Identification of the regulatory autophosphorylation site of autophosphorylation-dependent protein kinase (auto-kinase)

Evidence that auto-kinase belongs to a member of the p21-activated kinase family

Jau-Song YU*[1], Wei-Jen CHEN*, Mei-Hui NI*, Wen-Hsiung CHAN† and Shiaw-Der YANG†

*Department of Cell and Molecular Biology, Institute of Basic Medicine, Chang Gung University, Tao-Yuan, Taiwan, Republic of China, and †Department of Life Sciences, National Tsing Hua University, Hsinchu, Taiwan, Republic of China

Autophosphorylation-dependent protein kinase (auto-kinase) was identified from pig brain and liver on the basis of its unique autophosphorylation/activation property [Yang, Fong, Yu and Liu (1987) J. Biol. Chem. **262**, 7034–7040; Yang, Chang and Soderling (1987) J. Biol. Chem. **262**, 9421–9427]. Its substrate consensus sequence motif was determined as being -R-X-(X)-S*/T*-X_3-S/T-. To characterize auto-kinase further, we partly sequenced the kinase purified from pig liver. The N-terminal sequence (VDGGAKTSDKQKKKAXMTDE) and two internal peptide sequences (EKLRTIV and LQNPEK/ILTP/FI) of auto-kinase were obtained. These sequences identify auto-kinase as a C-terminal catalytic fragment of p21-activated protein kinase 2 (PAK2 or γ-PAK) lacking its N-terminal regulatory region. Auto-kinase can be recognized by an antibody raised against the C-terminal peptide of human PAK2 by immunoblotting. Furthermore the autophosphorylation site sequence of auto-kinase was successfully predicted on the basis of its substrate consensus sequence motif and the known PAK2 sequence, and was further demonstrated to be RST(*P*)MVGTPYWMAPEVVTR by phosphoamino acid analysis, manual Edman degradation and phosphopeptide mapping via the help of phosphorylation site analysis of a synthetic peptide corresponding to the sequence of PAK2 from residues 396 to 418. During the activation process, auto-kinase autophosphorylates mainly on a single threonine residue Thr^{402} (according to the sequence numbering of human PAK2). In addition, a phospho-specific antibody against a synthetic phosphopeptide containing this identified sequence was generated and shown to be able to differentially recognize the activated auto-kinase autophosphorylated at Thr^{402} but not the non-phosphorylated/inactive auto-kinase. Immunoblot analysis with this phospho-specific antibody further revealed that the change in phosphorylation level of Thr^{402} of auto-kinase was well correlated with the activity change of the kinase during both autophosphorylation/activation and protein phosphatase-mediated dephosphorylation/inactivation processes. Taken together, our results identify Thr^{402} as the regulatory autophosphorylation site of auto-kinase, which is a C-terminal catalytic fragment of PAK2.

INTRODUCTION

Autophosphorylation-dependent protein kinase (auto-kinase) was identified in pig brain and liver by Yang et al. [1,2] with the unique property that its activity is regulated by autophosphorylation. It is a cyclic nucleotide- and Ca^{2+}-independent protein serine/threonine kinase, and its apparent molecular mass is approx. 36 kDa by SDS/PAGE. The kinase is inactive as isolated but can be markedly activated by autophosphorylation in the presence of $MgATP^{2-}$. The autophosphorylated/activated auto-kinase can be further dephosphorylated and inactivated by treatment with protein phosphatase [1,2]. The activated auto-kinase can phosphorylate several proteins and enzymes *in vitro*, including myelin basic protein, histones, glycogen synthase, phosphorylase *b* kinase and intermediate filament protein vimentin [1–5]. Phosphorylation of glycogen synthase and phosphorylase *b* kinase by auto-kinase causes a profound activity change in these two enzymes [2,4,5]. As well as in pig brain and liver, a similar enzyme was also identified in bovine kidney by Guo et al. [6,7], which can phosphorylate and inactivate protein phosphatase 2A *in vitro*. Moreover the substrate consensus sequence motif for auto-kinase has been determined as Arg-X-(X)-Ser*/Thr*-X_3-Ser/Thr (where * is the kinase target site) [8], providing an important helpful clue in the further study of the biological function of the kinase.

During the past decade, several protein kinases whose activities have also been reported to be regulated by autophosphorylation have been discovered, such as a human placenta S6/H4 kinase [9], an interferon-induced RNA-dependent protein kinase [10] and a family of p21-activated kinases (PAKs) whose activities are dependent on GTP-binding protein Rac or Cdc42 [11–13]. Because the gene encoding auto-kinase has not yet been cloned, the interesting question of whether auto-kinase represents a newly described kinase or has any relationship to the other autophosphorylation-regulated kinases remains to be addressed. Furthermore the autophosphorylation sites of auto-kinase, which have a critical role in regulating its activity, have not yet been identified.

In the present study we have analysed the partial amino acid sequences of purified pig liver auto-kinase and found that all the partial sequences are either analogous to or 100% identical with the sequence of PAK2. We show that auto-kinase can be recognized by an antibody specific for PAK2 and is in fact a catalytic fragment of PAK2. Furthermore the autophosphorylation site responsible for the activation of auto-kinase has been identified as Thr^{402} (under the sequence numbering of human PAK2) by phosphoamino acid analysis, manual Edman degradation and phosphopeptide mapping with the help of phosphorylation-site analysis of a synthetic peptide corresponding to the sequence of PAK2 from residues 396 to 418. The regulatory role of Thr^{402} phosphorylation during both activation and inactivation of auto-kinase has also been evaluated by immuno-

Abbreviations used: auto-kinase, autophosphorylation-dependent protein kinase; GSK-3, glycogen synthase kinase 3; PAK, p21-activated kinase; PKA, cAMP-dependent protein kinase; Tos-Phe-CH_2Cl; 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one.

[1] To whom correspondence should be addressed (e-mail yusong@cguaplo.cgu.edu.tw).

blot analysis with a phospho-specific antibody against the identified phosphorylation-site sequence.

EXPERIMENTAL

Materials

[γ-^{32}P]ATP was purchased from Amersham. 1-Chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one (Tos-Phe-CH_2Cl)-treated trypsin, V8 protease, endoproteinase Lys-C and goat anti-(rabbit IgG) antibody conjugated with alkaline phosphatase were from Sigma. Anti-αPAK (C19) antibody was from Santa Cruz Biotechnology. Cellulose-coated TLC plates and trifluoroacetic acid were from Merck. Alkaline phosphatase conjugate substrate kit was from Bio-Rad. Syntide-3 peptide (RPRPAS-VPPSPSLSRHA) was synthesized with an automated Applied Biosystems peptide synthesizer 430A and purified on a preparative C_{18} reverse-phase HPLC column. STM-23 peptide (EQSKRSTMVGTPYWMAPEVVTRK) and phospho-STM-11-C peptide [SKRST(*P*)MVGTPYC] were synthesized by Chiron Technologies. Bicinchoninic acid protein assay reagent was from Pierce. PVDF membrane, Edman degradation reaction (Sequelon-AA) membrane, phenyl isothiocyanate and coupling buffer were from Millipore. Okadaic acid and CDP-Star® (a chemiluminescent substrate for alkaline phosphatase) were from Boehringer Mannheim. Molecular mass marker proteins (Mark12) were from Novel Experimental Technology. CNBr-activated Sepharose 4B and Protein A–Sepharose CL-4B were from Pharmacia.

Purification of enzymes and proteins

The inactive form of auto-kinase was purified to apparent homogeneity from pig liver by the procedure described by Yang et al. [1]. The active form of auto-kinase was isolated from pig liver basically as described by Yang et al. [1] with some modifications. First, the assay of auto-kinase during purification was performed with syntide-3 peptide instead of myelin basic protein, as a more specific substrate as previously described [8]. Secondly, syntide-3 was coupled to Sepharose 4B, and this coupled gel was used as the final step of auto-kinase purification. In brief, the inactive form of auto-kinase eluted from a phosvitin–Sepharose 4B column was concentrated to approx. 2–3 ml, diluted 10-fold with buffer A [20 mM Tris/HCl (pH 7.0)/15 mM 2-mercaptoethanol/4 mM EDTA/0.5 mM benzamidine/0.1 mM 7-amino-1-chloro-3-L-tosylamidoheptan-2-one/0.1 mM Tos-Phe-CH_2Cl] and then absorbed on a histone–Sepharose 4B column (1 cm × 8 cm) pre-equilibrated in buffer A. The column was washed with 50 ml buffer A and then eluted with a 150 ml linear salt gradient from 0 to 0.4 M NaCl in buffer A. The fractions containing auto-kinase were pooled, concentrated to approx. 1 ml and incubated with 0.2 mM ATP/20 mM Mg^{2+} at 30 °C for 20 min to activate the kinase fully. After activation, the reaction mixture was diluted 1:20 with buffer A and then reapplied to a second histone–Sepharose 4B column (1 cm × 8 cm). The column was washed with 50 ml of buffer A containing 0.2 M NaCl and then eluted with a 150 ml linear salt gradient from 0.2 to 0.6 M NaCl in buffer A. The active fractions were pooled and directly adsorbed on a syntide-3–Sepharose 4B column (1 cm × 5 cm) pre-equilibrated in buffer A. The column was washed with 50 ml of buffer A containing 0.5 M NaCl and then eluted with a 100 ml linear salt gradient from 0.5 to 1.5 M NaCl in buffer A. The active auto-kinase, eluted at approx. 1.0 M NaCl as a symmetrical peak, was collected, concentrated to approx. 1 ml, dialysed against 20 mM Tris/HCl (pH 7.0)/0.5 mM dithiothreitol containing 50 % (v/v) glycerol, and stored at −30 °C for further experiments. The purified auto-kinase was apparently homogeneous and gave a single major protein band at a molecular mass of approx. 36 kDa when analysed by SDS/PAGE. Analysis of the radioactively autophosphorylated kinase on the autoradiogram also revealed a single major phosphorylated protein band at a molecular mass of approx. 36 kDa. Approximately 20 μg of purified auto-kinase could be obtained from 600 g of pig liver, with a specific activity of approx. 1500 units/mg of protein. Myelin basic protein was purified from pig brain [14]. The catalytic subunit of cAMP-dependent protein kinase (PKA) [15] was purified from bovine heart. Protein kinase C [16], kinase F_A/glycogen synthase kinase 3α (GSK-3α) [17] and casein kinase 2 [18] were purified from pig brain. The catalytic subunit of protein phosphatase 2A was isolated from rabbit skeletal muscle [19].

Enzyme assays

The activity of auto-kinase was determined as described by Yang et al. [1,2] with syntide-3 (0.5 mM) as substrate. One unit of auto-kinase is defined as the amount of enzyme catalysing the incorporation of 1 nmol of phosphate/min into syntide-3 at 30 °C. Activities of the catalytic subunit of PKA and kinase F_A/GSK-3α were assayed in buffer B [20 mM Tris/HCl (pH 7.0)/0.5 mM dithiothreitol] containing 0.2 mM [γ-^{32}P]ATP and 20 mM Mg^{2+} ions at 30 °C for 10 min, with myelin basic protein (1 mg/ml) as substrate. Protein kinase C was assayed in buffer B containing 1 mM Ca^{2+} ions, 20 μg/ml phosphatidylserine, 0.2 mM [γ-^{32}P]ATP and 20 mM Mg^{2+} ions at 30 °C for 10 min, with histone III-S (1 mg/ml) as substrate. Casein kinase 2 was assayed in buffer B containing 0.2 M KCl, 0.2 mM [γ-^{32}P]ATP and 20 mM Mg^{2+} ions at 30 °C for 10 min, with the peptide RRRDDDSDDD (0.25 mM) as substrate, which was synthesized by Genosys Biotechnologies. One unit of each kinase is defined as the amount of enzyme catalysing the incorporation of 1 nmol of phosphate/min into substrate at 30 °C.

Peptide sequence analysis of auto-kinase

To obtain the N-terminal sequence of auto-kinase, the purified kinase was subjected to SDS/PAGE [10 % (w/v) gel], electrotransferred to PVDF membrane and stained with Amido Black; the stained protein band was then cut out for sequence analysis on an Applied Biosystems automated sequencer 473A. The sequence was determined as VDGGAKTSDKQKKKAXM-TDE. To obtain the internal peptide sequences of auto-kinase, peptides of auto-kinase were generated by chemical cleavage with CNBr or partial digestion by V8 protease. For CNBr cleavage *in situ*, auto-kinase was subjected to SDS/PAGE [10 % (w/v) gel], electrotransferred to PVDF membrane and stained with Amido Black. The stained protein band of auto-kinase was cut out and incubated with 150 μl of 70 % (v/v) formic acid containing 50 mg/ml CNBr at 37 °C for 36 h. The peptides released from the membrane were dried with a Speed-Vac concentrator and separated by 16.5 % (w/v) Tricine/SDS/PAGE [20]. The peptides were electrotransferred to PVDF membrane, stained with Amido Black and subjected to amino acid sequence analysis. The N-terminal sequence of one of the CNBr-cleaved peptides was determined as EKLRTIV. For partial digestion by V8 protease, purified auto-kinase was subjected to SDS/PAGE [10 % (w/v) gel] and stained with 4 M sodium acetate [21]. The kinase was cut out and partly digested by V8 protease in stacking gel followed by electrophoresis on a 17.5 % (w/v) SDS gel by the method of Cleveland et al. [22]. The peptides were electrotransferred to PVDF membrane, stained with Amido Black and analysed by automated amino acid sequencer. The N-terminal

sequence of one of the V8 protease-cleaved peptides was determined as LQNPEK/ILTP/FI.

Production of antibodies

The anti-PAK2 (C15) antibody was produced in rabbits by using the peptide TPLIMAAKEAMKSNR, corresponding to the C-terminal regions from residues 510 to 524 of the sequence of human and rabbit PAK2 [12,23] as the antigen. This peptide was synthesized by Genosys Biotechnologies. A cysteine residue was added to the N-terminus to facilitate coupling of the peptide to keyhole limpet haemocyanin by the procedure described by Reichlin [24], with glutaraldehyde as the cross-linker. The methods used for production and affinity purification of the anti-peptide antibody were detailed previously [25,26]. To produce the phospho-specific antibody against the phosphorylated/activated auto-kinase, the phospho-STM-11-C peptide SKRST(*P*)MVG-TPYC was coupled to keyhole limpet haemocyanin and used as the antigen to antibodies raised in rabbits as described above. The antiserum (10 ml) was first absorbed twice to an STM-23 peptide-coupled Sepharose 4B column (1 cm × 5 cm) to remove antibodies independent of the phosphorylation state of auto-kinase. Specific antibody against the phospho-STM-11-C peptide was then affinity-purified from the flow-through fractions by the phospho-STM-11-C peptide-coupled Sepharose 4B column (1 cm × 5 cm). The bound antibody was eluted from the column with 20 ml of 100 mM glycine at pH 2.5, and the eluted fractions (1 ml per tube) were collected in tubes containing 0.1 ml of 1 M Tris/HCl, pH 8.0. The purified antibody was concentrated to approx. 1 ml, dialysed against PBS containing 50 % (v/v) glycerol and 0.04 % NaN_3, then stored at −30 °C. This purified antibody can specifically recognize the phosphorylated/activated but not the non-phosphorylated/inactive auto-kinase on immunoblotting (see the Results section).

Immunoblots

Immunoblotting was performed essentially as described previously [25,26]. Affinity-purified anti-PAK2 antibody (1 μg/ml), commercial α-PAK (C19) antibody (0.2 μg/ml), or anti-(phospho-STM-11-C) antibody (1 μg/ml) was used to immunoblot proteins transferred from SDS gel to PVDF membrane. The proteins of interest were detected by using goat anti-(rabbit IgG) antibody conjugated with alkaline phosphatase and the alkaline phosphatase substrate kit or CDP-Star® (a chemiluminescent substrate for alkaline phosphatase) in accordance with the procedure provided by the manufacturer. For reprobing the same membrane by another kind of antibody, the blotted membrane was first incubated with 200 ml of stripping buffer [62.5 mM Tris/HCl (pH 6.7)/100 mM 2-mercaptoethanol/2 % (w/v) SDS] at 50 °C for 30 min, with occasional agitation to strip off the bound antibodies. After being washed three times in TTBS buffer [20 mM Tris/HCl (pH 7.4)/0.5 M NaCl/0.05 % (v/v) Tween 20], the stripped membrane was reprobed with another kind of antibody.

Two-dimensional phosphopeptide mapping, phosphoamino acid analysis and determination of phosphorylation site

To obtain the tryptic peptides from ^{32}P-labelled auto-kinase, the method of protease digestion of membrane-bound protein *in situ* [27] was used. In brief, ^{32}P-labelled auto-kinase was subjected to SDS/PAGE [10 % (w/v) gel] and electrotransferred to PVDF membrane. The bands of auto-kinase detected by autoradiography were cut out and incubated with 0.5 % poly-(vinylpyrrolidone) in 100 mM acetic acid for 1 h to block the free protein-binding sites. Membrane-bound proteins were digested by 10 μg of Tos-Phe-CH_2Cl-treated trypsin in 50 mM NH_4HCO_3 buffer at pH 8.0 for 48 h at 37 °C. Occasionally the membrane-bound proteins were subjected to oxidation by incubation in 100 μl of performic acid at 0 °C for 60 min before digestion with protease. The oxidized proteins were dried with a Speed-Vac concentrator (Savant), resuspended in 50 mM NH_4HCO_3 buffer, pH 8.0, and digested with trypsin as described above. After digestion, the reaction mixture was concentrated to dryness with a Speed-Vac concentrator and analysed by two-dimensional peptide mapping on cellulose-coated TLC plates [28]. The phosphopeptides detected by autoradiography were then scraped individually off the TLC plates, extracted with extraction solution [13.5 % (v/v) NH_4OH/10 % (v/v) propan-1-ol in water] and centrifuged at 100000 ***g*** for 10 min. The supernatants were removed and the pellets were extracted twice as described above. The extracted phosphopeptides were dried with a Speed-Vac concentrator and subjected to further analysis (see below).

Two-dimensional phosphoamino acid analysis was performed by the method described by Kamps and Sefton [29]. The positions of phosphoamino acids in plates were localized by ninhydrin staining of standards run in parallel. The dried plates were exposed to X-ray films for autoradiography or to an imaging plate of the Bio Imaging Analyser (Fuji Photo Film).

The phosphorylation site of each phosphopeptide was determined by sequential manual Edman degradation, essentially by the method of Laursen and Machleidt [30]. Briefly, ^{32}P-labelled peptide was coupled to Sequelon-AA membrane with carbodi-imide at room temperature for 20 min and washed with methanol and deionized water. The washed membrane was incubated with 50 μl of coupling buffer for 5 s followed by incubation with 50 μl of phenyl isothiocyanate at 56 °C for 17.5 min. The protected N-terminal amino acid was then cleaved from the membrane-linked peptide by incubation with 80 μl of trifluoroacetic acid at 56 °C for 10 min. The trifluoroacetic acid-treated membrane was removed, washed with 50 % (v/v) methanol and subjected to the next reaction cycle as described above. The radioactivity of the trifluoroacetic acid extracts from each reaction cycle was analysed by a liquid-scintillation counter for determination of the phosphorylation site.

Cell culture

The human hepatoma G2 cells were cultured at 37 °C in a water-saturated air/CO_2 (19:1) atmosphere in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 100 i.u./ml penicillin and 100 μg/ml streptomycin. Preparation of cell extracts was performed as described previously [25,26].

Analytical methods

Protein concentration was determined by using the bicinchoninic acid protein assay reagent from Pierce. SDS/PAGE was performed by the method of Laemmli [31]. Densitometric analysis was performed with a Computing Densitometer (Molecular Dynamics).

RESULTS

Partial amino acid sequence analysis of auto-kinase purified from pig liver

The N-terminus of auto-kinase purified from pig liver was not blocked and a 20-residue sequence (VDGGAKTSDKQKKK-AXMTDE) could be obtained by direct N-terminal sequencing

A.

```
  1 MSDNGELEDKPPAPPVRMSSTIFSTGGKDPLSANHSLKPLPSVPEEKKPR 50
 51 HKIISIFSGTEKGSKKKEKERPEISPPSDFEHTIHVGFDAVTGEFTGMPE 100
101 QWARLLQTSNITKLEQKKNPQAVLDVLKFYDSNTVKQKYLSFTPPEKDGF 150
151 PSGTPALNAKGTEAPAVVTEEEDDDEETAPPVIAPRPDHTKSIYTRSVID 200
201 PVPAPVGDSHVDGAAKSLDKQKKKPKMTDEEIMEKLRTIVSIGDPKKKYT 250
             VDGGAKTSDKQKKKAXMTDE    EKLRTIV
                I                II                III
251 RYEKIGQGASGTVFTATDVALGQEVAIKQINLQKQPKKELIINEILVMKE 300
         IV                V
301 LKNPNIVNFLDSYLVGDELFVVMEYLAGGSLTDVVTETCMDEAQIAAVCR 350
        VI                        VII
351 ECLQALEFLHANQVIHRDIKSDNVLLGMEGSVKLTDFGFCAQITPEQSKR 400
      VIII                    IX
401 STMVGTPYWMAPEVVTRKAYGPKVDIWSLGIMAIEMVEGEPPYLNENPLR 450
          X                XI
451 ALYLIATNGTPELQNPEKLSPIFRDFLNRCLEMDVEKRGSAKELLQHPFL 500
               LQNPEKLTPI
                  I  F
501 KLAKPLSSLTPLIMAAKEAMKSNR 524
```

B.

```
                          206                                  230
Rat      γPAK      VGDSHVDSGA..............KSSDKQKKKAKMTDE
Rabbit   PAKI      VGDSHVDSGA..............KSSDKQKKKTKMTDE
Human    hPAK65    VGDSHVDSGA..............KSSDKQKKKTKMTDE
Human    PAK2      VGDSHVDGAA..............KSLDKQKKKPKMTDE

Rat      βPAK      AAPNKEATPPSAENANSS...TLYRNTDRQRKKSKMTDE
Mouse    PAK3      AAPNKEDIPPSAENANST...TLYRNTDRQRKKSKMTDE

Rat      αPAK      PTRDVATSPISPTENNTTPPDALTRNTEKQKKKPKMSDE
Human    PAK1      PTRDVATSPISPTENNTTPPDALTRNTEKQKKKPKMSDE

Pig      AK             VDGGA..............KTSDKQKKKAXMTDE
```

Figure 1 Comparison of the peptide sequences of pig liver auto-kinase with the deduced amino acid sequence of human PAK2

(**A**) The three partial sequences of pig liver auto-kinase (AK) were aligned with the deduced amino acid sequence of human PAK2. Protein sequences are presented in single-letter code. The residues identical between the two protein sequences are underlined. Roman numbers above the sequence indicate the positions of the 11 conserved kinase subdomains. (**B**) The N-terminal sequence of auto-kinase was aligned with the deduced amino acid sequences of PAK isoforms from various species. The residues identical between these protein sequences are underlined.

of the purified kinase. To obtain information about the internal sequences of auto-kinase, the purified kinase was cleaved by CNBr or V8 protease and peptides were separated, isolated and partly sequenced as described in the Experimental section. Two partial sequences, EKLRTIV and LQNPEK/ILTP/FI, were obtained from CNBr- and V8 protease-cleaved peptides respectively. When we searched in sequence data banks, it was surprising to find that the three sequences are closely related or 100% identical with those of a member of the $p21^{cdc42/rac1}$-activated kinase (PAK) family, PAK2 (also termed hPAK65, γPAK or PAKI) [12,23,32,33,33a] (Figure 1A), indicating that auto-kinase purified from pig liver belongs to a member of the PAK family. Moreover, because the N-terminal sequence of auto-kinase is closely related to a fragment of peptide sequence of PAK2, which locates nearly at the central region of the PAK2 molecule from residues Val^{211} to Glu^{230} (Figures 1A and 1B), it seems likely that auto-kinase is a proteolytic fragment of PAK2.

Auto-kinase can be recognized by antibodies against PAK2

To further elucidate the relationship between auto-kinase and PAK, purified auto-kinase was subjected to immunoblotting by a commercial antibody raised against the C-terminal peptide of αPAK (also termed PAK1) [11], which also cross-reacts with βPAK (also termed PAK3) [34] and PAK2 respectively. As shown in Figure 2(A), purified auto-kinase can be recognized by this antibody on an immunoblot. Because this antibody cannot differentiate between PAK isoforms, we tried to produce an antibody specific for PAK2. An antibody against the 15-residue C-terminal peptide of human PAK2 was raised in rabbits and affinity-purified as described in the Experimental section. When extracts of human hepatoma G2 cells were immunoblotted with this anti-PAK2 (C15) antibody, a predicted 62 kDa protein band corresponding to PAK2 could be detected; detection of this protein band could be blocked by inclusion of the 15-residue C-terminal peptide of PAK2 during immunoblotting (Figure 2B, lanes 2 and 3). Although all of the three PAK isoforms can be recognized by the commercial antibody against PAK1, the anti-PAK2 (C15) antibody produced here has no obvious cross-reactivity to PAK1 (68 kDa) or PAK3 (65 kDa) as evidenced by immunoblotting (Figure 2B, lanes 1 and 2), demonstrating that the anti-PAK2 (C15) antibody can be used to immunoblot PAK2 specifically in hepatoma cell extracts.

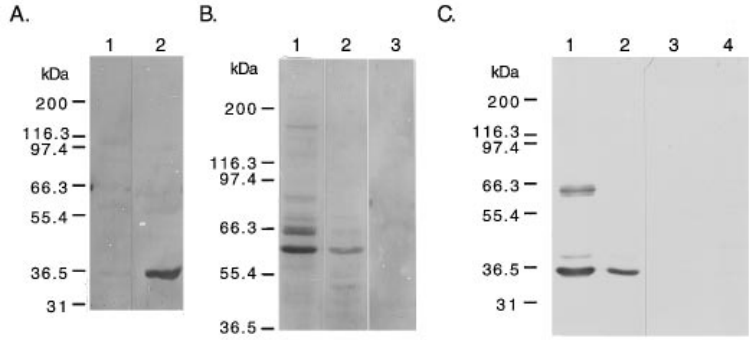

Figure 2 Immunoblot analysis of auto-kinase by using antibodies against PAK

(**A**) Purified auto-kinase was resolved by SDS/PAGE [10% (w/v) gel] and electroblotted on PVDF membrane. The membrane was then probed with preimmue rabbit serum (lane 1) or the commercial antibody against αPAK (C19) (lane 2). (**B**) Extracts (80 μg of protein) of human hepatoma G2 cells were resolved by SDS/PAGE [7.5% (w/v) gel] and electroblotted on PVDF membrane. The membrane was then probed with the commercial antibody against αPAK (C19) (lane 1) or the anti-PAK2 (C15) antibody in the absence (lane 2) or presence (lane 3) of the 15-residue C-terminal peptide of PAK2 (50 μg/ml). (**C**) Partly (lanes 1 and 3) and highly (lanes 2 and 4) purified auto-kinase were resolved by SDS/PAGE [10% (w/v) gel] and electroblotted on PVDF membrane. The membrane was then probed with the anti-PAK2 (C15) antibody in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of the 15-residue C-terminal peptide of PAK2 (50 μg/ml).

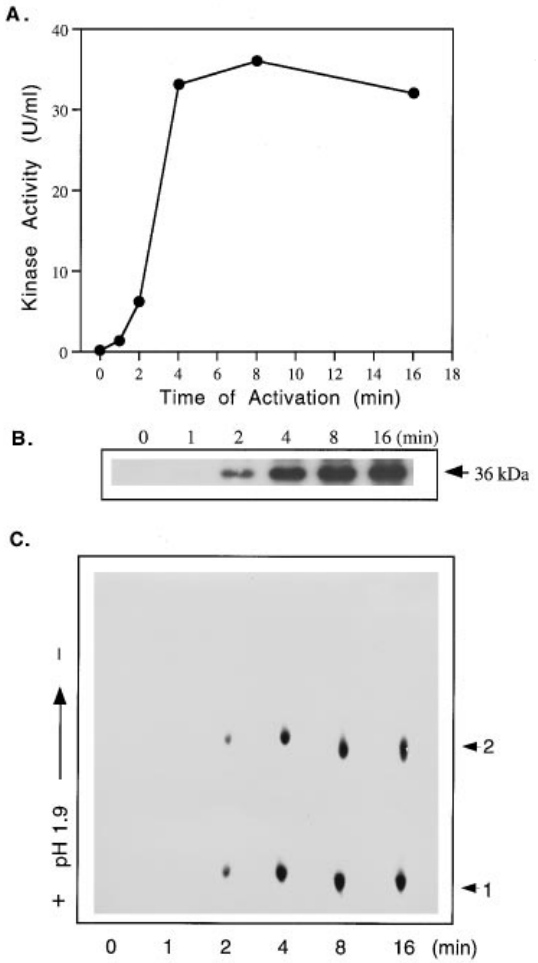

Figure 3 Tryptic phosphopeptide map analysis of auto-kinase during the autophosphorylation–activation process

Purified inactive auto-kinase (0.36 μg) was incubated in 30 μl buffer containing 20 mM Tris/HCl, pH 7.0, 0.5 mM dithiothreitol, 0.2 mM [γ-^{32}P]ATP and 20 mM Mg^{2+} ions at 30 °C for various durations as indicated. At each time point, aliquots of the reaction mixture were removed for kinase activity assay (**A**) or subjected to SDS/PAGE [10% (w/v) gel] and electroblotted on PVDF membrane followed by autoradiography (**B**). The position of the ^{32}P-labelled auto-kinase (36 kDa) is indicated by an arrow. Bands of ^{32}P-labelled auto-kinase were excised, digested exhaustively by trypsin, and subjected to one-dimensional phosphopeptide map analysis (**C**) as described in the Experimental section.

We further examined whether purified auto-kinase can be recognized by the anti-PAK2 (C15) antibody by immunoblotting. Indeed, in our hands a 36 kDa blotted band could be detected unequivocally in all the preparations of purified auto-kinase by this antibody, and inclusion of the 15-residue C-terminal peptide of PAK2 could prevent this detection. Two representative cases of this experiment are shown in Figure 2(C). In the preparation of partly purified auto-kinase, an additional blotted band at approx. 62 kDa could also be observed (Figure 2C, lane 1). It is highly possible that this 62 kDa band represents the intact form of PAK2, which is co-purified with auto-kinase.

Tryptic phosphopeptide analysis of auto-kinase during the autophosphorylation–activation process

To study the autophosphorylation site(s) of auto-kinase responsible for activation of the kinase, purified inactive auto-kinase was subjected to autophosphorylation in the presence of [γ-^{32}P]ATP. At various time points the reaction products were removed for kinase assay and for tryptic phosphopeptide map analysis of the ^{32}P-labelled auto-kinase. In agreement with previous reports [1,2], the inactive auto-kinase could be markedly activated to maximal level of activity within 4 min in the presence of $MgATP^{2-}$ (Figure 3A) and the increase in ^{32}P incorporation of auto-kinase paralleled directly the activation of the kinase (Figure 3B). Analysis of the tryptic phosphopeptides derived from the ^{32}P-labelled auto-kinase by one-dimensional high-voltage thin-layer electrophoresis revealed that two major ^{32}P-labelled peptide spots (1 and 2) appeared, in good correlation with the activity increase of auto-kinase during autophosphorylation (Figure 3C), indicating that the phosphorylation sites in the two peptides are responsible for the activation of auto-kinase. When the tryptic phosphopeptides derived from the kinase autophosphorylated at 4 min were further examined by two-dimensional peptide map analysis, in addition to spots 1 and 2, two extra phosphopeptide spots (3 and 4) were also detected (Figure 4A). It seemed that phosphopeptides 3 and 4 arose from 'spot doubling' of phosphopeptides 1 and 2 respectively because the mobility of phosphopeptides 3 and 4 on the TLC plate during electrophoresis was identical with that of phosphopeptides 1 and 2 respectively (Figure 4A). The possibility that 'spot doubling' of phosphopeptides 1 and 2 might have been due to different oxidation states of the two phosphopeptides was excluded by the observation that oxidation of the autophosphorylated auto-kinase immobilized on PVDF membrane by performic acid as described in the Experimental section before digestion with trypsin did not alter significantly the pattern of the phosphopeptide map (results not shown). To characterize the four phosphopeptides further, they were extracted from the TLC plates and subjected to phosphoamino acid analysis, phosphorylation site determination and amino acid sequence analysis. As shown in Figure 4(B), all four peptides were phosphorylated on the threonine residue. By sequential manual Edman degradation analysis of each phosphopeptide, the phosphorylation residue was determined to be at cycle 2 for both phosphopeptides 1 and 3 and at cycle 3 for both phosphopeptides 2 and 4 (Figure 4C). As mentioned by Boyle et al. [28], the phenomenon of 'spot doubling' in the chromatographic dimension of two-dimensional peptide map analysis is probably due to different extents of alkylation of a single peptide during the peptide-preparation process caused by the mould-release compound used during the manufacturing of the microcentrifuge tubes. On the basis of these observations, it is concluded that phosphopeptides 1 and 3 are derivatives of a single phosphopeptide, and phosphopeptides 2 and 4 are also derivatives of another single phosphopeptide. In contrast, the attempt to obtain the amino acid sequences of these extracted phosphopeptides was unsuccessful owing to the insufficient amount of peptides recovered from TLC plates.

Peptide synthesized to match a fragment of PAK2 sequence can serve as a specific substrate for auto-kinase

Previously the substrate consensus sequence motif for auto-kinase was determined as -Arg-Xaa-(Xaa)-Ser/Thr-Xaa_3-Ser/Thr-, where the first Ser or Thr residue in this sequence is the phosphorylation site [8]. As can be seen in Figure 4(C), the phosphorylation residue of the tryptic phosphopeptides derived from autophosphorylated auto-kinase is at cycle 2 or 3 by manual Edman degradation analysis, supporting the notion that these tryptic phosphopeptides might also possess this consensus sequence motif. Because our results have indicated that auto-kinase is a proteolytic fragment of PAK2 (see Figures 1 and 2),

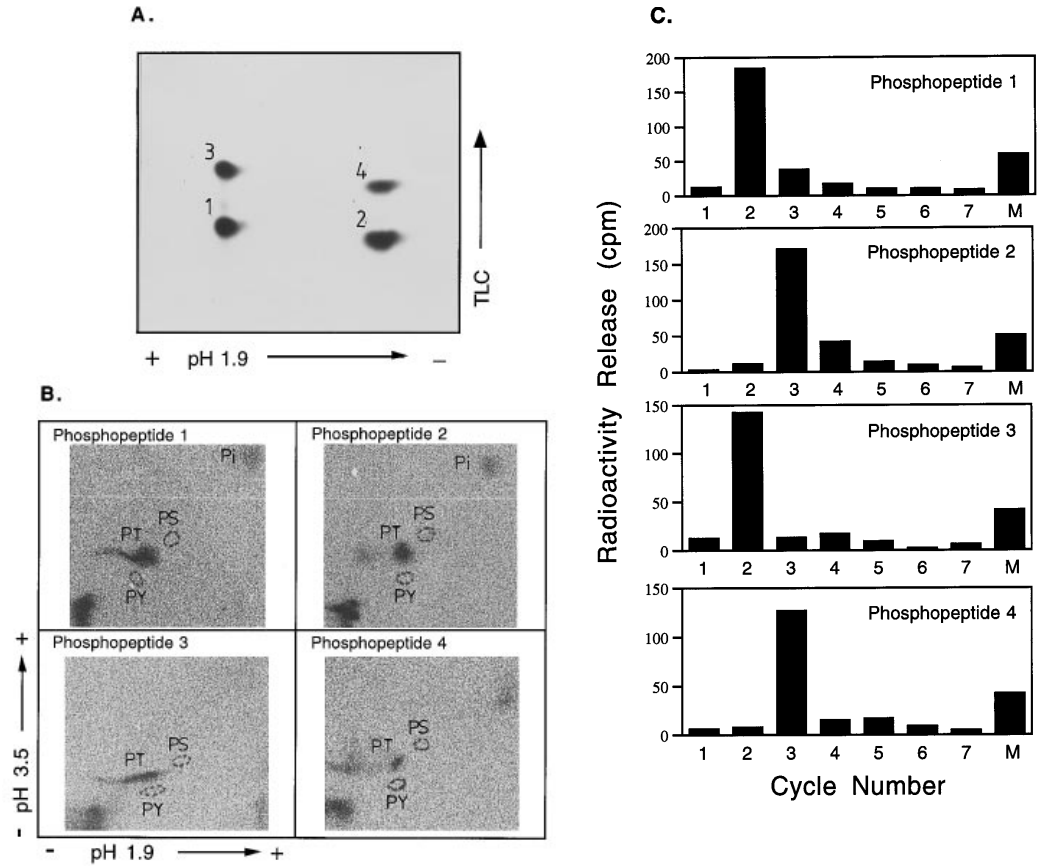

Figure 4 Characterization of the tryptic phosphopeptides derived from autophosphorylated auto-kinase

(**A**) The band of ^{32}P-labelled auto-kinase autophosphorylated at 4 min as depicted in Figure 3(B) was excised and exhaustively digested by 10 μg of trypsin at 37 °C for 48 h. The resulting tryptic phosphopeptides were subjected to two-dimensional phosphopeptide map analysis on a TLC plate followed by autoradiography as described in the Experimental section. (**B**) The tryptic phosphopeptides detected in (**A**) were extracted from TLC plate, respectively, and further submitted to phosphoamino acid analysis on a TLC plate as described in the Experimental section. The radioactive signals on the TLC plate were detected by an Imaging Plate of the Bio Imaging Analyser. The symbols used are: Pi, inorganic phosphate; PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine. (**C**) The tryptic phosphopeptides extracted from TLC plate as described in (**B**) were coupled individually to Sequelon-AA membranes and analysed further by manual Edman degradation as described in the Experimental section. Radioactivity released at each reaction cycle was determined by Čerenkov counting in a β-counter. Column M denotes the radioactivity remaining on the reaction membrane after seven cycles of reaction.

we turned to a search of the amino acid sequence of PAK2 that might have this consensus sequence motif for auto-kinase and found that a fragment of PAK2 sequence, RSTMVGT, from residues 400 to 406 completely fits the characteristic of this sequence motif (see Figure 1). A peptide, EQSKRSTMVGTP-YWMAPEVVTRK (STM-23 peptide), corresponding to the sequence of PAK2 from residues 396 to 418, was therefore synthesized and used as a substrate to test whether it could be phosphorylated by auto-kinase. As predicted, STM-23 peptide could be phosphorylated by auto-kinase (Figure 5A). When tested with several other serine/threonine kinases including PKA, protein kinase C, casein kinase 2 and glycogen synthase kinase 3α, it was found that, out of all the kinases tested, auto-kinase seemed to be the only one that could act significantly on STM-23 peptide (Figure 5B), indicating that STM-23 peptide is a specific substrate for auto-kinase. The result also implies that the phosphorylation site(s) of STM-23 peptide by auto-kinase could be the autophosphorylation site(s) of auto-kinase.

Phosphoamino acid analysis showed further that the threonine residue was phosphorylated in STM-23 peptide by auto-kinase (Figure 5C). Because there are three threonine residues in STM-23 peptide, manual Edman degradation of [^{32}P]STM-23 peptide was therefore performed to determine which of the threonine residues were phosphorylated. Because the N-terminal residue of STM-23 peptide, Glu, can react with the membrane disc used for Edman degradation through its side chain carboxy group, and this would hamper the progress of subsequent degradation cycles, ^{32}P-labelled STM-23 peptide phosphorylated by auto-kinase was first digested by endopeptidase Lys-C to remove its N-terminal EQSK fragment and then subjected to phosphorylation-site determination. As shown in Figure 5(D), we identified that of the three threonine residues in STM-23 peptide, only the first was phosphorylated by auto-kinase. Thus the phosphorylation-site sequence of STM-23 peptide catalysed by auto-kinase was determined as EQSKRST(*P*)MVGTPYWMAPEVVTRK.

Identification of the autophosphorylation site of auto-kinase

When the tryptic phosphopeptides derived from STM-23 peptide phosphorylated by auto-kinase were subjected to two-dimensional peptide map analysis, four phosphopeptide spots (spots a-d) were detected (Figure 6A). The phosphopeptide spots c and d seemed to arise from 'spot doubling' of the phosphopeptide spots a and b respectively. Manual Edman degradation

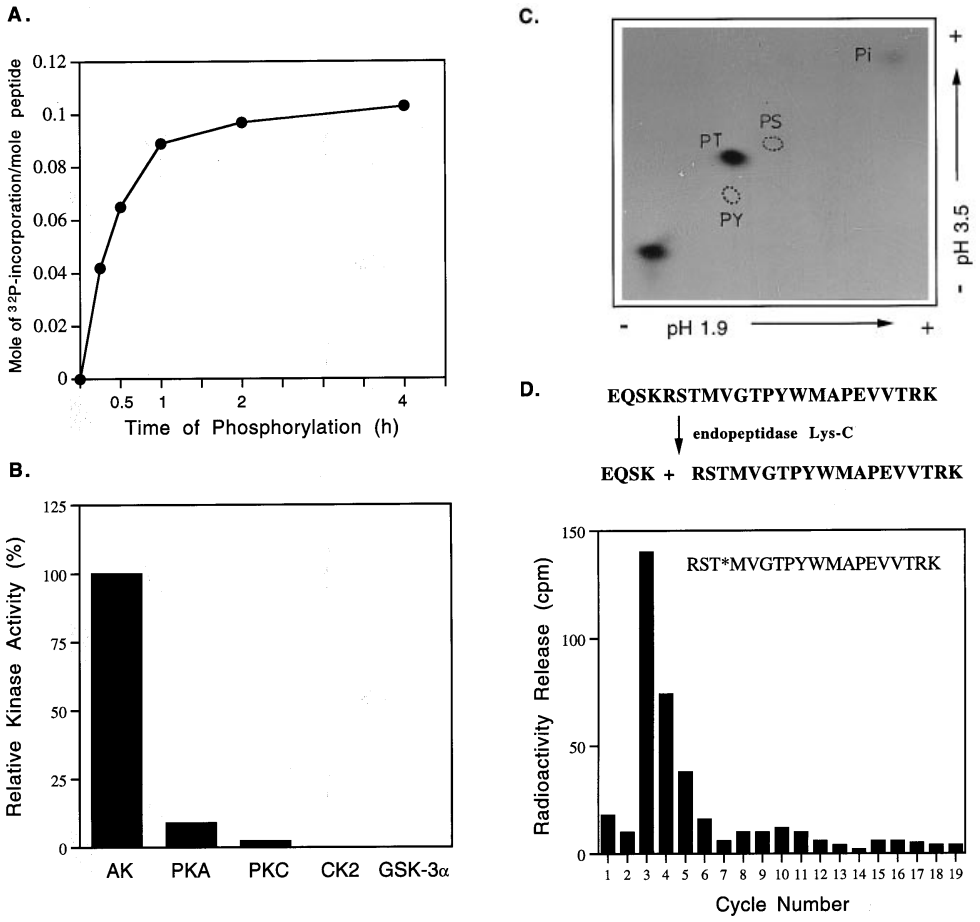

Figure 5 Specific phosphorylation of STM-23 peptide by auto-kinase and identification of the phosphorylation site in STM-23 peptide

(**A**) Phosphorylation of STM-23 peptide by auto-kinase. STM-23 peptide (120 μM) was phosphorylated by active auto-kinase (0.2 μg/ml) in buffer containing 20 mM Tris/HCl, pH 7.0, 0.5 mM dithiothreitol, 0.2 mM [γ-^{32}P]ATP and 20 mM Mg^{2+} ions at 30 °C for various durations as indicated. The incorporation of ^{32}P into STM-23 peptide was then determined as described in the Experimental section. (**B**) Specific phosphorylation of STM-23 peptide by auto-kinase. STM-23 peptide (120 μM) was phosphorylated by various protein kinases as indicated at 30 °C for 10 min under the conditions described in the Experimental section. The amount of each kinase used in this assay was as follows: 80 m-units of auto-kinase (AK), 120 m-units of the catalytic subunit of cAMP-dependent protein kinase (PKA), 90 m-units of Ca^{2+}/phospholipid-dependent protein kinase (PKC), 16 m-units of casein kinase 2 (CK2) and 27 m-units of GSK-3α. The activity of auto-kinase was taken as 100%. (**C**) Phosphoamino acid analysis of STM-23 peptide phosphorylated by auto-kinase. STM-23 peptide was ^{32}P-phosphorylated by auto-kinase at 30 °C for 10 min. STM-23 peptide labelled with ^{32}P was separated from auto-kinase by 16.5% (w/v) Tricine/SDS/PAGE. After electroblotting to PVDF membrane, the band of ^{32}P-labelled STM-23 peptide was excised and subjected to phosphoamino acid analysis as described in the Experimental section. The symbols used are the same as those used in Figure 4(B). (**D**) Manual Edman degradation analysis of STM-23 peptide phosphorylated by auto-kinase. The excised band of ^{32}P-labelled STM-23 peptide as described in (**C**) was cut into small pieces and incubated in 100 mM acetic acid containing 0.5% poly(vinylpyrrolidone) at 37 °C for 1 h. After washing several times with deionized water, the membrane-bound ^{32}P-labelled STM-23 peptide was then digested by 0.3 unit of endopeptidase Lys-C at 37 °C for 24 h in 100 μl buffer containing 50 mM NH_4HCO_3 at pH 8.0. The digested peptides released from membrane were collected, dried and subjected to manual Edman degradation for the determination of phosphorylation residue(s) as described in the Experimental section.

analysis further showed that the phosphorylation residue was at cycles 2 and 3 for phosphopeptide spots a and b respectively (results not shown). On the basis of this observation and the known sequence of STM-23 peptide, it is concluded that the sequences of phosphopeptide spots a and b are ST(*P*)MVGTPYWMAPEVVTR and RST(*P*)MVGTPYWMAPEVVTR respectively. Phosphopeptide b probably resulted from incomplete cleavage of STM-23 peptide by trypsin because it has been reported that trypsin does not efficiently cleave at an Arg residue in the sequence Arg-Xaa-Ser(*P*)/Thr(*P*) [28]. Most importantly, we found that the pattern of the map in Figure 6(A) is similar to that of the tryptic phosphopeptide map derived from autophosphorylated auto-kinase (see Figure 4A). To explore this further, the tryptic phosphopeptides derived from STM-23 peptide phosphorylated by auto-kinase and from autophosphorylated auto-kinase were mixed and then analysed by two-dimensional peptide mapping again. As shown in Figure 6(B), the two sets of tryptic phosphopeptides co-migrated precisely on a TLC plate. The result demonstrates that the tryptic phosphopeptides 1 and 2 derived from autophosphorylated auto-kinase in Figure 4(A) have sequences identical to those of phosphopeptide spots a and b respectively. As only the first threonine residue in STM-23 peptide is phosphorylated by auto-kinase (Figures 5C and 5D), the results taken together provide strong evidence that the regulatory autophosphorylation site sequence of auto-kinase is RST(*P*)MVGTPYWMAPEVVTR. The residue phosphorylated in this sequence is equivalent to Thr^{402} of human PAK2.

To examine further the role of phosphorylation of this identified threonine residue (Thr^{402}) in the activation process of auto-kinase, a phospho-specific antibody raised against a synthetic phosphopeptide SKRST(*P*)MVGTPYC (phospho-STM-11-C) in rabbits, and affinity-purified as described in the

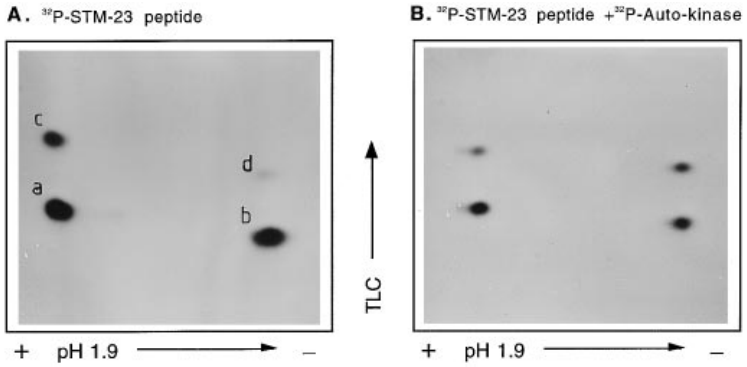

Figure 6 Comparison of the tryptic phosphopeptide pattern derived from ^{32}P-labelled STM-23 peptide with that from ^{32}P-autophosphorylated auto-kinase

The excised bands of ^{32}P-labelled STM-23 peptide and of ^{32}P-autophosphorylated auto-kinase as described in Figures 5(C) and 4(A) were each digested exhaustively by 10 μg of trypsin at 37 °C for 48 h. The tryptic phosphopeptides derived from ^{32}P-labelled STM-23 peptide were mixed without (**A**) or with (**B**) those derived from ^{32}P-autophosphorylated auto-kinase and subjected to two-dimensional phosphopeptide map analysis as described in the Experimental section.

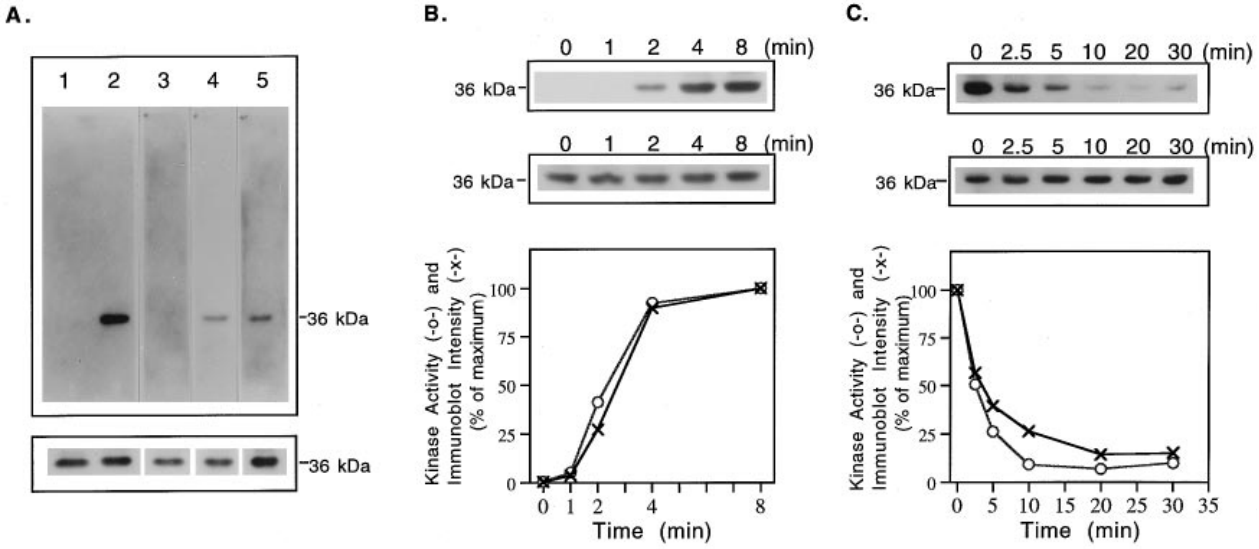

Figure 7 Immunoblot analysis of auto-kinase during the activation and inactivation processes with a phospho-specific antibody against the identified phosphorylation-site sequence

(**A**) Upper panel: purified inactive auto-kinase (0.36 μg) was incubated without (lane 1) or with (lanes 2–5) 0.2 mM ATP and 20 mM Mg^{2+} ions in 30 μl of buffer containing 20 mM Tris/HCl, pH 7.0, and 0.5 mM dithiothreitol at 30 °C for 8 min. The reaction products were resolved by SDS/PAGE [10% (w/v) gel] and electroblotted on PVDF membrane. The membrane was cut into strips, which were then probed with the affinity-purified antibody (1 μg/ml) raised against the phospho-STM-11-C peptide, as described in the Experimental section, without addition (lanes 1 and 2), or in the presence of 50 μg/ml phospho-STM-11-C peptide (lane 3), 50 μg/ml STM-23 peptide (lane 4) or 50 μg/ml phosphothreonine (lane 5). Lower panel: the bound antibodies were stripped from the blotted membrane strips described above by incubating with stripping buffer at 50 °C for 30 min as described in the Experimental section. After stripping, the membrane strips were then reprobed with the anti-PAK2 (C15) antibody to show the equal amount of auto-kinase protein in each lane. The position of auto-kinase (36 kDa) is indicated. (**B**) Purified inactive auto-kinase (0.36 μg) was incubated in 30 μl of buffer containing 20 mM Tris/HCl, pH 7.0, 0.5 mM dithiothreitol, 0.2 mM ATP and 20 mM Mg^{2+} ions at 30 °C for various durations as indicated. At each time point, one-third of the reaction mixture was removed for kinase activity assay (bottom panel, ○). The assay time was 1 min. The other two-thirds of the reaction mixture was mixed with an equal volume of 2% (w/v) SDS sample buffer, subjected to SDS/PAGE [10% (w/v) gel] and electroblotted on PVDF membrane followed by immunoblotting with the antibody raised against the phospho-STM-11-C peptide (top panel). The bound antibodies were stripped from the blotted membrane, which was then reprobed with the anti-PAK2 (C15) antibody (middle panel). The immunoblot intensity of phosphorylated auto-kinase in the top panel was quantified with a computing densitometer, and the result is shown in the bottom panel (×). In the bottom panel, both maximum kinase activity (1500 units/mg of protein) and maximum immunoblot intensity are taken as 100%. (**C**) Purified active auto-kinase (0.24 μg) was incubated with the catalytic subunit of protein phosphatase 2A (5 μg/ml) in 30 μl of buffer containing 20 mM Tris/HCl, pH 7.0, 0.5 mM dithiothreitol, 0.5 mg/ml BSA and 1 mM $MnCl_2$ at 30 °C for various durations as indicated. At each time point, okadaic acid (5 μM) was added to stop the phosphatase action, and one-third of the reaction mixture was removed for kinase activity assay (bottom panel, ○). The assay time was 1 min. The other two-thirds of the reaction mixture was mixed with an equal volume of 2% (w/v) SDS sample buffer, subjected to SDS/PAGE [10% (w/v) gel] and electroblotted on PVDF membrane followed by immunoblotting with the antibody raised against the phospho-STM-11-C peptide (top panel) or with the anti-PAK2 (C15) antibody (middle panel) as described in (**B**). In the bottom panel, both maximum kinase activity (1500 units/mg of protein) and maximum immunoblot intensity of phosphorylated auto-kinase detected in upper panel are taken as 100%.

Experimental section, was used to detect the phosphorylation level of Thr^{402} of auto-kinase during the activation and inactivation processes. This antibody could specifically recognize the autophosphorylated/activated auto-kinase but not the non-phosphorylated/inactive auto-kinase on immunoblotting (Figure 7A, upper panel, lanes 1 and 2). Furthermore this recognition could

be prevented by including the phospho-STM-11-C peptide during immunoblotting but not by including the non-phosphorylated STM-23 peptide or phosphothreonine (Figure 7A, upper panel, lanes 3–5). Note that all lanes in the upper panel of Figure 7(A) contained equal amounts of auto-kinase (Figure 7A, lower panel). The results indicate that the immunospecificity of this antibody is against the whole segment of the phosphopeptide antigen including the phosphate group introduced at Thr^{402}, but not against the peptide backbone or the phosphate group alone. The results clearly demonstrate that Thr^{402} is indeed phosphorylated during activation of auto-kinase. With this phospho-specific antibody as a studying tool, it was further found that the phosphorylation level of Thr^{402} of auto-kinase increased proportionally with the activation of the kinase during autophosphorylation (Figure 7B). Moreover, when the phosphorylated/activated auto-kinase was subjected to dephosphorylation by the catalytic subunit of protein phosphatase 2A, the decrease in the phosphorylation level of Thr^{402} of auto-kinase seemed to match well the inactivation of the kinase (Figure 7C). Taken together, the results indicate that the phosphorylation level of Thr^{402} of auto-kinase is a critical determining factor for its activity expression and identify Thr^{402} as the regulatory autophosphorylation site of auto-kinase.

DISCUSSION

By partial amino acid sequencing and immunoblot analysis, we demonstrate in this paper that the previously identified auto-kinase from pig liver belongs to a member of the PAK family discovered in 1994. PAKs were initially characterized by Manser et al. [11] as a set of 62–68 kDa proteins with the unique property that they can bind to small (21 kDa) GTPases (Rac and Cdc42) that regulate actin polymerization. After binding to active form of Rac or Cdc42, PAKs undergo an autophosphorylation/activation process and become active kinases capable of acting on exogenous substrates *in vitro* [11–13]. Three isoforms of PAK, namely α-PAK, β-PAK and γ-PAK (or PAK1, PAK3 and PAK2), have been found in mammalian tissues of various species [11–13,23,32,33,35]. All three PAKs have similar sequences and consist of an N-terminal regulatory region containing two to four Src-homology 3 (SH3)-binding domains and a p21-binding domain, a near central acid region, and a C-terminal serine/threonine kinase domain (reviewed in [34]). Among the three PAKs, several lines of evidence indicate that auto-kinase is a proteolytic fragment of a pig homologue of human PAK2. First, auto-kinase can be recognized on an immunoblot by a PAK2-specific antibody raised against the 15-residue C-terminal peptide of PAK2 (Figure 2). Secondly, although all three PAKs contain the peptide sequences corresponding to the two internal partial peptide sequences of auto-kinase (Figure 1A; see also [11–13]), only PAK2 contains the sequence closely related to the N-terminal sequence of auto-kinase (Figure 1B). Thirdly, the sequence around the phosphorylation site of auto-kinase is identical with a fragment of PAK2 located within the C-terminal kinase subdomain VIII of the kinase (Figures 1, 4, 5, 6 and 7). Fourthly, the molecular mass of auto-kinase is approx. 36 kDa, which is very close to the theoretical molecular mass (35242.4 Da) of a C-terminal fragment of PAK2 from Val^{211} to Arg^{524}. On the basis of these observations, it is concluded that auto-kinase represents a fragment of pig liver PAK2 from Val^{211} to Arg^{524} (by the sequence numbering of human PAK2). The N-terminal regulatory region of PAK2 is totally lost in auto-kinase.

It seems that auto-kinase results from a site-specific cleavage process of PAK2 protein because all the different preparations of auto-kinase that we have sequenced have the same N-terminal sequence starting from Val^{211} as described in Figure 1. In contrast, we notice that a similar kind of site-specific cleavage of PAK2 has recently been found to occur *in vivo*. Rudel and Bokoch [33] have shown that PAK2, but not PAK1 and 3, can be proteolytically cleaved by CPP32/caspase-3, an ICE/CED-3 family cysteine protease [36,37], in apoptotic Jurkat T cells induced by immunoglobulin M antibody against Fas or by tumour necrosis factor α. The resulting C-terminal fragment of PAK2 has a molecular mass of approx. 34 kDa and is an active kinase as evidenced by in-gel kinase assay. Moreover it is interesting to note that the starting residue of the N-terminal sequence of auto-kinase (Val^{211}) is adjacent to the cleavage site of PAK2 by CPP32/caspase-3 (Asp^{212}) [33]. As all caspases so far identified cleave their substrates after an Asp residue [38], auto-kinase could not be generated by caspase-mediated cleavage of PAK2 as reported by Rudel and Bokoch [33]. This raises a possibility that a protease distinct from the caspase family might also cause site-specific cleavage of PAK2 between its regulatory and catalytic domains. Immunoblot analysis showed that only the 62 kDa PAK2 band and not the 36 kDa band could be clearly detected in fresh extracts of pig liver, and both the 62 and 36 kDa bands could be observed in the peak fractions of auto-kinase obtained from the first purification step (DEAE-Sepharose) (results not further illustrated). The result seems to indicate that this kind of protease action might occur during the purification process, although several commonly used protease inhibitors were included throughout purification. It seems likely that the protease responsible for generating auto-kinase is not inhibited by the protease inhibitors used.

To facilitate the determination of the regulatory autophosphorylation site of auto-kinase, a peptide (STM-23) was synthesized in accordance with the substrate consensus sequence motif of auto-kinase and the known sequence of human PAK2. We show that the STM-23 peptide can serve as a specific substrate for auto-kinase because several other protein kinases with diverse preferences for substrate sequence motifs can hardly phosphorylate this peptide (Figures 5A and 5B). We identify only one threonine residue, which corresponds to Thr^{402} of PAK2, that is phosphorylated by auto-kinase in STM-23 peptide (Figures 5C and 5D). In fact, this residue in STM-23 peptide (–R-S-T*-M-V-G-T–) represents exactly the same one that is expected to be phosphorylated by auto-kinase on the basis of the substrate consensus sequence motif [-R-X-(X)-S*/T*-X_3-S/T-] of this kinase. The result provides initial evidence for the successful application of this identified sequence motif in searching the substrate for auto-kinase. More importantly, it was found by phosphopeptide map analysis that the phosphorylation site sequence in STM-23 peptide by auto-kinase is identical to the autophosphorylation site sequence of auto-kinase (Figure 6), providing strong evidence that Thr^{402} is the autophosphorylation site of auto-kinase. This notion is further supported by immunoblot analysis of auto-kinase during autophosphorylation/activation with a phospho-specific antibody raised against the identified phosphorylation site sequence (Figure 7A). Moreover, the successful generation of this phospho-specific antibody, which can differentially recognize the activated auto-kinase autophosphorylated at Thr^{402} but not the non-phosphorylated/inactive auto-kinase, allows us to address directly the regulatory role of Thr^{402} phosphorylation during both the activation and the inactivation processes of this kinase in a dynamic way. Our results clearly demonstrate for the first time that the activity of auto-kinase is determined by the state of phosphorylation of this single residue (Figures 7B and 7C).

We identified one single threonine residue (Thr^{402}) as the regulatory autophosphorylation site of auto-kinase. However,

we observed four spots in both tryptic phosphopeptide maps of autophosphorylated auto-kinase (Figure 4A) and phospho-STM-23 phosphorylated by auto-kinase (Figure 6A) analysed by two-dimensional electrophoresis/TLC. Although inspection of the sequence from residues 399 to 418 containing Thr^{402} [KRST-(*P*)MVGTPYWMAPEVVTRK] indicates that 'ragged' cleavage at the N-terminal KR and C-terminal RK basic residues by trypsin could theoretically generate four phosphopeptide fragments RST(*P*)–-RK, RST(*P*)–-R, ST(*P*)–-RK and ST(*P*)–-R, each containing the same phosphorylated Thr residue at cycle 2 or 3 of the manual Edman degradation, it seems unlikely that these four fragments represent the four spots observed in Figures 4(A) and 6(A). If this is so, ST(*P*)–-RK and ST(*P*)–-R should represent spots 1 and 3 (or 3 and 1), and RST(*P*)–-RK and RST(*P*)–-R should correspond to spots 2 and 4 (or 4 and 2) respectively, on the basis of the known cycle number of phosphorylation residue of the four spots (see Figure 4 for comparisons). However, because the expected charge-to-mass ratio of ST(*P*)–-RK at pH 1.9 used for electrophoresis is significantly greater than that of ST(*P*)–-R owing to the presence of an extra C-terminal Lys basic residue ($+2/2437$ compared with $+1/2291$, as calculated by the method of Boyle et al. [28]), it seems unlikely that the two phosphopeptides would co-migrate during electrophoresis at pH 1.9 as spots 1 and 3 do. The same reasoning can also be used to argue against the assumption that RST(*P*)–-RK and RST(*P*)–-R correspond to spots 2 and 4 (or 4 and 2). However, if modification of the phosphopeptides by small amounts of alkylating compounds in microcentrifuge tubes occurred during the peptide-preparation process, this would not significantly alter the charge-to-mass ratio and therefore the electrophoretic mobility of the modified peptides. Nevertheless, as the hydrophobicity of peptides would be greatly increased after modification by alkylation, the mobility of the modified peptides during chromatography on TLC plates would be significantly changed, which would lead to 'spot doubling' as mentioned by Boyle et al. [28]. Thus it is tempting to consider that incomplete cleavage at Arg in the sequence –-RST(*P*)–- by trypsin resulted in two phosphopeptide spots detected after electrophoresis and that modification of the phosphopeptides by alkylation further split the two phosphopeptides into four spots during chromatography.

The autophosphorylation sites of other forms of PAK have also been investigated. Benner et al. [39] reported that a trypsin-digested form of human placenta PAK2 termed p40 S6/H4 kinase could be activated by autophosphorylation at two sites: S^{401}(*P*)SMVGTPY (site 1) and S^{197}(*P*)VIDPVPAPVGDSHV-DGAAK (site 2). Phosphorylation of both sites is required for kinase activation. When these characteristics are compared with those of auto-kinase, several differences are evident. According to the known sequence of human PAK2, it seems that auto-kinase is shorter than p40 S6/H4 kinase in length and does not contain the phosphorylation site in the site 2 sequence. Unlike p40 S6/H4 kinase, phosphorylation at only one site (Thr^{402}) is required for auto-kinase activation. More importantly, it is Thr^{402} rather than Ser^{401} in the corresponding site 1 sequence that is phosphorylated during the activation process of auto-kinase. The reason for this discrepancy is not clear at present. However, when all the known sequences deduced from cDNA of PAKs from various mammalian species were inspected, it was found that the sequence around site 1 should be S^{401}TMVGTPY [11–13,23,32,33,35]. In contrast, another experiment with full-length recombinant PAK1 (αPAK) as the material under study, Manser et al. [40] showed that, in the presence of the active form of Cdc42, PAK1 could autophosphorylate at multiple sites in its N-terminal regulatory domain and at only one site within its C-terminal catalytic domain, which is equivalent to Thr^{402} of PAK2. By mutational analysis, they also reported that Thr^{402}, but not other phosphorylatable residues, is critical for regulating the activity of PAK1. This, taken together with our finding in the present paper, indicates that Thr^{402} might be the common regulatory autophosphorylation site of the PAK family enzymes, regardless of the forms in which they might exist as intact p21-dependent or proteolytic p21-independent.

PAK2/AK	386	DFGFCAQI...TPEQSKRS**T**MVGTPYWMAPE 413
PKA		DFGFAKRV......KGRTW**T**LCGTPEYLAPE
CDK2		DFGLARAF...GVPVRTY**T**HEVVTLWYRAPE
ERK2		DFGLARVADPDHDHTGFL**T**E**Y**VATRWYRAPE
GSK3		DFGSAKQL....VRGEPNVS**Y**ICSRYYRAPE

Figure 8 Comparison of the phosphorylation site(s) of various protein kinases in the region between conserved kinase subdomains VII and VIII

Sequences of various protein kinases whose activities are known to be regulated by phosphorylation are aligned in the region between the invariant DFG (in subdomain VII) and APE (in subdomain VIII) sequences. Residues identified as being phosphorylated are in bold and underlined. Abbreviations: AK, auto-kinase; CDK2, cyclin-dependent protein kinase 2; ERK2, mitogen-activated protein kinase 2.

Thr^{402} is located within 10 residues upstream of the consensus triplet Ala-Pro-Glu in the protein kinase subdomain VIII of PAK2. Subdomain VIII is a region that has been recognized as having an important role in catalysis [41]. Our finding is consistent with the previous observations that several protein serine/threonine kinases including PKA [42,43], mitogen-activated protein kinase [44], cyclin-dependent kinase 2 [45] and GSK-3 [46] have regulatory phosphorylation site(s) within this region (see Figure 8 for comparison), and phosphorylation of these sites in this region is required for the expression of activity of these kinases. For PKA, it is interesting that the residue (Thr^{197}) phosphorylated in this region is also an autophosphorylation site of the kinase. From crystal-structure analyses of PKA, mitogen-activated protein kinase 2 and cyclin-dependent protein kinase 2 [47–49], it has been recognized that the phosphorylation of residue(s) in this region will provide critical negative charges to co-ordinate catalytic residues into the correct conformation to promote catalysis. On the basis of these observations, it is conceivable that autophosphorylation at Thr^{402} might cause a marked conformational change in auto-kinase and thereby allow better access of exogenous substrates to the catalytic site of the kinase.

As noted, proteolytic cleavage/activation of PAK2 has been shown to occur *in vivo*, and the resulting catalytic fragment of PAK2 can be an important mediator involved in the receptor-mediated apoptotic signal transmission induced by anti-Fas and tumour necrosis factor α [33]. Because the catalytic fragment of PAK2 generated *in vivo* is almost identical with auto-kinase from the viewpoint of their N-terminal sequence analyses, it is conceivable that autophosphorylation at Thr^{402} would be needed to activate this catalytic fragment before it exerted its biological function(s) in apoptotic cells, and the level of phosphorylation of Thr^{402} could be useful for monitoring its activity in cells. In this model, the successful generation of a phospho-specific antibody, as reported here, that can differentially recognize the activated auto-kinase autophosphorylated at Thr^{402} but not the non-phosphorylated/inactive auto-kinase will allow us to address this important issue further in the future.

We thank Dr Wen-Chuan Lee for obtaining fresh pig livers. This work was supported by a grant (NSC87-2314-B-182-078) from National Science Council of Taiwan, ROC, and by a grant (CMRP-636) from Chang Gung University, Tao-Yuan, Taiwan, ROC.

REFERENCES

1 Yang, S.-D., Fong, Y.-L., Yu, J.-S. and Liu, J.-S. (1987) J. Biol. Chem. **262**, 7034–7040
2 Yang, S.-D., Chang, S.-Y. and Soderling, T.-R. (1987) J. Biol. Chem. **262**, 9421–9427
3 Huang, T.-J., Lee, T.-T., Lee, W.-C., Lai, Y.-K., Yu, J.-S. and Yang, S.-D. (1994) J. Protein Chem. **13**, 517–525
4 Yu, J.-S. and Yang, S.-D. (1995) Biochem. Biophys. Res. Commun. **207**, 140–147
5 Yu, J.-S., Lee, S.-C. and Yang, S.-D. (1995) J. Protein Chem. **14**, 747–752
6 Guo, H., Reddy, S. A. G. and Damuni, Z. (1993) J. Biol. Chem. **268**, 11193–11198
7 Guo, H. and Damuni, Z. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 2500–2504
8 Yang, S.-D. and Huang, T.-J. (1994) J. Biol. Chem. **269**, 29855–29859
9 Dennis, P. B. and Masaracchia, R. A. (1993) J. Biol. Chem. **268**, 19833–19841
10 Thomas, D. C. and Samuel, C. E. (1993) J. Virol. **67**, 7695–7700
11 Manser, E., Leung, T., Salihuddin, H., Zhao, Z.-s. and Lim, L. (1994) Nature (London) **367**, 40–46
12 Martin, G. A., Bollag, G., McCormick, F. and Abo, A. (1995) EMBO J. **14**, 1970–1978
13 Bagrodia, S., Taylor, S. J., Creasy, C. L., Chernoff, J. and Cerione, R. A. (1995) J. Biol. Chem. **270**, 22731–22737
14 Yu, J.-S. and Yang, S.-D. (1994) J. Neurochem. **62**, 1596–1603
15 Kinzel, U. and Kubler, D. (1976) Biochem. Biophys. Res. Commun. **71**, 257–264
16 Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S. and Nishizuka, Y. (1982) J. Biol. Chem. **257**, 13341–13348
17 Yu, J.-S. and Yang, S.-D. (1993) J. Protein Chem. **12**, 667–676
18 DePaoli-Roach, A. A. (1984) J. Biol. Chem. **259**, 12144–12152
19 Resink, T. J., Hemmings, B. A., Tung, H. Y. L. and Cohen, P. (1983) Eur. J. Biochem. **133**, 455–461
20 Schagger, H. and von Tagow, G. (1987) Anal. Biochem. **166**, 368–379
21 Higgins, R. C. and Dahmus, M. E. (1979) Anal. Biochem. **93**, 257–260
22 Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U. K. (1977) J. Biol. Chem. **252**, 1102–1106
23 Jakobi, R., Chen, C.-J., Tuazon, P. T. and Traugh, J. A. (1996) J. Biol. Chem. **271**, 6206–6211
24 Reichlin, M. (1980) Methods Enzymol. **70**, 159–165
25 Yu, J.-S. and Yang, S.-D. (1994) J. Biol. Chem. **269**, 14341–14344
26 Yu, J.-S. and Yang, S.-D. (1994) J. Cell. Biochem. **56**, 131–141
27 Luo, K., Hurley, T. R. and Sefton, B. M. (1990) Oncogene **5**, 921–923
28 Boyle, W. J., van der Geer, P. and Hunter, T. (1991) Methods Enzymol. **201**, 110–149
29 Kamps, M. P. and Sefton, B. M. (1989) Anal. Biochem. **176**, 22–27
30 Laursen, R. A. and Machleidt, W. (1980) in Methods of Biochemical Analysis, vol. 26 (Glick, D., ed.), pp. 201–284, John Wiley, New York
31 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
32 Teo, M., Manser, E. and Lim, L. (1995) J. Biol. Chem. **270**, 26690–26697
33 Rudel, T. and Bokoch, G. M. (1997) Science **276**, 1571–1574
33a Knaus, U. G., Morris, S., Dong, H.-J., Chernoff, J. and Bokoch, G. M. (1995) Science **269**, 221–223
34 Sells, M. A. and Chernoff, J. (1997) Trends Cell Biol. **7**, 162–167
35 Manser, E., Chong, C., Zhao, Z.-S., Leung, T., Michael, G., Hall, C. and Lim, L. (1995) J. Biol. Chem. **270**, 25070–25078
36 Fernandes-Alnemri, T., Litwack, G. and Alnemri, E. S. (1994) J. Biol. Chem. **269**, 30761–30764
37 Tewari, M., Guan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S. and Dixit, V. M. (1995) Cell **81**, 801–809
38 Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W. and Yuan, J. (1996) Cell **87**, 171
39 Benner, G. E., Dennis, P. B. and Masaracchia, R. A. (1995) J. Biol. Chem. **270**, 21121–21128
40 Manser, E., Huang, H.-Y., Loo, T.-H., Chen, X.-Q., Dong, J.-M., Leung, T. and Lim, L. (1997) Mol. Cell. Biol. **17**, 1129–1143
41 Hanks, S. K., Quinn, A. M. and Hunter, T. (1988) Science **241**, 42–52
42 Shoji, S., Titani, K., Demaille, J. C. and Fisher, E. H. (1979) J. Biol. Chem. **254**, 6211–6214
43 Steinberg, R. A., Cauthron, R. D., Symcox, M. M. and Shuntoh, H. (1993) Mol. Cell. Biol. **13**, 2332–2341
44 Payne, D. M., Rossomando, A. J., Martino, P., Erickson, A. K., Her, J.-H., Shabanowitz, J., Hunt, D. F., Weber, M. J. and Sturgill, T. W. (1991) EMBO J. **10**, 885–892
45 Morgan, D. O. (1995) Nature (London) **374**, 131–134
46 Hughes, K., Nikolakaki, E., Plyte, S. E., Totty, N. F. and Woodgett, J. R. (1993) EMBO J. **12**, 803–808
47 Knighton, D. R., Zheng, J., Ten Eyck, L. F., Ashford, V. A., Xuong, N.-H., Taylor, S. S. and Sowadski, J. M. (1991) Science **253**, 407–414
48 Zhang, F., Strand, A., Robbins, D., Cobb, M. H. and Goldsmith, E. J. (1994) Nature (London) **367**, 704–711
49 De Bondt, H. L., Rosenblatt, J., Jancarik, J., Johns, H. D., Morgan, D. O. and Kim, S. (1993) Nature (London) **363**, 595–602

Received 4 March 1998/8 May 1998; accepted 19 May 1998