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A Cardiolipin-activated Protein Kinase from Rat Liver Structurally Distinct from the Protein Kinases C*

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A cardiolipin- and protease-activated protein kinase (PAK) has been isolated from cytoplasmic extracts of rat liver. The enzyme (PAK-1) phosphorylates the ribosomal protein S6-(229–239) peptide analogue and can be activated by limited proteolysis. Partial amino acid sequences of tryptic peptides derived from both the purified 116-kDa PAK-1 holoenzyme and its active catalytic fragment reveal that the catalytic domain is most related (50–58% identity) to the protein kinase C family. PAK-1 has protein and peptide substrate specificities distinct from those of known protein kinase C isoforms and is insensitive to inhibition by the protein kinase C-α-(19–31) pseudosubstrate peptide. Phosphatidylserine, diacylglycerol, and phorbol ester do not activate PAK-1 toward the S6 peptide substrate. However, other acidic phospholipids, the most effective being cardiolipin, activate PAK-1 to a similar extent as trypsin. The PAK-1 catalytic activities generated through activation by cardiolipin or limited proteolysis were kinetically similar, with K_m values of 3.6 and 3.4 μM, respectively, for the S6-(229–239) peptide substrate. However, differences were observed in the catalytic activities with protamine sulfate and the glycogen synthase-(1–12) peptide analogue as substrates. It was concluded that PAK-1 is a phospholipid-regulated protein kinase with a primary structure, substrate specificity, and mechanism of regulation *in vitro* distinct from those of any known member of the protein kinase C superfamily.

Ribosomal protein S6 is phosphorylated at multiple sites (*e.g.* Refs. 1–3) in mammalian cells in response to growth-promoting and mitogenic stimuli (4–9). The anabolic responses can be largely attributed to a unique class of S6-specific protein kinases activated by direct phosphorylation on serine and threonine residues (reviewed in Ref. 8). There are indications that other protein kinases, including protein kinase C (PKC)[1] and a class of protease-activated protein kinase (PAK), can also contribute to insulin- and growth factor-dependent S6 phosphorylation responses, either directly or indirectly via the mitogen-activated protein kinases (7, 10). Several PAKs have been described during the past decade that are capable of phosphorylating ribosomal protein S6 and that have been implicated in anabolic regulation of mammalian cells (11–14), but most have not been characterized structurally. The contribution of PAKs to the overall growth responses has been difficult to assess, partly because of the uncertainty of their relationship with the PKC isoenzymes (*e.g.* Ref. 15), which can also be activated by mild proteolysis (16–18) and which have similar phosphorylation site specificities (18–20).

The aim of this study was to use proteolytic activation *in vitro* (11, 21) as a strategy for identifying and further characterizing potentially novel protein kinases in rat liver extracts capable of phosphorylating a peptide analogue of ribosomal protein S6 (*e.g.* Refs. 14 and 20). The mild conditions of proteolysis employed were designed to be effective in the removal of protein kinase regulatory domains containing autoinhibitory or pseudosubstrate inhibitory sequences without affecting the functional integrity of their catalytic domains (11, 22–24). A major form of hepatic PAK activity (referred to as PAK-1), previously shown to phosphorylate ribosomal protein S6 (14, 20), was purified and found to be activated by cardiolipin, but was determined to be structurally distinct from the previously described PKCs.

EXPERIMENTAL PROCEDURES

Materials—[γ-^{32}P]ATP was obtained from Bresatec Ltd. (Thebarton, Australia); chromatographic supports were from Pharmacia LKB (Uppsala, Sweden); TPCK-treated trypsin was from Worthington; alkylated trypsin was from Promega; ethanediol (Analar) was from Merck BDH; and protein substrates, phospholipids, diacylglycerol (diolein), protease inhibitors, 12-*O*-tetradecanoylphorbol-13-acetate (phorbol ester), and Brij-35 from Sigma. Synthetic peptide analogues (see Footnote 1) were synthesized (as C-terminal amides) on an Applied Biosystems Model 430A peptide synthesizer as previously described (19, 24). Threonine-Sepharose 4B was prepared as described by Kikkawa *et al.* (25). Protamine-CH-Sepharose 4B (2.1 mg of protamine/ml of resin) was prepared from activated CH-Sepharose 4B and protamine sulfate as described by the manufacturer (Pharmacia LKB). PKC (mixed isoenzymes) was prepared from rat brain as described by House *et al.* (19) and stored at −70 °C in 50% glycerol and 0.05% (v/v) Triton X-100.

Purification of PAKs from Rat Liver—PAKs were freshly prepared from the livers of 4–8-month-old Buffalo rats fed *ad libitum*; all opera-

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[1] The abbreviations used are: PKC, protein kinase C; PAK, protease-activated protein kinase; TPCK, tosylphenylalanyl chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride; FPLC, fast performance liquid chromatography; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; PKM, active catalytic fragment of brain PKC generated by limited proteolysis; S6-(229–239), synthetic peptide analogue of ribosomal protein S6, residues 229–239, Ala-Lys-Arg-Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala; [^{240}Cys]S6-(229–240), Ala-Lys-Arg-Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala-Cys; GS-(1–12), synthetic peptide analogue based on skeletal muscle glycogen synthase sequence, residues 1–12, Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys; EGFR-(650–658), epidermal growth factor receptor analogue, residues 650–658, Val-Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu; H4-(43–49), histone H4 analogue, residues 43–49, Val-Lys-Arg-Ile-Ser-Gly-Leu; PKC-α-(19–31) pseudosubstrate inhibitor peptide, Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val; PKI-(4–24), cAMP-dependent protein kinase inhibitor, residues 4–24, Glu-Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp.

tions were performed at 4 °C, and the liver post-microsomal (150,000 × *g*) supernatant fraction was chromatographed on DE52 as described previously (14). Fractions containing trypsin-activated protein kinase activity (14) were pooled, dialyzed *versus* buffer B (15 mM potassium phosphate, pH 6.4, 1 mM EDTA, 2 mM EGTA, 10 mM 2-mercaptoethanol, 2 mM benzamidine, and 0.25 mM PMSF), and loaded onto a CM-Sephadex column (50 ml/100 g of tissue) equilibrated in the same buffer. The column was washed with 5 column volumes of buffer B and eluted with an 8-volume linear gradient of 0–0.4 M KCl; 6-ml fractions were collected.

The PAK-1 fraction from CM-Sephadex was adjusted to 20 mM Tris-HCl, pH 7.5, plus 1 M ammonium sulfate and loaded onto phenyl-Sepharose 4B (1 ml of resin/15 mg of protein loaded) equilibrated in buffer C (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 mM EGTA, 10 mM 2-mercaptoethanol, 2 mM benzamidine, and 0.25 mM PMSF) containing 1 M ammonium sulfate. The column was then washed successively with 10-column volume batches of buffer C containing 1, 0.5, 0.2, or 0.1 M ammonium sulfate or no ammonium sulfate and finally with 10 volumes of buffer C containing 50% (v/v) ethanediol. The latter two washes containing PAK-1 activity were pooled, dialyzed against buffer D (buffer C containing 10% ethanediol, 0.01% (v/v) Brij-35, and 50 mM NaCl), loaded onto a 5-ml threonine-Sepharose 4B column equilibrated in buffer D, and eluted with a 120-ml 0.05–1 M NaCl linear gradient. Column fractions (2 ml) containing the peak of PAK-1 activity were pooled, diluted 1:1 with buffer D, and loaded onto a protamine-CH-Sepharose 4B column (5 ml) equilibrated in buffer D plus 0.2 M NaCl. The column was washed with 4 volumes of the same buffer followed by a 150-ml linear gradient of 0.2–2 M NaCl. The column fractions (2 ml) containing PAK-1 activity were pooled, dialyzed against buffer D, loaded onto a Pharmacia Mono Q 5/5HR column equilibrated in the same buffer, and chromatographed using a Pharmacia FPLC system. The single peak of PAK-1 activity was eluted with a linear gradient of 0.05–0.5 M NaCl (10 mM/ml) in buffer D; 0.5-ml fractions were collected.

Protein Kinase and Protein Assays—Column fractions were activated by mild trypsinolysis in the presence of PMSF-treated BSA (0.8 mg/ml) as described previously (Ref. 14; see also Refs. 7, 11, and 26). The resultant peptide kinase activity was assayed with 30 μM S6-(229–239) substrate in a reaction mixture (60 μl) containing 20 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 0.2 mM ATP (10–100 cpm/pmol), 40 mM KCl, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.18 mg/ml PMSF-treated BSA, and 2 μM PKI-(4–24) (27) for 5–15 min at 30 °C. The incorporation of ^{32}P-labeled phosphate into peptides was determined using the Whatman P-81 paper binding method (28). Protein kinase activities were assayed under the same reaction conditions, except that the S6 peptide substrate was replaced by protamine sulfate at 0.5 mg/ml or other proteins at 1 mg/ml as indicated. The ^{32}P radioactivity incorporated into protamine was also determined by the P-81 paper binding method, while other protein substrates were assayed with acid precipitation on Whatman No. 1 paper using 15% (w/v) trichloroacetic acid. ^{32}P radioactivity was determined by Cerenkov radiation using a liquid scintillation counter. For kinetic experiments, PAK activity was adjusted so that the reaction rates were constant with respect to time for all substrate concentrations employed. PAK-1 activity was adjusted so that no more than 10% of the peptide substrate was consumed. K_m and V_{max} values were determined by fitting data to the Michaelis-Menten equation as described previously (24).

PKC activity was assayed using 30 μM S6-(229–239) as substrate (19) as described above for peptide kinase activity with or without 100 μg/ml phosphatidylserine and 0.5 mM $CaCl_2$. The effects of cardiolipin and other phospholipids were investigated using the same reactions mixture as described for peptide kinase activity (see above), except that soybean trypsin inhibitor (0.1 mg/ml) was substituted for PMSF-treated BSA, and the assays were carried out in the presence and absence of sonicated phospholipid dispersions at the concentration specified in the text.

Standard procedures were used for Bradford protein determinations (29) (BSA as standard), SDS-PAGE using 7.5% resolving and 4% stacking gels (30), and staining with either Coomassie Brilliant Blue (30) or silver stain (Bio-Rad) (31). Stained protein bands were quantitated by densitometry using a Molecular Dynamics laser densitometer.

Isolation of PAK-1 and PKC Active Fragments—Preparations of phenyl-Sepharose-purified PAK-1 or brain PKC were dialyzed against buffer E (50 mM Tris-HCl, pH 8.0, 25 mM NaCl, 5 mM $MgCl_2$, 2 mM EGTA, 10 mM 2-mercaptoethanol, 10% ethanediol, and 0.01% (v,v) Brij-35), and after dialysis, PMSF-treated BSA was added to 0.5 mg/ml. The protein fraction was digested with TPCK-treated trypsin (1:100, w/w) for 3 min at 30 °C. The reaction was stopped with 32 × (w/w) excess of soybean trypsin inhibitor and 0.25 mM PMSF (on ice). The digest was loaded onto a 5-ml S6 peptide affinity column ([Cys^{240}]S6-(229–240) coupled to divinyl sulfone-activated Sepharose) equilibrated with buffer E containing 10 μg/ml soybean trypsin inhibitor. After washing with 5 volumes of buffer E (plus soybean trypsin inhibitor), the column was eluted with a 150-ml linear gradient of 0.025–0.525 M NaCl. Fractions containing protein kinase activity were pooled, dialyzed *versus* buffer E overnight, and then loaded onto a Mono Q 5/5HR column in buffer E using a Pharmacia FPLC system, and the proteins were eluted at 1 ml/min with a 45-ml linear gradient of 0.05–0.5 M NaCl; 0.5-ml fractions were collected.

Amino Acid Sequence Analysis—Purified PAK-1 (post-Mono Q fraction) or its catalytic fragments were dialyzed against 10 mM NH_4HCO_3 buffer containing 0.02% SDS and 20 mM 2-mercaptoethanol for 3 h, concentrated 50-fold, and alkylated with 2 μl of 4-vinylpyridine for 3 h at 37 °C. The protein was precipitated with 9 volumes of ethanol at −20 °C and digested with TPCK-treated trypsin (1:10, w/w) in 0.1 M NH_4HCO_3, 0.1 mM $CaCl_2$ buffer for 24 h at 37 °C. The tryptic peptides were separated by reversed-phase HPLC on a 5-μm Waters Delta-Pak ODS column (2.1 × 150 mm) developed with a linear gradient of 0–50% acetonitrile (0.5%/min) in aqueous 0.1% trifluoroacetic acid at 0.2 ml/min. In-gel digests of PAK-1 were performed by excising the Coomassie Blue-stained 116-kDa polypeptide band from SDS-polyacrylamide slab gels (1.5 mm thick), washing in deionized water (5 × 1 ml) for 2 h, and drying the gel piece under vacuum. The dried gel piece was hydrated in 0.4 ml of 0.1 M NH_4HCO_3 containing 0.1 mM $CaCl_2$ and 1 μg of alkylated trypsin (Promega) and incubated for 22 h at 37 °C. The digest was clarified by centrifugation before separating the tryptic peptides by reversed-phase HPLC using the Waters Delta-Pak ODS column in conjunction with acetonitrile gradient elution as described above. Peptides were sequenced on an Applied Biosystems Model 477A Sequencer with on-line phenylthiohydantoin-derivative analyses.

RESULTS

Resolution of Liver PAK Activities on CM-Sephadex—Liver PAK activities were assayed with the peptide analogue of the ribosomal S6-(229–239) sequence, containing two of the insulin- and growth factor-dependent phosphorylation sites (3, 32, 33). Three broad peaks of PAK activity were resolved following chromatography on CM-Sephadex (Fig. 1), with peaks eluting at ~100 mM KCl (termed PAK-1) and 250 mM KCl (PAK-2) (Fig. 1*a*). PAK-2 activity was only apparent when a range of protease inhibitors were included in the initial liver extraction buffer (see "Experimental Procedures"), and even with these precautions, the relative proportions of the PAK activities varied between experiments (compare activity profiles in Fig. 1, *a* and *b*). A peak of PKC activity eluted on the leading edge of the peak of PAK-1 activity (see Fig. 1 legend); this coincided with the only discrete peak of trypsin-dependent activity observed with the histone H1-rich Sigma Type III-S histone substrate, a substrate preferred by the PKCs (Fig. 1*a*) (17, 34).

Differential Sensitivities of PAKs to PKC Pseudosubstrate Inhibitor Peptide—The PAK activities resolved on CM-Sephadex exhibited differential sensitivities toward the PKC pseudosubstrate inhibitor peptide (PKC-α-(19–31)) (24). The later eluting PAK-2 activity was strongly inhibited, while the major component of the PAK-1 activity peak was relatively unaffected by the inhibitor peptide (Fig. 1*b*). The inhibitor-sensitive component of the leading edge of the PAK-1 activity peak coincided with the peak of phosphatidylserine/Ca^{2+}-dependent (data not shown) and histone H1 (Fig. 1*a*) kinase activities.

Purification of Liver PAK-1—Hydrophobic interaction chromatography of the CM-Sephadex pooled fractions of PAK-1 activity on phenyl-Sepharose resolved the major PAK-1 activity from several contaminating protein kinases, including the phosphatidylserine/Ca^{2+}- and cAMP-dependent protein kinase activities (data not shown). The PAK-1 recovered from phenyl-Sepharose was further purified by successive chromatographic steps on threonine-Sepharose 4B, protamine-CH-Sepharose 4B, and Mono Q (data not shown). The specific activity (Table I) and SDS-PAGE analyses (Fig. 2) showed that the protamine-CH-Sepharose step was the most effective in removing extra-

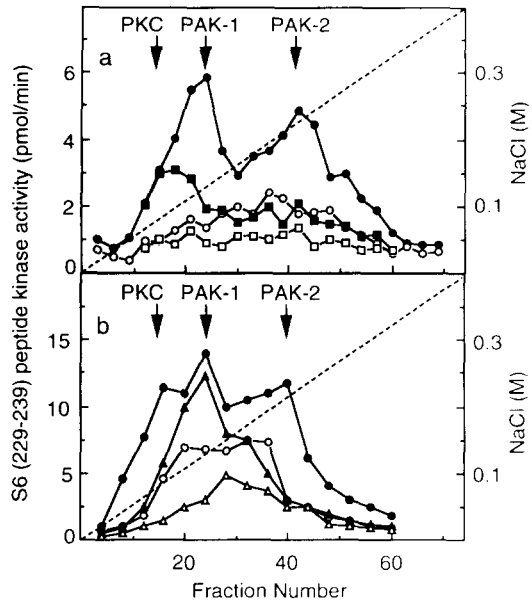

FIG. 1. **CM-Sephadex chromatography of hepatic protein kinases.** *a*, pooled fractions eluted from DE52-cellulose containing PAK and PKC activities were chromatographed on CM-Sephadex, and the column fractions were assayed for protein kinase activities with the S6-(229–239) peptide (●, ○) or histone Type III-S (■, □) as substrate (see "Experimental Procedures"). Assays with (●, ■) and without (○, □) trypsin treatment determined PAK activities. The position marked with an *arrow* as *PKC* denotes the position of elution of phosphatidylserine/Ca^{2+}-dependent S6-(229–239) peptide kinase (data not shown). Protein kinase activities were assayed in the presence of 2 μM PKI-(4–24) (28). *b*, chromatography of the post-DE52-cellulose preparation of PAK activities on CM-Sephadex was carried out as described for *a*. Column fractions were assayed for S6-(229–239) kinase activity with (●, ▲) and without (○, △) trypsin activation in the presence (▲, △) and absence (●, ○) of 40 μM PKC-α-(19–31) synthetic peptide.

neous protein. The final Mono Q step yielded a single peak of PAK-1 activity containing a highly purified preparation of a 116-kDa polypeptide as determined by SDS-PAGE (Fig. 2, *lane 6*). This estimate correlated with the apparent M_r of native PAK-1 of ~100,000 determined by gel permeation chromatography on Sephacryl S-200 (data not shown).

Purification of Proteolytically Generated Catalytic Fragment(s) of PAK-1—The active fragment(s) of PAK-1 was generated by mild trypsinolysis of the PAK-1 recovered from phenyl-Sepharose under the same digestion conditions employed in the PAK assay; SDS-PAGE analyses of the digestion products showed that ~70% of the 116-kDa PAK-1 polypeptide had been cleaved, generating fragments in the 43–55-kDa range (data not shown). The active catalytic fragments were purified by sequential chromatography on [Cys^{240}]S6-(229–240) peptide-Sepharose affinity (data not shown) and Mono Q (Fig. 3) columns. The catalytic fragment of PAK-1 eluted at a higher salt concentration than the active fragment(s) of brain PKC (referred to as PKM (17)) generated by the same proteolytic digestion protocol from both the peptide affinity (data not shown) and Mono Q (Fig. 3*B*) columns. SDS-PAGE analysis of Mono Q column fractions showed that the major PAK-1 polypeptide that copurified with the S6 peptide kinase activity was a 55-kDa species, with lesser quantities of 51- and 43-kDa species (Fig. 3*A*). All three species were distinct from any of the polypeptides associated with the PKM activity (≤45 kDa) (Fig. 3*B*). The intensity of silver-stained bands determined by densitometry compared with known protein standards provided an estimate of the amount of protein in the final peak fractions of PAK-1 fragments. On this basis, it was estimated that the specific activity of the purified PAK-1 fragments was ~5 μmol of $^{32}P_i$ transferred per min/mg of enzyme with the S6-(229–239) substrate. This high specific activity is within the same order of magnitude of the specific activities of cAMP-dependent protein kinase and PKC with their preferred peptide substrates.

Kinetic Properties of PAK-1—The kinetic properties of the intact preparations of PAK-1 were distinct from those of the proteolytically generated activity. The ATP substrate K_m for the holoenzyme was an order of magnitude higher than that observed for the isolated catalytic fragment (K_m = 200 and 12 μM, respectively). The K_m for the purified preparations of the holoenzyme with the S6-(229–239) substrate was estimated to be 110 μM, although some preparations of PAK-1 exhibited biphasic Lineweaver-Burk plots with the peptide substrate (data not shown), suggesting two catalytic activities with approximate K_m values of 3 μM (minor component) and 110 μM (major component). The low K_m value approximated to the K_m value of 3.4 μM determined for the trypsin-activated form of PAK-1 with the same substrate (20), suggesting that the low K_m component of the holoenzyme preparations relates to an activated form of the enzyme.

The V_{max} values estimated for the PAK-1 holoenzyme preparations with and without trypsin activation were 3.1 and 1.7 μmol/min/mg of PAK-1 protein, respectively, for the S6-(229–239) substrate. However, precise measurement of the V_{max} data was made difficult by peptide substrate inhibition of the trypsin-activated enzyme at high substrate concentrations (data not shown), similar to the inhibition of PKC by the myristylated alanine-rich C-kinase substrate peptide analogue (35).

Intact PAK-1 was also able to phosphorylate the protamine sulfate substrate without trypsin activation (Table II). The favorable kinetic properties of the enzyme with this substrate (K_m = 3.6 μM; V_{max} = 1.5 μmol/min/mg) resembled those of the proteolytically activated enzyme with the S6 peptide substrate. Surprisingly, however, the purified active catalytic fragment of PAK-1 exhibited only a relatively low level of protamine kinase activity (Table II). Consistent with this finding was the inhibitory effect of the trypsin pretreatment on the protamine kinase activity of the whole PAK-1 preparations (Table II). The residual protamine kinase activity associated with trypsin-treated PAK-1 preparations was at least partially attributable to the residual intact 116-kDa PAK-1 that survived the limited proteolysis (as discussed above).

Phospholipid Regulation of PAK-1—Intact preparations of purified PAK-1 exhibited basal S6 peptide kinase activity at 30 μM S6-(229–239) substrate, usually corresponding to 20–25% of the activity following trypsin treatment. The basal activity was not appreciably enhanced by the addition of Ca^{2+}/calmodulin, cAMP, or various combinations of phosphatidylserine, 12-*O*-tetradecanoylphorbol-13-acetate, diacylglycerol, and Ca^{2+} under conditions effective for activation of brain PKC (data not shown). Phosphatidylethanolamine and phosphatidylcholine also had no effect on PAK-1 activity (Fig. 4). However, cardiolipin activated the enzyme to a similar extent as trypsin (see Fig. 4 legend), generating a low K_m form of the enzyme both with respect to ATP (K_m = 20 μM) and S6-(229–239) (K_m = 3.6 μM) substrates. Thus, the cardiolipin- and protease-activated forms of PAK-1 were judged to be kinetically similar to both the S6-(229–239) peptide and ATP substrates. Other acidic phospholipids, with the exception of phosphatidylserine, also activated PAK-1, but from the dose-response curves, it was apparent that the enzyme was most sensitive to activation by cardiolipin (Fig. 4). A characteristic feature of the cardiolipin dose-response curve was the inhibition by higher concentrations of the lipid, with the maximum effect being observed at 10 μg/ml. Higher concentrations of phosphatidylglycerol, phosphatidylinositol, and phosphatidic acid were required for maxi-

TABLE I
Purification of PAK-1 from rat liver

PAK-1 was purified from the post-150,000 × *g* cytosol of 30 rat livers to apparent homogeneity. PAK-1 activity was assayed as described under "Experimental Procedures" and is expressed as the S6-(229–239) peptide kinase activity obtained with trypsin activation.

Purification step	Volume	Protein	Activity	Specific activity	Yield[a]	Purification
	ml	*mg/ml*	*nmol/min*	*nmol/min/mg*	%	*-fold*
DE52	250	12.3	426	0.1		
CM-Sephadex	285	0.4	619	5.4	100	1
Phenyl-Sepharose	59.5	0.25	241	16.2	39	3
Threonine-Sepharose	26	0.2	158	30.4	25	5.6
Protamine-CH-Sepharose	24.3	0.002[b]	84	1728	13	320
Mono Q	4.5	0.003[b]	33	2444	5	452

[a] Yield of PAK-1 is expressed as a percentage of the CM-Sephadex pool (100%).
[b] The protein concentrations of protamine-CH-Sepharose and Mono Q pools were estimated by densitometry of silver-stained PAK-1 in 7.5% SDS-polyacrylamide electrophoretograms using a standard curve generated from a separate gel loaded with 1–500 ng of BSA.

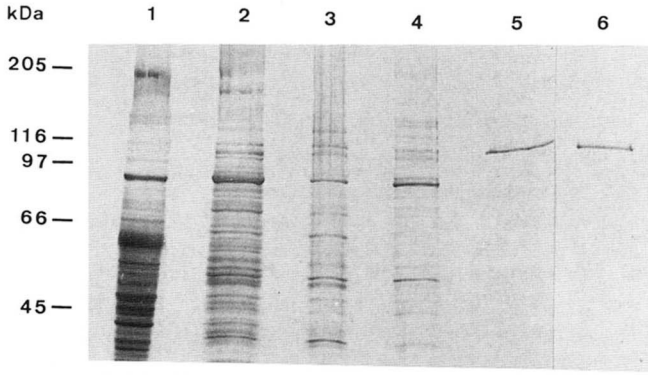

FIG. 2. **SDS-PAGE analysis of PAK-1 fractions during purification.** S6-(229–239) kinase peak activity fractions obtained at each step during purification of PAK-1 from the post-DE52-cellulose pool were analyzed by SDS-PAGE using 7.5% polyacrylamide gels and silver staining. The samples loaded were as follows: *lane 1*, 30 μg of post-DE52 fraction; *lane 2*, 19 μg of post-CM-Sephadex fraction (Fig. 1); *lane 3*, 6.3 μg of post-phenyl-Sepharose fraction; *lane 4*, 5 μg of post-threonine-Sepharose fraction; *lane 5*, 0.3 μg of post-protamine-CH-Sepharose fraction; *lane 6*, 0.4 μg of post-Mono Q fraction. The standard protein markers used were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase (97 kDa), BSA (66 kDa), and ovalbumin (45 kDa).

mal activation, and there was relatively little evidence of inhibitory effects of these lipids at even higher concentrations (Fig. 4). In all cases, phospholipid activation of PAK-1 was independent of calcium, phorbol ester, or diacylglycerol (data not shown).

Substrate Specificities of PAK-1—Appreciable differences were observed in the substrate preferences exhibited by the purified catalytic fragments of PAK-1 and brain PKC for a range of protein and peptide substrates (Table II). The most pronounced differences were observed with the synthetic peptide analogue of the glycogen synthase N-terminal sequence (based on residues 1–12), Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys (19), and the EGFR-(650–658) analogue of the epidermal growth factor receptor sequence, Val^{650}-Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu^{658} (19) (Table II). The glycogen synthase analogue was the best of all substrates tested (comparisons made at a peptide concentration of 100 μM) for the catalytic fragment of PAK-1. This analogue was a 6-fold better substrate than for PKM, apparently reflecting a difference in V_{max} as the K_m for PAK-1 of 32 μM was higher than that for brain PKC (4.1 μM; see Ref. 19). Conversely, the EGFR-(650–658) peptide was a relatively poor substrate for the PAK-1 fragment compared with PKM (3-fold difference).

The catalytic fragment of PAK-1 phosphorylated protamine sulfate at a rate 34 times slower than that for PKM despite the fact that the protamine kinase activity of intact PAK-1 was comparable to that of PKM (Table II). Also, the catalytic fragment of PAK-1 was severalfold less effective than PKM in the phosphorylation of the histone H1-enriched fraction (Sigma

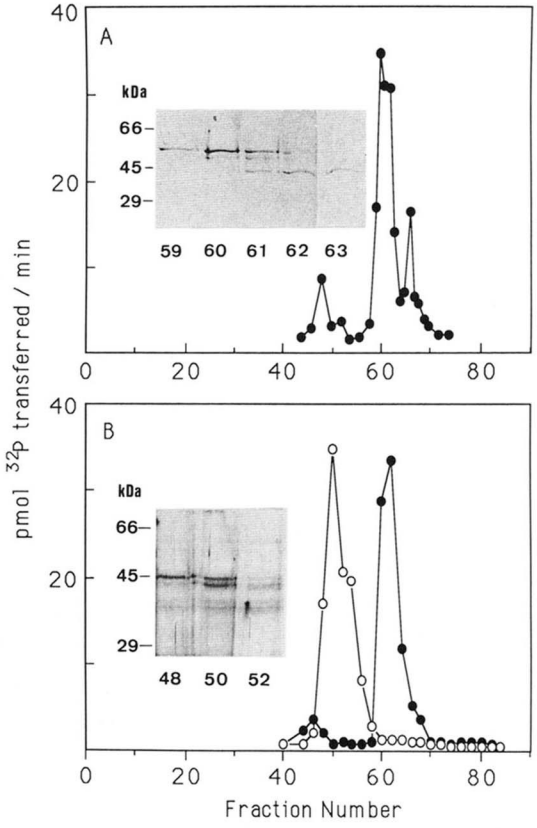

FIG. 3. **Chromatography of trypsin-generated fragments of PAK-1 and PKC (PKM) on Mono Q.** Preparations of the trypsin-generated active fragments of PAK-1 (●) and PKC (○) eluted from the S6 peptide affinity column were chromatographed on Mono Q (FPLC); 0.5-ml fractions were collected and assayed for S6-(229–239) peptide kinase activity. Separate preparations of PAK-1 fragments were chromatographed in *A* and *B*. In *A*, column fractions 59–63 were also analyzed by 10% SDS-PAGE and silver-stained (*inset*). In *B*, the PAK-1 and PKC (PKM) active fragments were generated and chromatographed under identical conditions. 0.5-ml column fractions were collected. Fractions 48, 50, and 52 were analyzed by SDS-PAGE (as described for *A*) for polypeptides associated with PKM activity (*inset*).

Type III-S histone fraction), thus explaining the absence of a clearly defined peak of PAK-1 activity when the CM-Sephadex chromatographic fractions were screened with this substrate (see Fig. 1*a*). Both PAK-1 and PKM displayed similarly low or no activity toward Sigma Type II-AS mixed histones, phosvitin, casein, or the H4-(43–49) analogue of histone H4 (Table II) (13).

The substrate specificities for the cardiolipin-activated PAK-1 and the purified catalytic fragment of the enzyme were generally similar. Exceptions were protamine sulfate, which was phosphorylated relatively well by the cardiolipin-activated enzyme, and the GS-(1–12) peptide substrate, which was a relatively better substrate for the catalytic fragment (Table II).

TABLE II
Comparison of substrate specificity of PAK-1

PAK-1 (post-protamine-CH-Sepharose pool), PAK-1 active fragments (consisting predominantly of the 55-kDa fragment; Fig. 3*A*), and protein kinase M (post-Mono Q; Fig. 3*B*) were assayed with synthetic peptide (S6-(229–239) at 30 μM and all other peptides at 100 μM) and protein (1 mg/ml) substrates for 10 min at 30 °C as described under "Experimental Procedures." Relative phosphotransferase activity refers to the activity as a percentage of that obtained from the S6-(229–239) substrate (in the case of PAK-1 minus trypsin, the activities are expressed as percentages of the PAK-1 plus trypsin S6-(229–239) activity). The absolute activities for the trypsin- and cardiolipin-activated forms of PAK-1 with the S6-(229–239) substrate were 26 and 25.5 pmol/min, respectively. The absolute activities (100%) of the purified catalytic fragments of PAK-1 and PKM were 25 and 27.5 pmol/min, respectively.

Substrate	Relative phosphotransferase activity				
	PAK-1 + trypsin	PAK-1 − trypsin	PAK-1 + CL[a]	Purified PAK-1 fragment	PKM
S6-(229–239)	100	21.5	100	100	100
Histone III-S	8.3	7.1	9.3	11.5	38.4
Histone II-AS	4.7	2.7	4.5	9.7	12.3
Phosvitin	1.5	1.5	1.6	1.4	0.8
Casein	0	0	0	0	1.3
Protamine sulfate	30.4	50.8	44.4	2.1	71.4
Myelin basic protein	11.1	8.9	ND	ND	ND
GS-(1–12) analogue	165.0	12.1	176.0	321	47.2
EGFR-(650–658)	29.3	6.6	20.6	19.3	42.6
H4-(43–49)	6.3	2.7	ND	1.0	0

[a] CL, cardiolipin; ND, not determined.

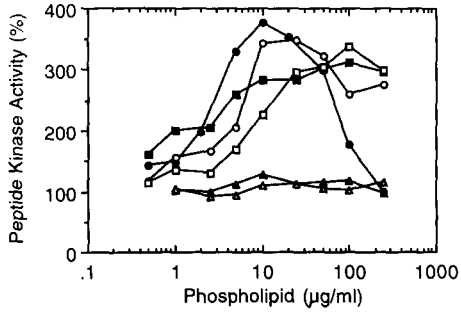

FIG. 4. **Dose-response curve for phospholipid activation of PAK-1.** The activity of purified liver PAK-1 was assayed with the S6-(229–239) peptide substrate in the presence of various concentrations of phospholipids as described under "Experimental Procedures." Activity is relative to that obtained in the absence of phospholipids (100%). The *x axis* refers to the concentration of individual phospholipids/milliliter of reaction mixture: cardiolipin (●), phosphatidylglycerol (○), phosphatidylinositol (■), phosphatidic acid (□), phosphatidylserine (▲), and phosphatidylcholine (△).

Inhibitors of PAK-1—PAK-1 exhibited differential sensitivities to a range of metal ions. While the enzyme exhibited a characteristic Mg^{2+} dependence for a protein kinase, with an optimal Mg^{2+} ion concentration in the range of 2–5 mM (data not shown), other divalent metal ions were inhibitory. Both the basal and protease-activated kinase activities were inhibited by Ca^{2+}, with an IC_{50} of 1.2 mM for the trypsin-activated PAK-1 (Table III). PAK-1 activity was inhibited by >98% by 10 mM Mn^{2+}, Cu^{2+}, Co^{2+}, or Zn^{2+} ions (data not shown). The enzyme was also sensitive to inhibition by NaF, glycerophosphate (mixed α- and β-isomer ions), staurosporine, and H-7 (1-(5-isoquinolinylsulfonyl)-2-methylpiperazine), with IC_{50} values of 30 mM, 54 mM, 30 nM, and 180 nM, respectively (Table III).

PAK-1 trypsin-activated S6 peptide kinase activity was also found to be very sensitive to inhibition by polyarginine and less sensitive to polylysine, with IC_{50} values of 0.3 and 3 μg/ml, respectively (Table III). However, PAK-1 protamine sulfate kinase activity was appreciably less sensitive to polyarginine, with an IC_{50} of 8 μg/ml, and was insensitive to inhibition by polylysine at concentrations up to 100 μg/ml of reaction mixture (Table III). A comparison of the inhibitor dose-response curves for the active catalytic fragments of PAK-1 and brain PKC with the S6-(229–239) substrate indicated that PAK-1 was 100-fold less sensitive to inhibition by the PKC-α-(19–31) pseudosubstrate inhibitor peptide (24), with IC_{50} values of 800 and 8 μM for PAK-1 and PKM, respectively (Table III).

TABLE III
Effects of inhibitors on PAK-1

Trypsin-activated PAK-1 was assayed for S6-(229–239) peptide kinase activities in the presence of various modifiers. For comparison, the IC_{50} for PKC-α-(19–31) inhibition of rat PKM with the S6-(229–239) substrate was 8 μM. PAK-1 (intact enzyme) was assayed for protamine kinase activity without trypsin treatment. The concentration of inhibitor at which the activity was reduced by 50% is given as the IC_{50}.

Modifier	IC_{50}
S6-(229–239) substrate	
Sodium glycerophosphate	54 mM
NaF	30 mM
$CaCl_2$	1.2 mM
PKC-α-(19–31)	0.8 mM
Staurosporine	30 nM
H-7	180 nM
Polyarginine	0.3 μg/ml
Polylysine	3.0 μg/ml
Protamine substrate	
Polyarginine	8 μg/ml
Polylysine	No inhibition (up to 100 μg/ml tested)

TABLE IV
Sequences of tryptic peptides derived from the 116-kDa PAK-1 and its active catalytic fragments

Tryptic peptides derived from the in-gel digest of the 116-kDa PAK-1 (peptides 1–4) and from the tryptic digest of the 55-kDa catalytic fragment of PAK-1 (peptides 1–7) were aligned with the protein kinase subdomains (I–XI) as described by Hanks and Quinn (36). Tryptic peptides 2 and 4–7 were also derived from pure preparations of the 43-kDa fragment. The 93 amino acid residues contained in the PAK-1 catalytic domain peptides 2 and 4–7 displayed an overall identity of 58% to the corresponding rat PKC-α sequences (37). The residues shown in boldface are identical to the corresponding PKC-α sequence.

Peptide	Sequence	Kinase subdomain
1	DFDFVAGGY	
2	**LDNLLLDTEGYVK**	VI
3	TLDWDALLAR	
4	**VLLSEFHSSGELFAIK**	I–II
5	**IADFGLCK**	VII
6	**TSTFCGTPEFLAPEVLTDTSYTR**	VIII
7	**AVDWWGLGVLLYEMLVGESPFPGD**	IX

Amino Acid Sequence Analysis of Tryptic Fragments of PAK-1—Automated sequence analyses of tryptic fragments of PAK-1 established that the active catalytic fragment of the enzyme generated by limited proteolysis was in fact derived from the 116-kDa polypeptide and that the enzyme was structurally distinct from any previously described protein kinase (Table IV). The sequences of several tryptic peptides generated from the 116-kDa PAK-1 polypeptide excised from SDS-polyacrylamide gels and purified by reversed-phase HPLC, although unique, as determined by screening the SwissProt and GeneBank data bases, could be aligned with the catalytic domains of known protein kinases (Table IV) (36). The same and additional tryptic peptides originating from the catalytic domain were also generated from purified preparations of the catalytically active 55- and 43-kDa PAK-1 fragments (Fig. 3*A*), confirming that these fragments are tryptic variants of the parent 116-kDa PAK-1 (Table IV). The tryptic peptide sequences that aligned with the catalytic domain consensus sequence (36) displayed the greatest homology to the PKC isoenzymes, ranging from 50–58%; for example, the PAK-1 tryptic peptides in Table IV displayed an

overall identity of 58% to the corresponding region of rat PKC-α (37).

DISCUSSION

PAK-1 is appreciably larger (apparent M_r of 116,000) than any of the defined PKCs, the largest being PKC-ε, with a M_r of 83,474 (apparent M_r of 95,700 by SDS-PAGE) (38). The amino acid sequences of its tryptic peptides, although related, do not match exactly the sequences of any previously defined protein kinases found by searching the SwissProt and GeneBank data bases (36, 39). The sequence comparisons showed that the PAK-1 tryptic sequences resembled the sequences of the PKC isoenzymes (50–58% sequence identity) to a greater degree than the corresponding regions of other protein kinases (<45% identity), suggesting that PAK-1 might be related to the PKC subfamily.

PAK-1 is >100-fold less sensitive than the brain PKCs to inhibition by the PKC-α-(19–31) pseudosubstrate inhibitor peptide (Table III) (24) and displays distinct substrate specificity. In particular, PAK-1 is relatively more active with the glycogen synthase peptide analogue and less active with histone H1 and the EGFR-(650–658) peptide substrates. However, PAK-1 and brain PKC share similar phosphorylation site specificity determinants within the S6-(229–239) substrate, particularly with respect to the beneficial influence of arginine residues closely situated on both sides of the preferred Ser^{236} phosphorylation site (19, 20). The affinity of the enzyme for sequences containing arginine residues was also reflected in the high activity of intact PAK-1 with the protamine sulfate substrate as well as the relatively low IC_{50} for inhibition of S6-(229–239) kinase activity by polyarginine, even though the inhibitor was less effective with the protamine substrate (Table III).

Rat liver PAK-1 can also be distinguished from other protease-activated protein kinases capable of phosphorylating ribosomal protein S6. Rabbit reticulocyte PAK II (11) displays similar activity toward ribosomal protein S6 *in vitro* (11, 12) and sensitivity to inhibition by Ca^{2+} (15, 26), but unlike liver PAK-1, is activated by a mixture of phosphatidylserine and diolein (15), has a relatively high K_m for ATP of 56 μM (26) compared with 12 μM for PAK-1, and is relatively more active with histone H1 as substrate. The lymphosarcoma PAK (13) and a similar enzyme from human placenta (40–42), which also phosphorylates ribosomal protein S6, can be distinguished from PAK-1 by their preference for the histone H4-(43–49) peptide substrate, which is relatively a very poor substrate for PAK-1 (Table II). While S6/H4 kinase resembles PAK-1 in that it phosphorylates the S6-(229–249) analogue and is unaffected by the PKC-α-(19–31) inhibitor (40, 41), it is smaller (66 kDa) (42) and is relatively more effective with both histone H4 and myelin basic protein substrates (40, 41).

So far, we have been unable to demonstrate the phosphorylation of protein substrates for PAK-1 other than ribosomal protein S6 in liver extracts, indicating a narrow range of substrate specificities. The preferred phosphorylation site for PAK-1 in S6 is Ser^{236} (14, 20), which is also phosphorylated *in vivo* (2, 6, 32, 33) and which is the initial site phosphorylated by *Xenopus* S6 kinase II in the ordered sequence of phosphorylation sites on 40 S ribosomal subunits (3). While PAK-1 does not efficiently catalyze the phosphorylation of sites other than Ser^{236} (20), the possibility remains that PAK-1 contributes to S6 phosphorylation *in vivo* in other physiological contexts.

The favorable kinetics of phosphorylation of protamine sulfate by intact PAK-1, without proteolytic or cardiolipin activation (K_m = 3.6 μM), indicate that this substrate overcomes intramolecular inhibition of the catalytic activity, possibly due to displacement of an intramolecular inhibitory sequence from the active site of the enzyme by the highly basic protamine (24, 39). The arginine-rich S6-(229–239) substrate also appears to be able to overcome this form of competitive inhibition at high substrate concentrations. The need to overcome the intramolecular competitive inhibition would explain the relatively high K_m observed with this peptide substrate in the absence of enzyme activation (110 μM) compared with the low K_m for the catalytic fragment (3.4 μM) (20) or cardiolipin-activated enzyme (3.6 μM). Protamine sulfate can also overcome the normal requirements for PKC activation (39, 43, 44). However, a striking difference between PAK-1 (Table II) and the PKCs (16) is the relative inability of the purified catalytic fragment of PAK-1 to phosphorylate protamine compared with the PKM activity. This implies an influence of the proteolytically cleaved regulatory domain of PAK-1 on substrate specificity analogous to that reported for PKC-ε (16). A further complexity is that the relatively high activity of the cardiolipin-activated enzyme with the protamine substrate, compared with the low activity of the catalytic fragment with this substrate, suggests that the favorable influence of the regulatory domain on protamine phosphorylation is retained in the phospholipid-activated enzyme.

The moderate abundance of PAK-1 in liver suggests an important role in hepatic regulation. Activation through interaction with some lipophilic regulator seems likely given the resemblance between several properties of PAK-1 and the PKCs (17, 39). Consistent with this possibility is the activation of PAK-1 by low concentrations of cardiolipin and, to varying degrees, several other acidic phospholipids. However, the unresponsiveness of the PAK-1 S6 peptide kinase activity to various combinations of phosphatidylserine, phorbol ester, and Ca^{2+} distinguishes PAK-1 from the previously defined members of the PKC family, even after taking into account the differential sensitivities of the individual PKC isoenzymes to the different ligands (17, 34, 39, 43, 44). Cardiolipin also activates the PKCs, although the sensitivities of individual PKC isoenzymes to this phospholipid varies considerably, with little activation of the classic PKC-α, PKC-βII, and PKC-γ (45) compared with relatively good activation of PKC-ε (38, 46) and PKC-ζ (43). The physiological significance of the cardiolipin effects *in vitro* is unclear given the almost exclusive location of the lipid within the inner mitochondrial membrane. While the possibility of a role of PAK-1 in mitochondrial regulation warrants further investigation, potential regulatory roles in other subcellular locations must also be considered, possibly involving other lipid activators of PAK-1 such as phosphatidic acid, which has recently been implicated in a range of signal transduction processes (*e.g.* Ref. 47).

The isolation, partial structural characterization, and observed activation of PAK-1 by phospholipids should provide the basis for elucidating the physiological role(s) of this enzyme in future investigations.

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