Cdc42-independent activation and translocation of the cytostatic p21-activated protein kinase γ-PAK by sphingosine

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Received 20 July 2001; revised 18 September 2001; accepted 21 September 2001

First published online 9 October 2001

Edited by Guido Tettamanti

Abstract Autophosphorylation of p21-activated protein kinase γ-PAK is stimulated at 10 μM sphingosine in vitro and is maximal at 100 μM. Sites autophosphorylated on γ-PAK in response to sphingosine are identical to those obtained with Cdc42(GTP). Autophosphorylation is paralleled by stimulation of γ-PAK activity as measured with peptide and protein substrates. In 3T3-L1 cells, sphingosine stimulates the autophosphorylation and activity of γ-PAK associated with the membrane-containing particulate fraction by 2.8-fold, but does not stimulate the activity of the soluble enzyme. Thus, γ-PAK is activatable via a Cdc42-independent mechanism, suggesting sphingosine has a role in γ-PAK activation under conditions of cell stress.

Key words: Sphingosine; Protein kinase; p21-activated protein kinase; Cytostasis

1. Introduction

Sphingosine is a lipid produced in cellular membranes as a result of sphingolipid catabolism in response to different signals such as insulin, PDGF, phorbol esters, tumor necrosis factor-α, and γ-irradiation [1–3]. Sphingosine can stimulate the proliferation of different cell types, but also has cytostatic and apoptotic properties [3–7].

γ-PAK (also known as PAK2) is a ubiquitous member of the family of p21-activated protein kinases (PAKs) [8–10]. Binding of Cdc42(GTPγS) stimulates the autophosphorylation and activity of γ-PAK. γ-PAK, but not α-PAK or β-PAK, is activated by caspase cleavage during apoptosis [11–13]. In contrast to α-PAK, which is activated by growth factors and insulin [14–16], γ-PAK has been shown to have cytostatic activity [17,18] and is activated by stimuli leading to cytostasis or apoptosis [11,12,18–20]. Hyperosmolarity, γ-radiation and treatment with the DNA-damaging agent AraC result in activation of γ-PAK; in contrast, both α-PAK and γ-PAK are activated in response to UV and cisplatin [19,20].

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Abbreviations: PAK, p21-activated protein kinase

The protein kinase activity of γ-PAK is regulated by changes in autophosphorylation [21–23]. Eight autophosphorylation sites have been identified in γ-PAK, seven serines located in the regulatory domain, and one threonine in the catalytic domain [23]. Activation of the enzyme correlates with autophosphorylation of two serines (Ser-141 and Ser-165) in the regulatory region and threonine (Thr-402) in the activation loop.

Recently, sphingosine has been shown to induce autophosphorylation and activation of α-PAK towards exogenous substrates [24]. In the present study, γ-PAK is shown to be autophosphorylated and activated in response to sphingosine. In 3T3-L1 cells, sphingosine stimulation of γ-PAK activity occurs in the membrane-containing particulate fraction and is accompanied by increased phosphorylation of γ-PAK.

2. Materials and methods

2.1. Materials

Histone 4 was from Boehringer Mannheim; sphingosine from bovine brain sphingomyelin (S6879), phosphatidylinositol, phosphatidylserine, diacylglycerol and C_6-ceramide were from Sigma. Antibody specific for γ-PAK (RR-1) was prepared in rat [17] or was obtained from Santa Cruz Biotechnology, Inc. (N19). Peptide S3 (AKRESAA) was synthesized and purified as described [25]. GTPγS was from Boehringer Mannheim; [γ-^{32}P]ATP was from Dupont NEN. GST-Cdc42 was expressed in *Escherichia coli* and purified on glutathione-Sepharose 4B [26].

2.2. Autophosphorylation of γ-PAK

The GST fusion protein of rabbit γ-PAK (M_r 88 kDa) was expressed in insect cells (TN5B-4) and purified on glutathione-Sepharose 4B. GST-γ-PAK has the same enzyme activity as γ-PAK [13,22]. Stock solutions of sphingosine and C_6-ceramide (10 mM) were prepared in DMSO and diluted in 20 mM Tris–HCl, pH 7.4. GST-γ-PAK (0.10–0.16 μg) was incubated in 25 μl containing 20 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 30 mM 2-mercaptoethanol, and 0.2 mM [γ-^{32}P]ATP (1000–2000 cpm/pmol), with sphingosine or ceramide (0–400 μM), or with Cdc42(GTPγS) [26]. Incubation was for 15 min at 30°C. Autophosphorylation of γ-PAK was analyzed on 12.5% polyacrylamide gels, followed by autoradiography, and quantified by liquid scintillation counting.

2.3. Activity assays for γ-PAK

Following autophosphorylation of γ-PAK with sphingosine or C_6-ceramide, as described above, the synthetic peptide S3 (AKRESAA, 1 mM) and bovine serum albumin (0.4 mg/ml) were added to the reaction in a final volume of 28 μl. Incubation was for 15 min at 30°C; these were kinetically valid conditions and phosphorylation was linear over time. The reactions were terminated with 5 μl of 100 mM ATP at 0°C and an aliquot (3–5 μl) was subjected to thin-layer electrophoresis on cellulose plates [25]. The ^{32}P-labeled S3 peptide was identified by autoradiography and quantified by liquid scintillation counting. Assays with histone 4 or myelin basic protein (1 μg) were carried out as

PII: S0014-5793(01)02965-9

described [25] and analyzed by SDS–PAGE on 15% polyacrylamide gels.

2.4. Phosphopeptide mapping and phosphoamino acid analysis

γ-PAK (2.0 μg) was autophosphorylated in the presence of 200 μM sphingosine or Cdc42(GTPγS) and tryptic phosphopeptide maps were obtained by two-dimensional peptide gel electrophoresis [23]. Phosphoamino acid analysis was carried out by thin-layer electrophoresis [21].

2.5. Cell culture and preparation of cell extracts

Exponentially growing 3T3-L1 mouse preadipocytes (gift of Dr. Charles S. Rubin, Albert Einstein College of Medicine) at 60% confluency were treated for the indicated times with 15 μM sphingosine (stock solution 10 mM in DMSO) or mock-treated with DMSO (Control). Cells were washed, harvested, and stored at −80°C as indicated [19]. Extracts were prepared in 0.5 ml of freshly prepared lysis buffer containing phosphatase and protease inhibitors as described previously [19]. After 10 min on ice, the lysate was centrifuged at $16\,000\times g$ for 10 min at 4°C. The supernatant (soluble fraction) was collected, and the pellet was solubilized by sonication on ice in 0.5 ml of lysis buffer containing 1% NP40. After centrifugation at $16\,000\times g$ for 10 min at 4°C, the supernatant was collected and designated as the solubilized particulate fraction. Protein concentrations were determined using the Bradford assay [19].

2.6. Assay for γ-PAK activity in immunoprecipitates

γ-PAK was immunoprecipitated from the soluble and particulate fractions (100 μg of protein each) using 1 μg of N19 antibody [19] and assayed for 30 min with 2 μg of histone 4 in a final volume of 30 μl as described above. Radiolabeled histone 4 was quantified using a PhosphorImager system. Western blot analysis of γ-PAK was as described with horseradish peroxidase-linked secondary antibodies and enhanced chemiluminescent detection [10,20].

3. Results

3.1. Stimulation of autophosphorylation and activity of γ-PAK by sphingosine

Recombinant γ-PAK was autophosphorylated with [γ-^{32}P]-ATP using increasing concentrations of sphingosine. Autophosphorylation was stimulated at concentrations as low as 10 μM and maximal stimulation was obtained at 100–400 μM (Fig. 1). Increased autophosphorylation resulted in progressively slower migrating forms of γ-PAK on SDS–PAGE. Only a low level of autophosphorylation was observed with C_6-ceramide, as compared to sphingosine. Other lipids, such as phosphatidylserine, phosphatidylinositol or diacylglycerol, did not stimulate autophosphorylation (data not shown). At low concentrations of sphingosine (≤50 μM), up to 3 mol/mol of phosphate was incorporated into γ-PAK. Maximal autophosphorylation was 6–7 mol of phosphate incorporated/mol of γ-PAK and was comparable to the level of autophosphorylation obtained with Cdc42(GTPγS). These values correlate with the eight autophosphorylation sites identified previously for γ-PAK [23].

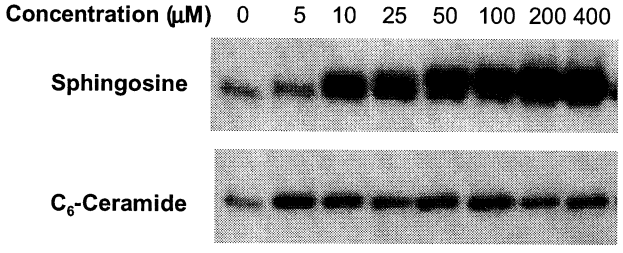

Fig. 1. Autophosphorylation and γ-PAK activity in response to sphingosine and C_6-ceramide. GST-γ-PAK (0.14 μg) was autophosphorylated for 15 min with [γ-^{32}P]ATP at the indicated concentrations of sphingosine or C_6-ceramide and analyzed by SDS–PAGE. The autoradiogram is shown.

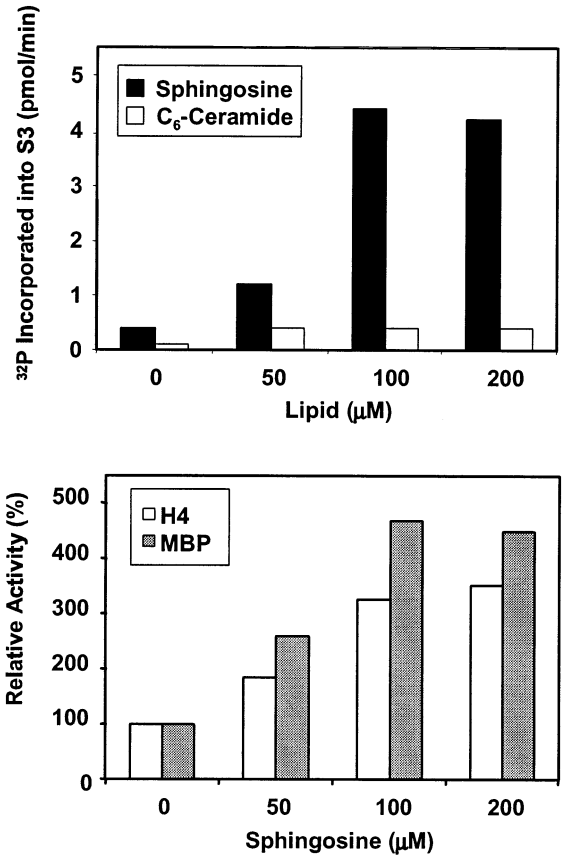

Fig. 2. Stimulation of γ-PAK activity by sphingosine. Upper panel: GST-γ-PAK (0.14 μg) was autophosphorylated at the indicated concentrations of sphingosine or C_6-ceramide and assayed with peptide S3. Lower panel: GST-γ-PAK (0.16 μg) was autophosphorylated and assayed with histone 4 (H4) or myelin basic protein (MBP). Phosphorylation in the absence of sphingosine was set at 100% (1.9 pmol for H4, 1.0 pmol for MBP). Data are representative of three experiments.

To examine the effects of autophosphorylation on γ-PAK activity, the samples were assayed with the peptide S3 (AKRESAA). At ≤50 μM sphingosine, there was a 3-fold stimulation of γ-PAK activity (Fig. 2, upper panel). γ-PAK activity was stimulated 11-fold at concentrations of sphingosine where maximal phosphorylation was observed (100–200 μM). Similar experiments with C_6-ceramide showed little stimulation of γ-PAK activity.

When phosphorylation of histone 4 and myelin basic protein was examined with sphingosine-activated γ-PAK (Fig. 2, lower panel), the rate of phosphorylation was optimal at 100–200 μM sphingosine. Phosphorylation of histone 4 was stimulated 3–4-fold, and myelin basic protein 4–5-fold. Phosphorylation obtained in the absence of sphingosine was due to substrate-level activation of γ-PAK [26].

3.2. Phosphopeptide mapping of sphingosine-activated γ-PAK

To analyze the sites autophosphorylated in response to sphingosine, tryptic digests of ^{32}P-labeled γ-PAK were analyzed using two-dimensional peptide PAGE [23]; the tryptic phosphopeptide pattern showed autophosphorylation at mul-

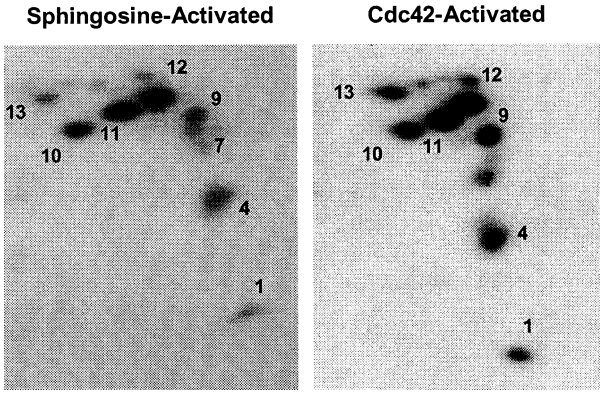

Fig. 3. Comparison of tryptic phosphopeptides of γ-PAK activated by sphingosine or Cdc42(GTPγS). γ-PAK (0.14 μg) was autophosphorylated with [γ-^{32}P]ATP in the presence of 100 μM sphingosine or 1.6 μM Cdc42(GTPγS) and subjected to tryptic digestion. The phosphopeptides were analyzed as described in Section 2. Left panel: autoradiogram of γ-PAK activated by sphingosine. Right panel: γ-PAK activated by Cdc42(GTPγS) (from Gatti et al. [23]).

tiple sites. Under these conditions, 6–7 mol of phosphate was incorporated/mol of γ-PAK (Fig. 3, left panel). The phosphopeptide pattern was similar, if not identical, to that observed upon autophosphorylation with Cdc42(GTPγS) (Fig. 3, right panel), and consistent with the autophosphorylation pattern of active γ-PAK. As shown previously, in the absence of an activator, γ-PAK was autophosphorylated, but not activated [21]. Phosphoamino acid analysis of γ-PAK activated with sphingosine or Cdc42(GTPγS) indicated that the majority (~90%) of the phosphate was on serine, with less than 10% on threonine. Without activators, only serine was observed (data not shown).

3.3. Co-activation of γ-PAK by Cdc42 and sphingosine

To examine whether sphingosine activated γ-PAK through a mechanism similar to that of Cdc42, the effects of these activators on γ-PAK activity were measured at suboptimal conditions, where there is a linear relationship between the activator concentration and degree of activation, and at optimal concentrations to observe maximal activity. As shown in Table 1, at low concentrations of Cdc42 (0.16 and 0.32 μM) or sphingosine (10 μM), the level of activation of γ-PAK was ≤37% of the maximal activity observed at optimal concentrations of Cdc42. When both activators were present at low concentrations, activation was additive. At optimal concentrations of Cdc42 (1.6 μM), where 6–7 mol of phosphate were incorporated into γ-PAK, the level of activation attained with Cdc42 was not augmented further by addition of suboptimal or optimal concentrations of sphingosine. This would be expected if activation of γ-PAK in response to sphingosine and Cdc42 was via the same mechanism, autophosphorylation.

3.4. Stimulation of γ-PAK activity in 3T3-L1 cells by sphingosine

Exponentially growing 3T3-L1 cells were treated for up to 1 h with 15 μM sphingosine. This treatment induced cell rounding and inhibited cell growth if sustained for more than 12 h (data not shown). γ-PAK was immunoprecipitated from the soluble and solubilized particulate fractions with N19 antibody and γ-PAK activity was measured with histone 4. Fig. 4A (upper panel) shows no significant changes in γ-PAK activity in the soluble fraction during 30 min of exposure to sphingosine. After 1 h of exposure to sphingosine, the activity of γ-PAK in the soluble fraction was reduced by 56% as compared to the non-treated control cells. In contrast, sphingosine induced a 2.8-fold increase in γ-PAK activity in the membrane-containing particulate fraction at 15 min (Fig. 4A, lower panel). The effects of sphingosine could be detected at 5 min, and γ-PAK activity was maximal at 15 min of treatment. After 1 h, γ-PAK activity was reduced to the level of the control. Similar results were obtained with an in-gel kinase assay of γ-PAK using histone as substrate (data not shown).

As shown by Western blotting with N19 antibody, there was little change in protein in the soluble fraction, while the level of γ-PAK protein was significantly increased in the particulate fraction at 15–30 min following addition of sphingosine to the cells (Fig. 4B). A second anti-γ-PAK antibody

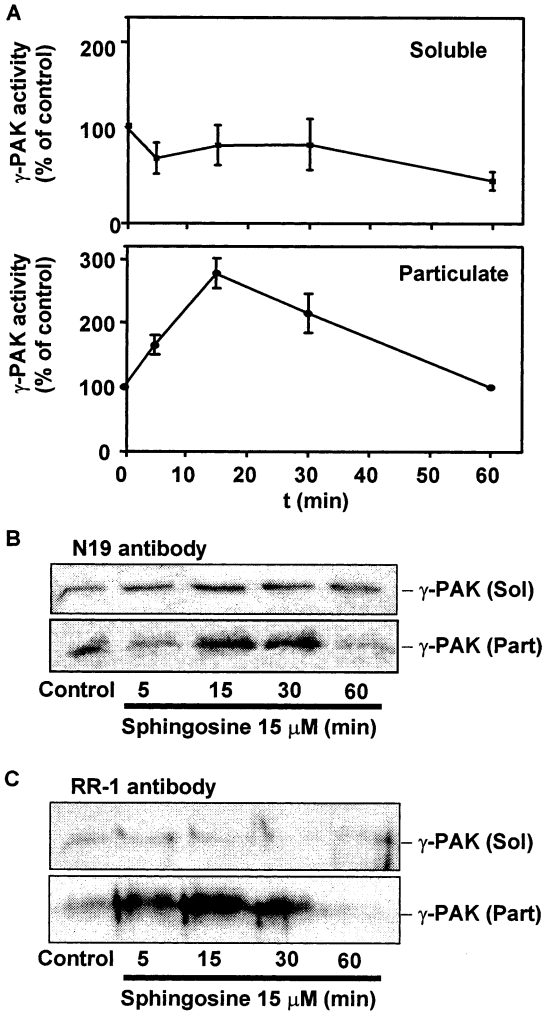

Fig. 4. Sphingosine-induced phosphorylation and activation of γ-PAK in 3T3-L1 cells. 3T3-L1 cells were treated for the indicated times with 15 μM sphingosine in DMSO or with DMSO alone (control). A: γ-PAK activity in the soluble and particulate fraction was assayed with histone 4 following immunoprecipitation with N19 antibody. The mean ± S.E.M. of three separate experiments is shown. The activity of the control cells was set as 100% for each fraction. B: γ-PAK protein was determined by Western blotting with N19, using equivalent amounts of soluble (30 μg) or particulate protein (110 μg). C: Immunoreactivity of γ-PAK with RR-1 antibody.

Table 1
Effects of sphingosine and Cdc42(GTPγS) on activation of γ-PAK

Cdc42		Sphingosine		Cdc42+sphingosine
Concentration (μM)	Activity[a] (%)	Concentration (μM)	Activity (%)	Activity (%)
0.16	11	10	9	24
0.32	37	10	13	48
1.60	100	10	11	95
		20	28	98
		100	76	95

[a]γ-PAK activity was measured with peptide S3. Maximal activity (14.0 pmol/min) obtained with 1.6 μM Cdc42(GTPγS) was set at 100%.

(RR-1) recognizes the regulatory domain of γ-PAK and reacts preferentially with highly autophosphorylated forms of activated γ-PAK [20]. In the absence of sphingosine, little if any reactivity of RR-1 with γ-PAK was observed. Within 5 min following addition of 15 μM sphingosine, significant immunoreactivity was observed with the RR-1 antibody and γ-PAK in the particulate fraction (Fig. 4C). The immunoreactivity was optimal between 15 and 30 min, indicating autophosphorylation of γ-PAK, and coincided with the stimulation of γ-PAK activity (Fig. 4A). Little if any reactivity with RR-1 was observed with γ-PAK in the soluble fraction before or after sphingosine treatment. Taken together, the data indicate that sphingosine stimulated the phosphorylation and activation of γ-PAK in the membrane-containing particulate fraction of 3T3-L1 cells, but not in the soluble fraction.

4. Discussion

γ-PAK has been shown to be activated by binding of the small GTPase protein Cdc42(GTP) and by cleavage with caspase 3 (CPP32) [22–24]. Here we show that sphingosine induces autophosphorylation and activation of γ-PAK both in vitro and in vivo. Autophosphorylation of γ-PAK in vitro is stimulated at concentrations of sphingosine as low as 10 μM and is optimally activated at 100–400 μM, with a concomitant stimulation of protein kinase activity, as assayed with peptide S3 and two protein substrates.

The tryptic phosphopeptide map of γ-PAK autophosphorylated in response to sphingosine is identical to that obtained with γ-PAK autophosphorylated in response to Cdc42(GTPγS) and coincides with the activation of γ-PAK. Activation in response to sphingosine includes autophosphorylation at Ser-141, Ser-165 and Thr-402, which are autophosphorylated only when γ-PAK is activated by Cdc42 [23]. This supports a mechanism of activation of γ-PAK in which sphingosine acts by stimulating autophosphorylation. This could occur through disruption of the autoinhibitory interaction between the regulatory and the catalytic domains of γ-PAK, in a manner similar to that described with α-PAK activated by Cdc42 [9,27,28]. Data showing that the effects of sphingosine and Cdc42 on γ-PAK activity are additive at suboptimal concentrations, and saturable at high concentrations, support further the idea that the lipid and Cdc42 activate γ-PAK through a similar mechanism, with either stimulus fully activating γ-PAK at optimal concentrations.

Previously, recombinant α-PAK has been shown to be activated both in vitro and in vivo by sphingosine [24]. α-PAK is activated when associated with membranes [24], and some of the biological effects of α-PAK activation can be mimicked by artificial targeting of the protein kinase to the plasma membrane [29].

In the present study, we show that sphingosine has a direct effect on γ-PAK in vivo, stimulating the translocation and activity of γ-PAK to the particulate fraction of 3T3-L1 cells. This increased activity is concomitant with enhanced phosphorylation of γ-PAK in this fraction, as shown by Western blotting with an antibody which reacts preferentially with active autophosphorylated γ-PAK [20]. The effects observed in vivo thus support those observed in vitro, i.e. activation of γ-PAK by sphingosine requires autophosphorylation. Thus, activation of PAK by sphingosine in the membrane may be a general mechanism of regulation of the activity of this family of protein kinases in response to specific stimuli.

Acknowledgements: This research was supported by NIH Grant GM26738. The authors thank Dr. Andrea Gatti for performing the phosphopeptide mapping.

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