Cleavage and Activation of p21-activated Protein Kinase \( \gamma \)-PAK by CPP32 (Caspase 3)

EFFECTS OF AUTOPHOSPHORYLATION ON ACTIVITY*

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p21-activated protein kinase \( \gamma \)-PAK (Pak2, Pak 1) is cleaved by CPP32 (caspase 3) during apoptosis and plays a key role in regulation of cell death. In vitro, CPP32 cleaves recombinant \( \gamma \)-PAK into two peptides; 1–212 contains the majority of the regulatory domain whereas 213–524 contains 34 amino acids of the regulatory domain plus the entire catalytic domain. Following cleavage, both peptides become autophosphorylated with \([\gamma-32P]ATP\). Peptide 1–212 migrates at 27,000 daltons (p27) upon SDS-polyacrylamide gel electrophoresis and at 32,000 daltons following autophosphorylation on serine (p27P); the catalytic subunit migrates at 34,000 daltons (p34) before and after autophosphorylation on threonine. Following caspase cleavage, a significant lag (~5 min) is observed before autophosphorylation and activity are detected. When \( \gamma \)-PAK is autophosphorylated with ATP(Mg) alone and then cleaved, only p27 contains phosphate, and the enzyme is inactive with exogenous substrate. After autophosphorylation of \( \gamma \)-PAK in the presence of Cdc42(GTP\( \gamma \)S) or histone 4, both cleavage products contain phosphate and \( \gamma \)-PAK is catalytically active. Mutation of the conserved Thr-402 to alanine greatly reduces autophosphorylation and protein kinase activity following cleavage. Thus activation of \( \gamma \)-PAK via cleavage by CPP32 is a two-step mechanism wherein autophosphorylation of the regulatory domain is a priming step, and activation coincides with autophosphorylation of the catalytic domain.

In response to a variety of death stimuli, the caspase family of cysteinyl aspartate-directed proteases becomes activated. The caspases play integral roles in apoptosis or programmed cell death, which include a number of morphological changes such as nuclear condensation, membrane and cytoskeletal rearrangement, and formation of apoptotic bodies (for review, see Ref. 1). CPP32 (caspase 3) is cleaved to p20 and p10 (2) and has been shown to participate in apoptosis through cleavage of a number of proteins including DNA-activated protein kinase (3) and protein kinase C-related kinase (4), among others (5).

Recently, Rudel and Bokoch (6) and Lee et al. (7) have shown that the p21-activated protein kinase \( \gamma \)-PAK\(^1\) (Pak2, Pak 1) is constitutively activated via cleavage by CPP32 following induction of apoptosis in Jurkat cells.

\( \gamma \)-PAK is the ubiquitous isoform of a family of protein kinases activated by the small G proteins Cdc42 and Rac. The G protein binding site is in the amino-terminal regulatory domain, whereas the carboxyl terminus consists of a highly conserved catalytic domain (see Refs. 8 and 9 for review). \( \gamma \)-PAK was initially purified as an inactive holoenzyme from rabbit reticulocytes that could be activated by limited digestion with trypsin (10). It has a calculated molecular weight of 58,015 and migrates at 58–60 kDa on SDS-PAGE (11). \( \gamma \)-PAK has been shown to phosphorylate multiple substrates including histones H2B and H4 (10), myosin light chain (12, 13), and Rous sarcoma virus protein NC (14, 15).

\( \gamma \)-PAK has also been implicated in cytostasis, as shown by injection of subfemtomole amounts of \( \gamma \)-PAK into 1 blastomere of 2-cell frog embryos, whereupon cleavage of the injected blastomere is inhibited while the noninjected blastomere continues through mid-cleavage (16). A protein of 58,000 Da that reacts with antibody to \( \gamma \)-PAK from rabbit is present in high amounts in frog oocytes and is greatly diminished following fertilization, suggesting \( \gamma \)-PAK is involved in maintaining oocytes in a non-dividing state (16). In dividing 3T3-L1 cells, \( \gamma \)-PAK is primarily in an inactive form. Active \( \gamma \)-PAK becomes the predominant form in the cytoplasm when 3T3-L1 cells are serum-starved or enter the quiescent state.

Inactive \( \gamma \)-PAK and two endogenously active forms have been purified from rabbit reticulocytes,\(^2\) whereas wild type and mutant forms of recombinant \( \gamma \)-PAK from rabbit have been expressed in insect cells (17). In the studies described herein, the requirements for activation of recombinant \( \gamma \)-PAK via cleavage with recombinant CPP32 have been analyzed. Cleavage produces 2 peptides, p27 containing the majority of the regulatory domain and p34 containing part of the regulatory domain and the entire catalytic domain, both of which become autophosphorylated. A comparison of autophosphorylation and activity of wild type and mutant \( \gamma \)-PAK shows autophosphorylation is a bipartite mechanism, with the regulatory domain (p27) autophosphorylated at multiple residues, while activa-

\(^1\) The abbreviations used are: Pak2, p21-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; GTP\( \gamma \)S, guanosine 5'-3-O-(thio)triphosphate; CHAPS, 3-[[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; p35, autophosphorylated p35, PKA, cAMP-dependent protein kinase; Cdk2, cell division kinase 2

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Expression and Purification of CPP32 from BL21(DE3) Cells—A construct containing an open reading frame for the CPP32 gene at genetic background in BL21(DE3) was used for protein expression. Recombinant CPP32 was obtained from 500 ml of culture induced with isopropyl-1-thio-β-D-galactopyranoside for 3–4 h at 37 °C. The supernatant was chromatographed on 0.3 ml of TALON-NX metal resin. The protein was eluted with a final concentration of 500–2000 dpm/pmol. Incubation coincides with autophosphorylation of the catalytic domain of γ-PAK at Thr-402.

EXPERIMENTAL PROCEDURES

Materials—TALON-NX metal resin was from CLONTECH, Immobilon PSQ was from Millipore. Histone 4 and GTPγS were from Boehringer Mannheim. γ-32P]ATP was from NEN Life Science Products; human thrombin and other chemicals were from Sigma. Antibody prepared in rat to a highly conserved sequence in the catalytic region of STE20 from Saccharomyces cerevisiae was purchased from Upstate Biotechnology. pAcG2T vector and the regulatory domain, and with an- ti-STE20 antibody, which reacted with the catalytic domain, γ-PAK (p58), GST-γ-PAK (p90), and the cleavage products are indicated by molecular mass. Lane 1, CPP32 and ATP omitted; lane 2, CPP32 omitted, ATP added; lane 3, CPP32 added, ATP omitted; and lane 4, CPP32 and ATP added.

Microsequence analysis of γ-PAK—GST-γ-PAK (10 μg) was cleaved with 100 units of CPP32 for 30 min at 37 °C in a final volume of 100 μl, and the products were separated by SDS-PAGE. The peptides were transferred to Immobilon PSQ in 10 mM CAPS (pH 11) and 10% methanol by semi-dry blotting. The proteins were stained with Coomassie Blue for 20 s, destained with 50% methanol, and washed with water. The p34 peptide was excised and sequenced using an Applied Biosystems Procise Sequencer.

Identification of the Cleavage Products of γ-PAK—Microsequence analysis of p34 was carried out as described under “Experimental Procedures.” The identified sequence is compared with the sequence calculated from the cDNA (11). The diagram of γ-PAK shows the cleavage site for CPP32 (p34) and the major trypsin cleavage site (p37). The positions of mutations used herein are identified.

Activity assays were carried out under kinetically valid conditions with the synthetic peptide S3 (AKRESAA; 1.0 mM), as described previously (18). The reactions were terminated by addition of 7 μl of 100 mM nonradioactive ATP, and phosphorylated S3 was analyzed on P81 phosphocellulose paper. Under these conditions, less than 10% of the substrate was phosphorylated and phosphate incorporation was linear with time.

Microsequence analysis of γ-PAK—GST-γ-PAK (10 μg) was cleaved with 100 units of CPP32 for 30 min at 37 °C in a final volume of 100 μl, and the products were separated by SDS-PAGE. The peptides were transferred to Immobilon PSQ in 10 mM CAPS (pH 11) and 10% methanol by semi-dry blotting. The proteins were stained with Coomassie Blue for 20 s, destained with 50% methanol, and washed with water. The p34 peptide was excised and sequenced using an Applied Biosystems Procise Sequencer.
were detected with peroxidase-conjugated secondary antibody and by chemiluminescence with the Amersham Pharmacia Biotech ECL detection kit.

**RESULTS**

Cleavage of Purified γ-PAK by CPP32—γ-PAK (p58) and GST-γ-PAK (p90) cloned from rabbit, and expressed in insect cells, were subjected to cleavage by the caspase CPP32. As shown by Coomassie Blue stain, p58 was cleaved into two peptides, p27 and p34, whereas cleavage of p90 resulted in peptides migrating at p53 and p34 on SDS-PAGE (Fig. 1, lanes 0-18). 32P-labeled peptides were detected by phosphor screen autoradiography.
To identify the cleavage products, antibodies prepared to amino acids in the catalytic region of STE20, or to purified γ-PAK from rabbit, which reacted specifically with the regulatory region, were used. The STE-20 antibody reacted with p58 and GST-γ-PAK p90; following cleavage with CPP32, an additional band was detected that migrated with a molecular weight of 34,000 (p34), indicating p34 contained the catalytic domain. Antibody prepared to rabbit γ-PAK reacted with p58 and p90. Upon cleavage of p58, a single peptide migrating with a molecular weight of 27,000 (p27) was detected; a peptide of 53,000 daltons (p53) was detected with GST-γ-PAK, which contained GST-p27.

The p34 peptide was subjected to microsequence analysis, and the sequence obtained was identical to the sequence calculated from the rabbit cDNA for γ-PAK beginning with amino acid 213 (Fig. 2, top panel). Thus, p34 contained the entire catalytic domain of γ-PAK, which began at amino acid 247, as well as 34 amino acids of the regulatory domain (Fig. 2, bottom panel). The cleavage site was consistent with the sites identified previously for caspase 3 and coincided with that identified for γ-PAK during apoptosis using peptide inhibitors and the mutant D212N (6). The CPP32 cleavage site was 16 amino acids from the major site of trypsin cleavage at arginine 195 which produced the active peptide p37 (11).

Autophosphorylation of γ-PAK following Cleavage by CPP32—γ-PAK and GST-γ-PAK p90 were cleaved by CPP32, then autophosphorylated, and then compared with the non-cleaved PAK. Western blotting was carried out with antibody to the regulatory and catalytic domains as shown in Fig. 1, lanes 2 and 4. No Coomassie Blue-stained peptides were observed in the absence of protease. Antibody to the catalytic domain detected a single peptide of 34,000 Da. With antibody to the regulatory domain, a single peptide of 32,000 Da was detected (p27P) when γ-PAK was autophosphorylated following caspase cleavage. This indicated that all of the p27 peptide had become autophosphorylated which resulted in retarded migration on SDS-PAGE. A slightly retarded migration was also detected with autophosphorylated p53 (p53P) cleaved from GST-γ-PAK p90.

To examine the effects of autophosphorylation on protein kinase activity, γ-PAK was cleaved with CPP32 and then incubated with radiolabeled ATP for up to 15 min, and analyzed by autoradiography following SDS-PAGE. As shown in Fig. 3, top panel, some autophosphorylation of both p27 and p34 was observed at 5 min, after which autophosphorylation of both peptides increased with time. The increased level of autophosphorylation was concomitant with an increased retardation of the migration of p27 in SDS-PAGE gels.

To measure γ-PAK activity, the heptapeptide S3, which was shown previously to be a specific substrate for PAK (18), was added to the reaction mixture at 0 time or after 15 min of autophosphorylation. The rate of phosphorylation of S3 correlated with the extent of autophosphorylation of γ-PAK. As shown in Fig. 3, bottom panel, no significant phosphorylation of S3 was observed until a sufficient level of autophosphorylation had been reached at 5 min. After that time, the rate of phosphorylation was linear with time. In contrast, the rate of phosphorylation of S3 with γ-PAK prephosphorylated for 15 min was essentially linear for the entire 15 min incubation period.

Autophosphorylation of γ-PAK Prior to Cleavage by CPP32—As shown previously, γ-PAK can be activated by binding of Cdc42(GTPγS) or by the substrate histone 4 (17, 21). To examine the effects of autophosphorylation of γ-PAK on cleavage by CPP32, the protein kinase was preincubated with ATP(Mg) alone, with histone 4, with Cdc42, or with Cdc42(GTPγS). When γ-PAK p58 was autophosphorylated with ATP(Mg) alone

![Fig. 5. Correlation of autophosphorylation of γ-PAK prior to cleavage with protein kinase activity. γ-PAK (p58) was autophosphorylated in the presence and absence of Cdc42(GTPγS) for 15 min and then incubated in the presence or absence of CPP32. Top panel, samples (30 µl) were analyzed by SDS-PAGE; the autoradiogram is shown. Middle panel, the same samples (30 µl) were assayed for protein kinase activity with peptide S3 for 10 min. Bottom panel, a time course of protein kinase activity was carried out with γ-PAK prephosphorylated prior to cleavage and compared with γ-PAK autophosphorylated after cleavage (taken from Fig. 3).]
and then subjected to cleavage, only the p27 peptide contained phosphate and migrated with a molecular mass of p32 (p27P) (Fig. 4, left panel). The identity of this peptide was confirmed by Western blotting and phosphopeptide mapping (see below). In the presence of histone 4, autophosphorylation of p58 was increased 14-fold over that obtained with ATP(Mg) alone, and caspase cleavage showed both the catalytic and regulatory domains contained phosphate. In the presence of Cdc42 alone, the results were identical to those obtained with ATP(Mg). With Cdc42(GTPγS), autophosphorylation of γ-PAK p58 was highly stimulated. Following cleavage, both p27 and p34 were phosphorylated, along with a slightly smaller fragment. In contrast, with GST-γ-PAK, only a small amount of autophosphorylation was observed with ATP(Mg) alone or with Cdc42 (Fig. 4, right panel). Cleavage with CPP32 showed this phosphate was in p53P. In the presence of histone 4 or Cdc42(GTPγS), p90 was highly autophosphorylated; upon cleavage, both p53 and p34 contained phosphate.

To analyze the effects of autophosphorylation of γ-PAK on activity following caspase cleavage, γ-PAK was prephosphorylated in the presence or absence of Cdc42(GTPγS) and then incubated in the presence and absence of CPP32. Activity assays with peptide S3 were carried out for up to 10 min. As shown in Fig. 5, top panel, prephosphorylation in the presence of Cdc42(GTPγS) prior to substrate addition at 15 min resulted in a high level of phosphate in p58 or in p27 and p34. In contrast, upon prephosphorylation in the absence of Cdc42, the amount of phosphate present in p58 or p27P at 0 time was significantly less, and little or no phosphate was present in p34. Incubation of the cleaved products with ATP(Mg) resulted in both p27 and p34 becoming highly autophosphorylated within 1 min (data not shown). Thus, autophosphorylation of the regulatory domain in the absence of Cdc42(GTPγS) primed the protein kinase for rapid autophosphorylation of the catalytic domain following cleavage.

When protein kinase activity was measured at the 10 min time point, little phosphorylation of peptide S3 was observed with p58 prephosphorylated with ATP(Mg) alone (Fig. 5, middle panel). A significant stimulation of activity (3-fold) was observed with p58 prephosphorylated in the presence of Cdc42(GTPγS). Upon cleavage of γ-PAK prephosphorylated in the presence and absence of Cdc42(GTPγS), the activity was significantly higher, a 10-fold stimulation over the noncleaved enzyme. A time course showed that at 2.5 min, the activity of cleaved γ-PAK prephosphorylated in the presence and absence of Cdc42(GTPγS) was identical. No lag phase was observed with any of the prephosphorylated samples (Fig. 5, bottom panel). This coincided with the rapid autophosphorylation observed following cleavage. Similar results were obtained within 1 min of cleavage (data not shown). Thus autophosphorylation of the regulatory domain was the priming step in activation, but phosphorylation of Thr-402 on the regulatory domain was required for activity with exogenous peptide substrates.

**Autophosphorylation Sites Required for Activity**—Multiple sites of autophosphorylation have been observed with γ-PAK (17, 21). When the CPP32 cleavage products of γ-PAK were autophosphorylated, six phosphopeptides were observed with p27P and two phosphopeptides with p34 (Fig. 6). The additive
data were similar to the phosphopeptide maps obtained with native and recombinant γ-PAK p58 activated by Cdc42(GTPγS) (17, 21), except that the migration of phosphopeptide 9 in p27 was altered after CPP32 cleavage. This suggested that cleavage altered the migration of one of the tryptic phosphopeptides. Phosphoamino acid analysis of p27 showed autophosphorylation was only on serine. When the two phosphopeptides obtained with p34 were analyzed individually, only phosphothreonine was observed (Fig. 7).

To examine the effects of autophosphorylation further, mutants of γ-PAK prepared by site-directed mutagenesis were incubated in the presence and absence of CPP32 and then autophosphorylated. As shown in Fig. 8, left panel, no autophosphorylation was observed with the knock-out mutation K278R replacing a conserved lysine in the active site. With T402A, autophosphorylation of p27 and p34 was greatly inhibited, indicating that autophosphorylation of Thr-402 was required for autophosphorylation of the cleaved regulatory domain. With the mutants S401A, S490A, and S490D, both p27 and p34 were autophosphorylated to a similar extent as the recombinant wild type γ-PAK.

Assays of the protein kinase activity of the γ-PAK mutants with peptide S3 showed no substrate phosphorylation with K278R (Fig. 8, right panel). With T402A, only 2% of the activity of recombinant wild type γ-PAK was observed, whereas significant activity was obtained with S401A, S490A, and S490D. Taken together, the data indicated that Thr-402 was the only detectable site of autophosphorylation in p34 and that autophosphorylation of Thr-402 was critical for autophosphorylation of p27 and for phosphorylation of the peptide substrate.

**DISCUSSION**

Activation of γ-PAK can be initiated under different conditions, by binding of small G proteins, in particular Cdc42(GTPγS) (11), by association with histone 4 (17, 21), by cleavage with trypsin to form p37 (11), and by cleavage with the caspase CPP32. Cleavage by CPP32 produces two peptides, one containing the majority of the regulatory domain (p27) and the other containing the entire catalytic domain and 34 amino acids of the regulatory domain (p34). Following cleavage, both peptides become highly autophosphorylated, which results in a shift of migration of p27 from 27 to 32 kDa upon SDS-PAGE, as shown by Coomassie Blue staining, Western blotting, and radiolabeling. Autophosphorylation of p34 does not alter the migration pattern on SDS-PAGE but is required for activity, as shown with the peptide S3 (AKRESAA).

Prior to cleavage, autophosphorylation of γ-PAK with ATP(Mg) alone or with Cdc42 in the absence of GTPγS results in a similar shift in p27 during SDS-PAGE; however, no autophosphorylation of p34 is observed. Under these conditions, there is little protein kinase activity. Incubation in the presence of Cdc42(GTPγS) stimulates autophosphorylation of γ-PAKp58 by 2.5-fold, and the phosphate is found in both p27 and p34 following cleavage. With Cdc42(GTPγS), protein kinase activity as determined with peptide S3 is observed both before and after cleavage. There is a 3-fold stimulation of p58 activity and a 10-fold stimulation following caspase cleavage. Similar results are obtained following autophosphorylation of p58 and the two cleavage products in the presence of histone 4. These data indicate that the cleaved enzyme has a higher level of activity than the Cdc42-activated enzyme.

Autophosphorylation of the regulatory domain is the priming step in activation, and autophosphorylation of p34 on Thr-402 is critical for phosphorylation of peptide substrates. Autophosphorylation is a bipartate mechanism; autophosphorylation of multiple sites in the regulatory domain is a priming step and can occur in the absence of any effectors. Upon caspase cleavage or addition of Cdc42(GTPγS) or histone 4, Thr-402 in the catalytic domain is autophosphorylated, resulting in stimulation of protein kinase activity with exogenous substrates. Based on a comparison of sequences of γ-PAK with the highly studied cAMP-dependent protein kinase (PKA) and cell division kinase 2 (Cdk2), Thr-402 corresponds to the highly conserved threonine, which is located in the p + 1 loop adjacent to the activation loop, as determined by x-ray crystallography (22–25). In PKA and Cdk2, phosphorylation of this threonine is required for activation, as is shown herein for γ-PAK.

Autophosphorylation of the regulatory domain of the holoenzyme p58 can occur independently of phosphorylation of Thr-402, but autophosphorylation of Thr-402 is required for phosphorylation of exogenous substrates. Following caspase cleavage, autophosphorylation of p27 becomes dependent on a phosphothreonine at position 402, because p27 is now an exog-
enous substrate.

Previously Rudel and Bokoch (6) showed that γ-PAK cleavage was correlated with the onset of apoptosis and that the inhibitor of CPP32 cleavage (DEVD-ald) inhibited cleavage of γ-PAK in Jurkat T cells undergoing apoptosis. The site of cleavage, aspartate 212, was identified using recombinant CPP32 or apoptotic cell lysates which cleaved γ-PAK into two fragments, but did not cleave the mutant D212N. That data was confirmed herein by direct sequencing of the CPP32 cleavage site generated in vitro.

In other studies, γ-PAK activity was shown to be high in the cytosol of quiescent and serum-starved cells and to be diminished in dividing cells. Thus regulation of γ-PAK under conditions of growth and quiescence is different from regulation of γ-PAK during apoptosis. Cleavage by CPP32 allows γ-PAK to be constitutively active leading to cell death, whereas regulation by Cdc42(GTP) produces γ-PAK cycling between active and inactive forms.

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