15 min at 30 °C.

Cdc42(GTPγS) (Table 4). In these studies, PAK was autophosphorylated in the presence of Cdc42(GTPγS), the metal ion was removed by washing the beads, and the appropriate metal ion was added with the substrate peptide AKRESAA and Cdc42(GTPγS). When γ-PAK was autophosphorylated in the presence of ATP(Mn), and ATP(Mn) was used to phosphorylate the peptide, little or no phosphate was incorporated into the peptide. The rate of phosphorylation of the peptide was the highest with γ-PAK autophosphorylated with ATP(Mg), and when ATP(Mg) was also utilized to phosphorylate the peptide, there was a 20-fold increase over that observed with ATP(Mn). When PAK was autophosphorylated by ATP(Mn) in the presence of Cdc42 (GTPγS) and ATP(Mg) was used in the second reaction, substrate phosphorylation was increased 15.5-fold. This indicated that magnesium in the second reaction could overcome the effects of manganese in the first. However, γ-PAK did not utilize ATP(Mn) for phosphorylation of exogenous substrates. When ATP(Mg) was present in the first reaction and manganese in the second, γ-PAK activity was inhibited.

Tryptic Phosphopeptide Mapping and Phosphoamino Acid Analysis of γ-PAK Autophosphorylated with ATP(Mg) and ATP(Mn). Tryptic phosphopeptide mapping and phosphoamino acid analysis of γ-PAK autophosphorylated with ATP(Mg) or ATP(Mn) were carried out in the absence and presence of Cdc42(GTPγS). Tryptic phosphopeptide maps obtained with the activated form of γ-PAK autophosphorylated with ATP(Mg) in the presence of Cdc42(GTPγS) contained 9 major phosphopeptides (Figure 3). In the absence of Cdc42, two major phosphopeptides, 4 and 9, and three minor phosphopeptides 1, 5, and 6, were detected. These results were different from the data obtained with γ-PAK autophosphorylated with ATP(Mn); seven phosphopeptides were observed when γ-PAK was autophosphorylated in the presence or absence of Cdc42(GTPγS), four major phosphopeptides (3, 4, 5, and 9), and three minor ones. The two missing phosphopeptides were 7 and 8.

Phosphoamino acid analysis showed that γ-PAK autophosphorylated with ATP(Mg) in the presence of Cdc42-(GTPγS) was primarily on serine residues, with ~10% of the phosphate on threonine. γ-PAK phosphorylated with ATP(Mg) alone or ATP(Mn) in the presence or absence of Cdc42(GTPγS) was phosphorylated only on serine (Figure 4).

By using a recombinant mutant of γ-PAK in which the threonine at position 402 was replaced with alanine (T402A), we carried out autophosphorylation with ATP(Mg) and

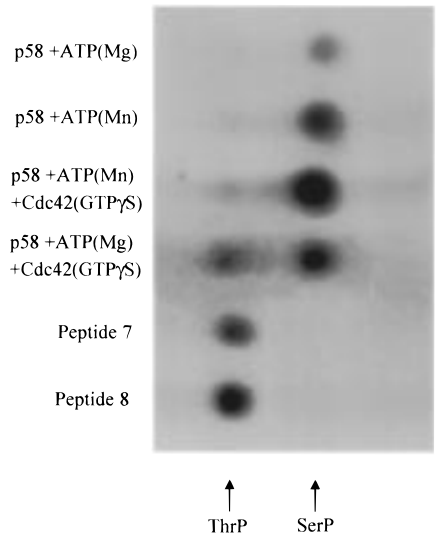

FIGURE 4: Phosphoamino acid analysis of autophosphorylated γ-PAK. Autophosphorylation was carried out in the presence or absence of Cdc42(GTPγS) with 10 mM ATP(Mg) or 6 mM ATP(Mn). Peptides 7 and 8 were excised from maps, and phosphoamino acid analysis was carried out as described under Experimental Procedures. The autoradiograms are shown.

Cdc42(GTPγS) which resulted in the same phosphopeptide pattern as the native γ-PAK autophosphorylated with ATP(Mn) in the presence of Cdc42(GTPγS); peptides 7 and 8 were missing (Figure 3). Thus, it can be concluded that threonine 402 was not autophosphorylated with ATP(Mn), although autophosphorylation of serine could occur.

Effects of Magnesium and Manganese on Stimulation of Autophosphorylation by Basic Proteins. In other studies we have shown that histone 4 and 2B stimulated autophosphorylation and activation of γ-PAK in the absence of Cdc42(GTPγS) (*15*). Thus, it was of interest to examine the effects of magnesium and manganese on stimulation of autophosphorylation by addition of these proteins to the autophosphorylation reactions. As shown in Figure 5, in the absence of added protein, there was a 5-fold increase in the rate of autophosphorylation of γ-PAK with ATP(Mn) as compared with ATP(Mg). Upon addition of histone 4 and 2B, there was a significant increase in autophosphorylation (20- and 9-fold, respectively) with ATP(Mg). With ATP(Mn) there was little effect on the rate of autophosphorylation as compared with ATP(Mn) in the absence of added protein, 1.1- and 1.3-fold, respectively, for histone 4 and 2B. Addition of bovine serum albumin, polylysine, or Cdc42 in the absence of GTPγS resulted in little or no stimulation of autophosphorylation with either ATP(Mg) or ATP(Mn). Thus, the stimulation of autophosphorylation observed with histone 4 and 2B occurred only with ATP(Mg) and not with ATP(Mn).

DISCUSSION

γ-PAK from rabbit reticulocytes can be autophosphorylated using ATP(Mg) or ATP(Mn). In the absence of Cdc42, the rate of autophosphorylation is 4−5-fold greater with ATP(Mn) than with ATP(Mg) (Table 1). In the presence of Cdc42(GTPγS), autophosphorylation is enhanced under both conditions. However, in the presence of magnesium, autophosphorylation resulting from binding of Cdc42(GTP) results in activation of γ-PAK and phosphorylation of exogenous substrates. The level of autophosphorylation can be directly correlated with the level of protein kinase activity (Table 1). In contrast, autophosphorylation of γ-PAK with ATP(Mn) in the presence or absence of Cdc42(GTPγS) does not activate the protein kinase toward exogenous substrates. The rate of phosphorylation with ATP(Mg) is up to 20-fold greater as compared to that with ATP(Mn). Proteins and peptides that are excellent substrates for active γ-PAK are not phosphorylated in the presence of ATP(Mn) using γ-PAK autophosphorylated with ATP(Mn) or ATP(Mg). However, PAK autophosphorylated with ATP(Mg) or ATP(Mn) can phosphorylate substrates in the presence of ATP(Mg) and

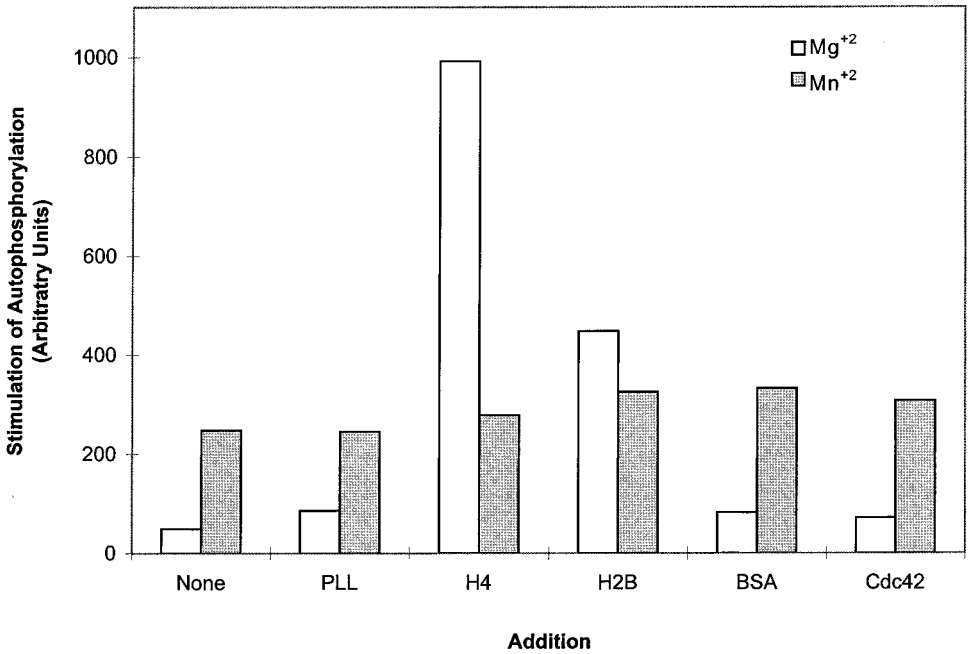

FIGURE 5: Effects of exogenous proteins and polylysine on autophosphorylation of γ-PAK. Autophosphorylation of γ-PAK (5 units) was carried out in the presence of 10 mM ATP(Mg) and 6 mM ATP(Mn), of 1 μM histones 4 (H4) and 2B (H2B) bovine serum albumin (BSA), Cdc42, or polylysine (PLL) (0.1 μM).

Cdc42(GTPγS) (Table 4). Thus, although γ-PAK can utilize either ATP(Mg) or ATP(Mn) for autophosphorylation, it has a requirement for magnesium in the phosphorylation of exogenous substrates.

Tryptic phosphopeptide maps with ATP(Mg) and Cdc42-(GTPγS) show 9 phosphopeptides, two more than are observed with ATP(Mn) in the presence and absence of Cdc42(GTPγS). Autophosphorylation of γ-PAK with ATP-(Mg) in the presence of Cdc42(GTPγS) is predominantly on serine residues, with about 10% of the phosphorylation on threonine. When autophosphorylation is carried out with ATP(Mn), autophosphorylation is exclusively on serine. To determine whether the two missing phosphopeptides are due to autophosphorylation of a threonine in the catalytic domain conserved in most other protein kinases, we used recombinant γ-PAK containing a substitution of alanine for threonine 402 (T402). The tryptic phosphopeptide map of T402 autophosphorylated with ATP(Mg) shows a profile similar to that of native PAK autophosphorylated with ATP(Mn) in that peptides 7 and 8 are missing. As shown by site-directed mutagenesis of recombinant γ-PAK, the only phosphothreonine in activated γ-PAK p58 is amino acid 402, and this threonine is responsible for full protein kinase activity. This is consistent with the lack of phosphorylation and activity of γ-PAK with ATP(Mn) and indicates that phosphorylation of threonine is required for activation of γ-PAK. This is supported by the observation that Mn-autophosphorylated PAK, wherein peptides 7 and 8 are missing, cannot phosphorylate substrate with ATP(Mn) in the presence of Cdc42-(GTPγS), but becomes activated with ATP(Mg) in the presence of Cdc42(GTPγS), and is capable of phosphorylating the substrate. Under these conditions threonine 402 becomes autophosphorylated as indicated by tryptic phosphopeptide mapping. These data are consistent with the studies of Walter et al. (*14*) showing that phosphorylation of threonine 402, following cleavage of γ-PAK into a p27 regulatory domain and a p34 catalytic domain by CPP32 (caspase 3), is required for γ-PAK activity.

γ-PAK binds both ATP(Mg) and ATP(Mn) in its inactive conformation and utilizes ATP for autophosphorylation with both metal ions. Thus, ATP(Mn) appears to be in the proper position for autophosphorylation on serine, but is not able to transfer phosphate to threonine 402 or to an exogenous substrate, even in the presence of Cdc42(GTP). With ATP-(Mg) in the presence of Cdc42(GTPγS), both serine and threonine are autophosphorylated, the latter on a site in the catalytic domain shown to be T402, which results in activation of the protein kinase activity with exogenous substrates (*15*). It has been shown that phosphorylation of the conserved threonine adjacent to the activation loop is required for activation of a number of protein kinases and is important in interacting with the catalytic loop (*32*−*34*).

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