Molecular Cloning and Sequencing of the Cytostatic G Protein-activated Protein Kinase PAK I*

Rolf Jakobi, Chang-Jui Chen, Polygena T. Tuazon, and Jolinda A. Traugh†

From the Department of Biochemistry, University of California, Riverside, California 92521

The serine/threonine protein kinase PAK I (p21-activated protein kinase), a ubiquitous multipotential protein kinase of 58–60 kDa, has been shown to have cytosolic properties. Data from our laboratory show that PAK I is highly active in oocytes and quiescent and serum-starved cells, and injection of active PAK I into one blastomere of two-cell frog embryos inhibits cleavage of the injected blastomere. To clone the cDNA encoding PAK I, purified peptides from rabbit PAK I were sequenced, degenerate oligonucleotides were used to isolate PAK I clones from a rabbit spleen library, and the 5′-terminus was obtained by polymerase chain reaction. The entire cDNA sequence extends over 4471 nucleotides, with an open reading frame for a protein of 524 residues and a 3′-noncoding region of 2826 nucleotides. Clones with the same open reading frame but with 3′-noncoding regions of 1055 and 2478 nucleotides were isolated, suggesting the generation of different transcripts by alternative termination of transcription. The amino acid sequence of PAK I shows high homology to the p21-activated protein kinases from human placenta and rat brain and to yeast STE20. PAK I is activated by the p21-activated protein kinase C and the catalytic subunit of cAMP-dependent protein kinase, have no effect on cleavage.

The cloning and sequencing of the cDNA encoding PAK I, described herein, has revealed a mode of regulation of PAK I activity. The N-terminal regulatory domain contains a G protein-binding region similar to that of other protein-activated protein kinases including PAK65 (12), human, placenta PAK65 (13), and yeast STE20 (14, 15). Binding of the small G protein Cdc42 stimulates autophosphorylation and protein kinase activity, as shown by phosphorylation of H4.

EXPERIMENTAL PROCEDURES

Materials—32P-Labeled and 35S-labeled nucleotides as well as Hybrid-M membranes were purchased from Amersham Corp. Immobilon-P was obtained from Millipore Corp., and ProBlott was obtained from Applied Biosystems Inc. Endopeptidase Asp-N was purchased from Boehringer Mannheim. The Vydac reverse-phase C18 column was obtained from The Separations Group. Pyrostase was obtained from Molecular Genetic Resources, and the nested deletion kit was from Pharmacia Biotech Inc. The messenger RNA isolation kit, the rabbit spleen Uni-ZAP library, Stratscript RTNase H reverse transcriptase, and cloned Psi DNA polymerase were purchased from Stratagene. T4 polynucleotide kinase, T4 DNA ligase, Klenow DNA polymerase, and restriction enzymes were from New England Biolabs Inc. The 5′-Amplifier™ RACE kit was obtained from CLONTECH, and the Sequenase Version 2.0 DNA sequencing kit was from U. S. Biochemical Corp. The Qiaprep spin kit and the plasmid midi kit were from QiAGEN Inc.; the Gene-dean kit was from BIO 101, Inc. Expression clones for Rac1, Cdc42Hs, and RhoA as GST fusion proteins were generously provided by Dr. Channing Der (University of North Carolina, Chapel Hill, NC).

Purification and Analysis of PAK I Peptides—The inactive PAK I holoenzyme was purified to apparent homogeneity from rabbit reticulocytes. The active PAK I was isolated by combination of CNBr and endopeptidase Asp-N.

For in situ CNBr cleavage, PAK I was subjected to electrophoresis on a 12.5% SDS-polyacrylamide gel (16) and transferred to an Immobilon-P membrane. PAK I was cleaved on the membrane with 4 mg/ml CNBr for 15 h at room temperature, and the peptides were eluted with 100 μl of 70% isopropanol alcohol and 3% trifluoroacetic acid for 3 h at room temperature, followed by 100 μl of 40% aceticitrile for 1 h at 37 °C. The extracts and the original cleavage solution were dried in a Speed Vac concentrator and separated by electrophoresis on a 16.5% SDS-polyacrylamide gel (16). The peptides were transferred to a ProBlott.
tott membrane and subjected to amino acid sequence analysis. The N-terminal sequence of CNBr peptide 1 (20 kDa) was PEQWARL-GTNSXTRLQKK, and that of CNBr peptide 2 (8 kDa) was XXXXSFSTGGKDPLSANHXL.

To obtain the amino-terminal sequence of the 37 peptide, PAK I was partially digested with 4 μg/ml trypsin on ice for 1 min as described by Tahara et al. (1), with the addition of bovine serum albumin. Peptides were separated by electrophoresis on a 12% SDS-polyacrylamide gel (16) and transferred to Immobilon-P, and the sequence was determined to be SVIDPAPVPQGSHV.

For CNBr/endopeptidase Asp-N cleavage, PAK I was cleaved with 10 mg/ml CNBr for 15 h at room temperature, and the peptides were then dialyzed, reduced, and carboxymethylated as described (17). The CNBr peptides were digested with endopeptidase Asp-N, and the digest was acidified with glacial acetic acid and separated by reverse-phase high pressure liquid chromatography on a Vydac C18 column with a acetonitrile gradient from 0 to 100%. Amino acid sequence analysis and mass spectrometry were performed at the Biotechnology Instrumentation Facility. The N-terminal sequences of six CNBr/endopeptidase Asp-N peptides were as follows: CNBr/Asp-N peptide 1 (5497 Da), DGFPSGAPALTNKXVXTASVVT; CNBr/Asp-N peptide 2 (4319 Da), VEGEPYFLNPEPFLRLYIAIQ; CNBr/Asp-N peptide 3 (3479 Da), DVALGQCEAQNLOKPKPELI; CNBr/Asp-N peptide 4 (3479 Da), DVEKRGSAKELLQHPFF; CNBr/Asp-N peptide 5 (3226 Da), DEXGLAAAEXRXQKEFQGANQVHR; and CNBr/Asp-N peptide 6 (1438 Da), ELIKUNP.

_cDNA Cloning by Reverse Transcription and Polymerase Chain Reaction—_Poly(A)^9 RNA was isolated from rabbit liver, spleen, kidney, and brain using the messenger RNA isolation kit. One μg of poly(A)^9 RNA was reverse-transcribed with StrataScript RNaseH^- reverse transcriptase using an oligo(dT) primer. The resulting cDNA was amplified over 35 cycles in a polymerase chain reaction (18) using Pyrobest, the 48-fold degenerate sense oligonucleotide primer (5′-ATGGAYGARCCARATHGC-3′ (1 μm)), and the 512-fold degenerate antisense oligonucleotide primer (5′-CCNCKYTTYTCTNCRTCTCAT-3′ (5 μm)). A PCR product of 431 bp was subcloned into pBluescript SK−.

_cDNA Library Screening—_For high density screening in a rabbit spleen library, 5−10^9 plating units were plated on Escherichia coli XL-1Blue. Two replicates were prepared from each plate on Hybond-N membranes. The 431-bp PAK I cDNA was labeled by the multipriming procedure (19) with [α-32P]dCTP. Hybridization at 30°C and washes at 55°C were carried out as described elsewhere (20). After autoradiography, positive clones were purified over two more rounds of screening, excised in vivo from the Uni-ZAP vector as pBluescript SK−_ clones, and analyzed by Southern blotting (21). Cloning of the 5′-cDNA Region by RACE-PCR—_The 5′-end of the PAK I cDNA was obtained by RACE-PCR using the 5′-AmplifierTM RACE kit. Total RNA from rabbit spleen was isolated by the guanidinium thiocyanate method (22), and poly(A)^9 RNA was isolated by two rounds of oligo(dT)-cellulose chromatography (23). For the cDNA synthesis, 10 μL of E. coli RNA polymerase (2 μg) was extended with reverse transcriptase using the oligonucleotide primer P1 (5′-GCCTGTAAACACTCTCTGCA-3′). The synthesized cDNA was purified and ligated to a synthetic vector. PCR was carried out over 35 cycles with donor Pfu DNA polymerase using the oligonucleotide primer P2 (5′-CTTATCCATGCAGGTTTCCTGTAC-3′) and a primer corresponding to the anchor sequence. A PCR product of ~1150 bp was subcloned into pBlueScript SK−.

Sequence Analysis of PAK I cDNA Clones—_Plasmid DNA was isolated with the plasmid midi kit; subclones were constructed using internal EcoRI and PstI sites, and nested deletions were created with the double-stranded nested deletion kit. DNA sequencing was carried out using the dideoxy termination method of Sanger et al. (24) using α-35S-dATP and T7 DNA polymerase. Nucleotide sequences were aligned to form the full-length cDNA sequence, and the sequence was analyzed with the University of Wisconsin Genetics Computer Group Package Version 8 (25).

Stimulation of PAK I Autophosphorylation and Activity by G Proteins—_GST fusions of Rac1, Cdc42Hs, and RhoA were expressed individually in E. coli DH5α; the bacteria were sonicated in the presence of the protease inhibitors aprotinin, leupeptin, and phenylmethylsulfonyl fluoride (0.5 mM), and the fusion proteins were purified on glutathione-Sepharose beads. GST-Rac1, GST-Cdc42Hs, or GST-RhoA (1 μg) was preloaded with 0.18 mM GTPyS or 0.18 mM GDP in 20 mM Tris-HCl, pH 7.5, and 50 mM NaCl for 10 min at 30°C in a volume of 20 μL. The GST proteins were incubated with PAK I (0.1 μg) in 70 μL of phosphorylation buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 30 mM 2-mercaptoethanol) with 0.2 mM [γ-32P]ATP (4000 dpm/pmol) at 30°C. Aliquots were removed at 5, 10, and 15 min; autophosphorylation was analyzed by electrophoresis on a 12.5% SDS-polyacrylamide gel followed by autoradiography.

The effects of autophosphorylation on activity were measured by preincubation of G protein with PAK I for 10 min as described above. A 20-μL sample was assayed in a final volume of 70 μL containing phosphorylation buffer, 0.2 mM [32P]ATP (2000 dpm/pmol) by incubation for 15 min at 30°C. Phosphorylation of H4 was analyzed by electrophoresis on a 15% SDS-polyacrylamide gel followed by autoradiography (26) and confirmed by scintillation counting of the excised H4 band.

RESULTS

Preparation of a PAK I-specific Hybridization Probe—_Peptides from the inactive PAK I holoenzyme purified from rabbit reticulocytes were obtained by cleavage with CNBr, CNBr/ endopeptidase Asp-N, or trypsin. The peptides were separated, purified, and partially sequenced as described under "Experimental Procedures." Two CNBr peptides of 20 and 8 kDa and six CNBr/endopeptidase Asp-B peptides ranging from 5497 to 1438 Da were isolated. A peptide of 37 kDa, isolated following limited proteolysis with trypsin, contained the active catalytic domain. All nine peptides were subjected to partial sequence analysis as described under "Experimental Procedures." The N-terminal sequences of CNBr/Asp-N peptides 2–5 could be located within the alignment of the catalytic domains of other protein kinases as described by Hanks et al. (27, 28).

Following reverse transcription of mRNA from rabbit spleen and brain, degenerate oligonucleotides corresponding to CNBr/ Asp-N peptides 4 and 5 were used to amplify a PAK I-specific polynucleotide. After 35 cycles of amplification, PCR products of 431 bp were subcloned and analyzed by DNA sequencing. The clones from spleen and brain were identical and contained the complete amino acid sequences of CNBr/Asp-N peptides 2, 4, and 5.

Cloning of the cDNA Encoding PAK I—_The partial 431-bp cDNA from rabbit spleen was used to screen a rabbit spleen cDNA library, and nine clones were detected within 5 × 10^9 plaque-forming units. Restriction analysis and partial cDNA N-terminal sequencing showed that all nine μg of H4, and containing the same open reading frame, but were incomplete at the 5′-end. Differences in the length of the 3′-untranslated region were observed. PAKI-4.1 was the clone that contained the longest open reading frame and had the longest 3′-untranslated region (Fig. 1). The 5′-end of the cDNA for PAK I was obtained by RACE-PCR from rabbit spleen mRNA. Six positive clones with an insert of ~1150 bp were characterized by restriction analysis and partial DNA sequencing. All clones overlapped with the 5′-end of PAKI-4.1 and contained the same open reading frame starting with ATG; only the lengths of the 3′-untranslated region were different. The longest clone isolated by RACE-PCR (clone 1150-4) contained the longest 5′-untranslated region.

Complete Sequence Analysis of PAK I cDNA—_Both strands of the library clone PAKI-4.1 and the RACE-PCR clone 1150-4 were sequenced completely. For the sequence analysis of PAKI-4.1, subclones E1100 and P2900 were constructed using internal EcoRI and PstI sites (Fig. 1). Overlapping nested deletions were created to sequence 2665 nucleotides from the 3′-end of the noncoding strand of PAKI-4.1, the entire coding strand of subclone P2900, and both entire strands of subclone E1100 and of RACE-PCR clone 1150-4.

The complete cDNA sequence of PAK I consists of 4471 bp, including a poly(A) tail of 18 nucleotides (Fig. 2). The cDNA sequence contains an open reading frame from nucleotides 74 to 1645, 73 nucleotides of 5′-untranslated region, and 2826 nucleotides of 3′-untranslated region. The 3′-untranslated regions of other clones isolated by screening of the rabbit spleen library were shorter than that of PAKI-4.1, but could be aligned.

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is part of the conserved DFG motif and chelates the Mg²⁺.
Nucleotide binding begins at residue 256; residue 278
contains part of the regulatory domain (from residues 197
and an N-terminal regulatory domain, including a region
encoding a polypeptide of 524 amino acid residues with a calcu-
lated molecular mass of 58,027 Da. All nine partial amino acid
sequences, obtained by microsequence analysis of purified pep-
tides of PAK I, could be aligned within the deduced amino acid
sequence (Fig. 2). The peptide beginning with SVID at residue
197 indicates the N terminus of the p37 peptide generated by
limited trypsin digestion, which contains the catalytic domain.
The beginning of the catalytic domain at residue 247 was
determined by comparison with the sequences of other protein
kinases using the alignment of Hanks et al. (27, 28). Peptide
p37 contains part of the regulatory domain (from residues 197
to 246) and all 11 conserved subdomains characteristic of the
catalytic domain of protein kinases. The GXGXXG motif in-
volved in nucleotide binding begins at residue 256; residue 278
is the invariant lysine that contacts the α- and β-phosphates of the
ATP; residue 368 is the aspartate that acts as a general base
for the removal of a proton from the hydroxyl group of the
protein substrate; residue 370 is the lysine that binds to the
γ-phosphate of the ATP; and residue 386 is the aspartate that
is part of the conserved DFG motif and chelates the Mg²⁺
bound to the ATP. A putative binding site for Rho-like G
proteins was identified in the regulatory domain between res-
ides 73 and 107 by sequence comparison with PAK 65 from rat
brain and human placenta and yeast STE20 (12–15).

Amino acid sequence alignment of the total proteins revealed
95% identity between rabbit PAK I and human PAK 65 and
78% identity between rabbit PAK I and rat PAK 65 (Fig. 3).
Within the catalytic domain, rabbit PAK I has 99% identity
with human PAK 65, 92% with rat PAK 65, and 65% with yeast
STE20. In the regulatory domain, PAK I has significantly less
homology with STE20 and rat PAK 65, except for the G protein-
binding region. Compared with rat PAK 65, PAK I was 20
residues shorter because of five gaps in the regulatory domain
ranging from 1 to 10 residues. Human PAK 65 was 18 residues
shorter than rabbit PAK 1 because of differences at the N
terminus over the first 30 amino acid residues; the rest of the
amino acid sequence was very similar to that of rabbit PAK 1.

G Protein Activation of PAK I—Previously, a number of
compounds known to modulate protein kinase activity have been
examined as possible physiological regulators of PAK I;
none of these had any effect on PAK I activity. To examine the
effects of the G proteins Rac1, Cdc42Hs, and RhoA on auto-
phosphorylation and activation of purified inactive PAK I, GST
fusion proteins expressed in E. coli were purified on glutathione-Sepharose beads and preloaded with GTPγS. A low basal
level of autophosphorylation of inactive PAK I was detected in
the absence of G protein (Fig. 4). Upon addition of GST-Rac1,
a slight stimulation of autophosphorylation over the basal level
was observed over a 15-min period of incubation, while GST-
RhoA had little effect. Stimulation of autophosphorylation
upon addition of GST-Cdc42Hs was observed within 5 min. The
rate of autophosphorylation was linear up to 10 min and began
to level off by 15 min.

To examine the effects of autophosphorylation on activation
of PAK I, protein kinase activity was measured by phosphoryl-
ation of the substrate H4 (Fig. 5). PAK I was incubated under
autophosphorylation conditions for 10 min, either alone or with
GTPγS. GST-Rac1 (GTPγS), or GST-RhoA (GTPγS) preloaded with
GTPγS or GDP, and then assayed with H4. Phosphorylation of
H4 was dependent on the level of autophosphorylation of PAK
I. Without addition of G protein, a low level of autophosphoryl-
ation and PAK I activity with H4 was observed, which was
subtracted from the data obtained in the presence of G protein.
Addition of GST-Cdc42Hs(GTPγS) resulted in an increased
autophosphorylation of PAK I of 0.97 pmol/min and a stimulation
of the rate of phosphorylation of H4 of 18.261 pmol/min/mg.
With GST-Cdc42Hs(GDP), stimulation of autophosphoryl-
ation was 0.12 pmol/min, and the activity was 2154 pmol/min/mg.
These values were ~8-fold lower than those observed with
Cdc42Hs(GTPγS). GST-Rac1(GTPγS) resulted in only a modest
stimulation, ~10-fold lower than that observed with Cdc42Hs-
(GTPγS); autophosphorylation and activity were 0.09 pmol/min
and 1515 pmol/min/mg, respectively. GST-RhoA(GTPγS) had
no stimulatory effect on either autophosphorylation or activity.

DISCUSSION

The cDNA isolated by screening of a rabbit spleen cDNA
library and by 5'-RACE-PCR using rabbit spleen mRNA con-
tains the information for the complete sequence of PAK I. The
deduced amino acid sequence contains the catalytic domain
and an N-terminal regulatory domain, including a region
shown previously to bind Rho-like G proteins. All of the partial amino acid sequences determined by microsequencing of nine peptides from PAK I could be aligned with the deduced amino acid sequence. The calculated molecular mass of 58,027 Da is in accordance with the apparent molecular mass of 58–60 kDa displayed by native PAK I on SDS-polyacrylamide gels (5, 6).

The N-terminal amino acid residue of the proteolytically activated p37 fragment of PAK I is shown (first ). Also shown is the start of the catalytic domain (second ) as determined by comparison with the sequence of other protein kinases (31, 32).

Sequence comparison shows high sequence homology to the yeast protein kinase STE20 (14, 15) and PAK65 from rat brain and human placenta (12, 13), suggesting that they belong to a family of related protein kinases. Rat PAK65 appears to be a brain-specific isoform; a protein with the corresponding molecular mass was detected only in brain extracts, but not in other
tissues, by overlay assays with radiolabeled Rac1(GTP) and Cdc42Hs(GTP) and by a specific antibody raised against the N-terminal region of rat brain PAK65 (12, 13). Unlike brain-specific rat PAK65, inactive PAK1 was purified from a number of tissue and cell types, including rabbit reticulocytes, liver, and skeletal muscle; chicken gizzard, liver, and brain; bovine liver; mouse 3T3-L1 cells (1–6); and frog oocytes and embryos. 2 The protein reacted with antibodies raised against PAK1 from rabbit reticulocytes. PAK1 from rabbit spleen and PAK65 from human placenta have a high degree of sequence homology, but the N-terminal 30 amino acid residues are completely different. Human PAK65 lacks 18 amino acid residues, and the remaining 12 amino acid residues cannot be aligned with either rabbit PAK I or rat PAK65. Therefore, it is unlikely that the two protein kinases are homologous enzymes, but they appear to be closely related isoforms.

**Fig. 3.** Multiple sequence alignment of p21-activated protein kinases. The deduced amino acid sequences of rabbit PAK I, human PAK65, rat PAK65, and the GTP/p21-binding and catalytic domains of yeast STE20 were aligned using the programs Pileup, Lineup, and Pretty of the University of Wisconsin Genetics Computer Group Package Version 8 (25). The consensus sequence indicates identical residues for at least two of the protein sequences. Deviations from the consensus sequence are indicated by lower-case letters; gaps are indicated by dots. The positions of the 11 conserved subdomains of the catalytic domain of the protein kinases are shown below the alignment in Roman numerals.

![Alignment of PAK1](image_url)

**Fig. 4.** G protein-stimulated autophosphorylation of PAK I. PAK I (0.1 μg) was incubated with [γ-32P]ATP for the times indicated, alone or in the presence of the GST fusion protein of Rac1, Cdc42Hs, or RhoA bound to GTP·S. Autophosphorylation was analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. The autoradiogram is shown.

![Autophosphorylation of PAK1](image_url)
activity in actively dividing cells. PAK I activity is also high in mature frog oocytes, but is greatly diminished following fertilization. Injection of active PAK I into two-cell embryos has been shown to inhibit cell division in the injected blastomere (11). Thus, PAK I appears to be a candidate for regulation of the signaling cascade that is activated in response to stress.

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Fig. 5. Stimulation of PAK I activity by autophosphorylation. PAK I (0.1 μg) was autophosphorylated for 10 min in the presence of the GST fusion protein of Cdc42Hs, Rac1, or RhoA preloaded with GTP-Y-S or GDP. Following autophosphorylation, assays with H4 were carried out and analyzed on a 15% SDS-polyacrylamide gel, followed by autoradiography and scintillation counting of the excised H4 bands. Phosphorylation of H4 in the absence of G protein was used as background and subtracted from the data.

Rac1 and Cdc42, but not RhoA; autophosphorylation resulted in activation as shown by stimulation of phosphorylation of the universal substrate, myelin basic protein (12, 13, 29, 30). Binding of the GTP-bound form of GST-Cdc42Hs, but not GST-Rac1 or GST-RhoA, greatly induced autophosphorylation of PAK I from rabbit reticulocytes, and autophosphorylation resulted in activation of the protein kinase.

STE20 in budding yeast plays a key role in signal transduction pathways connecting membrane receptors with the MAP kinase cascade (33). In the mating differentiation pathway, a pheromone initiates differentiation by activation of STE20, which activates the MAP kinase cascade. STE20 and MAP kinases are also involved in other signal transduction pathways in yeast, including the invasive growth response and pseudohyphal development pathways initiated by nutritional starvation. Different isoforms of STE20 and MAP kinases could have alternative functions in regulating different signal transduction pathways (33). PAK I and other PAK kinases, as the mammalian counterparts of yeast STE20 protein kinases, also appear to be involved in the coordination of signal transduction pathways. It has recently been hypothesized (34, 35) that the Rac1/Cdc42-regulated PAK enzymes are a key point regulator of the stress-activated protein kinase or Jun kinase signaling pathway, which regulates cyclin activity. Biochemical evidence from our laboratory suggests that PAK I is involved in regulation of cytostasis as shown by the high level of activity in quiescent and serum-starved cells and lower levels of PAK I activity in actively dividing cells. PAK I activity is also high in mature frog oocytes, but is greatly diminished following fertilization. Injection of active PAK I into two-cell embryos has been shown to inhibit cell division in the injected blastomere (11). Thus, PAK I appears to be a candidate for regulation of the signaling cascade that is activated in response to stress.
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Kinase PAK 1
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