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Enzyme-Linked Immunosorbent Assay for the Determination of p21-Activated Kinase Activity[1]

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An enzyme-linked immunosorbent assay (ELISA) for the measurement of p21-activated kinase (PAK) activity is described. The development of this method takes advantage of the fact that a phospho-epitope-specific antibody against the regulatory autophosphorylation site sequence of PAK was successfully produced, and after being phosphorylated by PAK, a cross-linked peptide containing the autophosphorylation site of PAK could be recognized on immunoblot by this antibody. This procedure involves coating the cross-linked peptide on microtiter plates, phosphorylating the cross-linked peptide by adding active PAK plus ATP·Mg^{2+}, and detecting peptide phosphorylation using the phospho-epitope-specific antibody and secondary antibody conjugated with alkaline phosphatase followed by reaction with *p*-nitrophenyl phosphate (for colorimetric detection) or fluorescein diphosphate (for fluorimetric detection). The PAK activity detected by this method was linearly proportional to the amount of kinase used in the reaction and to the duration of the kinase reaction. Furthermore, fluorimetric detection proved more sensitive than colorimetric detection in terms of both detection limit and signal magnitude. Kinase inhibitor assay revealed that the IC_{50} value of staurosporine obtained by this ELISA was very close to that obtained in radioassay. Besides staurosporine, the inhibitory activity of several kinase inhibitors was also tested by the PAK ELISA. The results taken together demonstrate the feasibility and efficacy of this solid phase method for the measurement of PAK activity in a non-radioactive way. Development of this method can be helpful in further high-throughput screening of potential inhibitors of this kinase.

Key words: antibody, ELISA, PAK, peptide, phosphorylation.

p21-activated kinases (PAKs) were initially characterized by Manser *et al.* (*1*) as a set of 62–68 kDa proteins with the unique property of being able to bind to the small (21 kDa) guanosine triphosphatases (GTPases) Rac and Cdc42 that regulate actin polymerization. Three isoforms of PAK, termed α-, β-, and γ-PAK (or PAK1, 3, and 2), have been identified in mammalian tissues, and all have similar sequences containing an N-terminal regulatory region with a p21-binding site and a C-terminal kinase domain (see Ref. *2* for review). After binding to the active form of Rac or Cdc42, PAKs undergo an autophosphorylation/activation process and become active kinases capable of acting on exogenous substrates *in vitro* (*1*, *3*). Another regulatory mechanism of PAKs involves the proteolytic removal of its N-terminal regulatory region (*4*, *5*). The resulting C-terminal catalytic fragment can then be activated by autophosphorylation at certain specific site(s) (*4*, *6*). Recent studies have indicated that PAKs are involved in modulating diverse cell functions, including cytoskeleton rearrangement (*7*, *8*), apoptotic cell death (*9–11*), and cell cycle progression (*12*, *13*). In addition, although the exact mechanisms and functional consequences are largely unknown, PAKs can be activated in cells by various extracellular stimuli such as growth factors (*14*, *15*), chemoattractants (*16*), thrombin (*17*), angiotensin II (*18*), and CD28 (*19*), and can act as upstream regulators of the MAPK, JNK, and p38 MAPK pathways (*20–22*). These observations suggest that PAKs are important enzymes that participate in multiple cellular signaling pathways. At present, however, potent and specific inhibitors of PAKs, which could provide powerful tools for elucidating the regulatory mechanisms and physiological roles of this kinase family, have not yet been found. Therefore, a non-radioactive, convenient kinase assay suitable for high-throughput screening of inhibitors of this enzyme appears to be essential.

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Abbreviations: BSA, bovine serum albumin; CL-STM-23, cross-linked STM-23 peptides; ELISA, enzyme-linked immunosorbent assay; FDP, fluorescein diphosphate; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; PAK, p21-activated kinase; pNPP, *p*-nitrophenyl phosphate; PVDF, polyvinylidene fluoride; STM-23-BSA, STM-23 peptide-BSA conjugates.

The phospho-epitope-specific antibodies that are highly selective in distinguishing phosphorylated and non-phosphorylated forms of a variety of protein kinases and their substrates have been demonstrated to be powerful and convenient tools for measuring kinase activity and substrate phosphorylation in a non-radioactive way (New England Biolabs). While most phospho-epitope-specific antibodies are used to detect kinase activities by immunoblotting or immunostaining, some have been employed to develop enzyme-linked immunosorbent assays (ELISA) for this purpose (*23–25*). Recently, we identified Thr^{402} as the regulatory autophosphorylation site of the catalytic fragment of PAK2 *via* the help of a phospho-epitope-specific antibody against the regulatory autophosphorylation site sequence of PAK (*6*). This phospho-epitope-specific antibody recognizes the activated catalytic fragment of PAK2 phosphorylated at Thr^{402}, but not the non-phosphorylated/inactive enzyme, and the kinase activity is dependent on the extent of Thr^{402} phosphorylation (*6*). In that study, we also found that a synthetic 23-mer peptide (STM-23) containing Thr^{402} of PAK2 can serve as a rather specific substrate for the enzyme, and showed that only one threonine residue, which corresponds to Thr^{402} in PAK2, is phosphorylated by PAK2. The success in producing the phospho-epitope-specific antibody against active PAK and the identification of STM-23 as a good peptide substrate for PAK prompted us to develop a non-radioactive ELISA for the measurement of PAK activity.

MATERIALS AND METHODS

Materials—[γ-^{32}P]ATP was purchased from Amersham. Polystyrene 96-well microtiter plates were from Corning. Staurosporine, H7, H89, K252a, KT5720, olomoucine, PD98059, SB203580, and genistein were from Calbiochem. Bovine serum albumin (BSA) and goat anti–rabbit IgG antibody conjugated with alkaline phosphatase were from Sigma. Glutaraldehyde and *p*-nitrophenyl phosphate (pNPP) were from E. Merck. STM-23 peptide (EQSKRSTMVGTPYWMAPEVVTRK) and phospho-STM-11-C peptide ($SKRST_{(P)}MVGTPYC$) were synthesized by Chiron Technologies. The BCA protein assay reagent was from Pierce. Fluorescein diphosphate (FDP) was from Molecular Probes. Polyvinylidene fluoride (PVDF) membranes were from Millipore. CDP-Star™ (a chemiluminescent substrate for alkaline phosphatase) was from Boehringer Mannheim. Molecular weight marker proteins (Mark12) were from Novel Experimental Technology.

Purification of the Catalytic Fragment of PAK2—The catalytic fragment of PAK2 was purified to apparent homogeneity from pig liver according to the procedure previously described (*6*). The purified catalytic fragment of PAK2 was apparently homogeneous and gave a single major protein band at a molecular weight ~36,000 when analyzed by SDS-PAGE. Analysis of the radioactively autophosphorylated kinase on the autoradiogram also revealed a single major phosphorylated protein band at a molecular weight ~36,000.

Preparation of Cross-Linked Peptides and Peptide-BSA Conjugates—Cross-linking of the STM-23 peptide was performed at 4°C as follows using glutaraldehyde as the cross-linker. STM-23 peptide was dissolved in 3 ml of ice-cold phosphate-buffered saline (PBS) at 6 mg/ml. An equal volume of freshly prepared glutaraldehyde solution (2%, v/v in PBS) was added drop-wise, and the reaction mixture was stirred for 1 h. The reaction was stopped by adding 1 ml of glycine (1 M in PBS), and the reaction mixture was stirred for another 1 h. The resulting peptide conjugates were dialyzed extensively against PBS for 3 days. Insoluble materials were removed by centrifuged at 10,000 $\times g$ for 10 min, and the supernatant was stored in aliquots at −30°C for further experiments. To prepare the peptide-BSA conjugates, phospho-STM-11-C peptide (4 mg) or STM-23 peptide (4 mg) was mixed with BSA (12 mg) and then dissolved in 3 ml PBS. The peptides were then coupled to BSA by the procedure described above.

Production of a Phospho-Epitope-Specific Antibody against Active PAK—The phospho-specific antibody against the phosphorylated/activated PAK2 was produced in rabbits using the phospho-STM-11-C peptide ($SKRST_{(P)}MVGTPYC$) as the antigen and affinity-purified as previously described (*6*). This purified antibody can recognize specifically the phosphorylated/activated but not the non-phosphorylated/inactive PAK2 on immunoblots (*6*).

Radioactive PAK Assay—The activity of the catalytic fragment of PAK2 was assayed in a 40 μl reaction mixture containing 50 ng of purified kinase, 20 mM Tris-HCl, pH 7.0, 0.5 mM dithiothreitol, 0.2 mM [γ-^{32}P]ATP , 20 mM $MgCl_2$, and STM-23 peptide (0.25 mM), cross-linked STM-23 peptide (1.25 mg/ml), or STM-23-BSA conjugates (1 mg/ml) at 30°C for 10–40 min. Half of each reaction mixture was spotted onto P81 paper (Whatman, Maidstone, UK) and processed as previously described (*6*); the other half was mixed with an equal volume of 2× Laemmli sample buffer and analyzed by 8% SDS-PAGE (for STM-23-BSA conjugates) or 16.5% SDS-PAGE in the tricine-tris buffer system (for STM-23 peptide and cross-linked STM-23 peptides) (*26*). After electrophoresis, the gels were stained, destained, dried and subjected to autoradiography.

ELISA Procedure for PAK Assay—Cross-linked peptides or peptide-BSA conjugates were diluted in PBS at various concentrations and coated on 96-well microtiter plates (50 μl/well) at 37°C for 1 h. The plates were washed 3 times with 200 μl of PBS, incubated with blocking solution (1 mg/ml BSA in PBS, 200 μl/well) at 37°C for 1 h, and then washed 3 times with PBS. The kinase reaction mixtures (50 μl each) containing 20 mM Tris-HCl (pH 7.0), 0.5 mM dithiothreitol, 0.2 mM ATP, 20 mM $MgCl_2$, 50 μg BSA, and various amounts of purified catalytic fragment of PAK2 were added to the wells, and the kinase reaction was performed at 37°C for 15–60 min. For the kinase inhibitor assay, staurosporine or other compounds at various concentrations were included in the reaction mixtures before starting the kinase reaction. The reaction was stopped by removing the reaction mixtures and washing the plates 3 times with TTBS buffer (20 mM Tris-HCl at pH 7.4, 0.5 M NaCl, and 0.05% Tween 20). The washed plates were incubated with the anti-phospho-STM-11-C antibody (5 μg/ml in TTBS, 50 μl/well) at 4°C for 16 h. The plates were washed four times with TTBS, and 50 μl of goat anti–rabbit IgG antibody conjugated with alkaline phosphatase (diluted at 1:2,000 in TTBS) was added to the wells, and the plates were incubated at 37°C for 1 h. The plates were then washed four times with TTBS and incubated with alkaline phosphatase substrate solution (1 mg/ml pNPP, 10 mM $MgCl_2$, and 50 mM Tris-HCl, pH 9.5, 50 μl/well) at 37°C for

1 h. The formation of 4-nitrophenolate was measured at 405 nm using an ELISA reader (MR5000, Dynatech). For fluorescence assays, 5 μM of fluorescence diphosphate (FDP, Molecular Probes) in alkaline phosphate substrate solution was added to the assay plates and incubated at 37°C for 15 min. The formation of fluorescein was measured using a fluorescence plate reader (Wallac Victor 4200, $\lambda_{excitation}$: 485 nm, $\lambda_{emission}$: 535 nm). All assays were performed at least two times in duplicate. The IC_{50} value was defined as the drug concentration that causes 50% inhibition of kinase ac-tivity.

Immunoblots—Immunoblotting was carried out essentially as previously described (*6*). Affinity-purified anti–phospho-STM-11-C antibody (1 μg/ml) was used to probe phospho-peptides and phospho-peptide-BSA conjugates transferred from the SDS-gel to the PVDF membrane. The bound antibody was detected by goat anti–rabbit IgG antibody conjugated with alkaline phosphatase and CDP-Star™ (a chemiluminescent substrate for alkaline phosphatase) according to the procedure provided by the manufacturer.

Analytic Methods—Protein concentration was determined by the BCA protein assay reagent from Pierce. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according the method of Laemmli (*27*).

RESULTS

Cross-Linked Peptides and Peptide-BSA Conjugates as Substrates for PAK—Previously we have shown that a 23-mer peptide, STM-23, can be phosphorylated by the catalytic fragment of PAK2 at a single threonine residue that corresponds to the regulatory autophosphorylation site of PAK2 (*6*). To develop an ELISA for PAK activity measurement, we tried to use the STM-23 peptide as the immobilized peptide substrate for PAK because the phosphorylated STM-23 peptide could be, theoretically, recognized by our previously produced phospho-epitope-specific antibody against the regulatory autophosphorylation site sequence of PAK (*6*). Considering that the accessibility of small peptide substrates to kinase may be reduced or blocked by the spatial hindrance resulting from immobilizing the peptide to a microtiter plate, we attempted to modify the STM-23 peptide by cross-linking the peptide itself or coupling the peptide to BSA. Both modifications were successful as evidenced by analyzing the modified products by SDS-PAGE followed by Coomassie blue staining (Fig. 1A). According to the apparent molecular weights of the modified products observed in gels, it was estimated that ~2–4 mol of STM-23 peptide were incorporated into one mole of the major cross-linked STM-23 (CL-STM-23) and STM-23–BSA products, respectively. We then examined whether these peptide derivatives can still serve as substrates for PAK. As shown in Fig. 1B, ^{32}P-incorporation into both CL-STM-23 and STM-23-BSA could be catalyzed by PAK, and phosphorylation of both modified peptide derivatives increased proportionally with the reaction time. It was noted that BSA could not be phosphorylated by PAK (Fig. 1B). The results demonstrate that both cross-linked STM-23 peptides and STM-23–BSA conjugates can serve as substrates for PAK.

The Phosphorylated, Immobilized CL-STM-23 Peptides and STM-23–BSA Conjugates Can Be Recognized by the Phospho-Epitope-Specific Antibody Against Active PAK—Although we have previously shown that a phospho-epitope-specific antibody can differentiate the active form of PAK2 catalytic fragment from its inactive form by immunoblotting (*6*), it remains uncertain whether this antibody can specifically recognize the phosphorylated CL-STM-23 peptides and STM-23-BSA conjugates immobilized on solid supports such as PVDF membranes for immunoblotting and microtiter plates for ELISA. Therefore, CL-STM-23 peptides and STM-23–BSA conjugates were phosphorylated by the catalytic fragment of PAK2 and subjected to SDS-PAGE followed by immunoblotting on a PVDF membrane with the phospho-epitope-specific antibody. As shown in Fig. 2, the two phosphorylated, but not the non-phosphorylated, modified peptide derivatives could be clearly detected by the antibody on immunoblot. To test the immunoreactivity of modified peptide derivatives immobilized on microtiter plates, both STM-23–BSA and phospho-STM-11–BSA conjugates prepared as described under "MATERIALS AND METHODS" were coated on the plates, and the interaction between the phospho-epitope-specific antibody

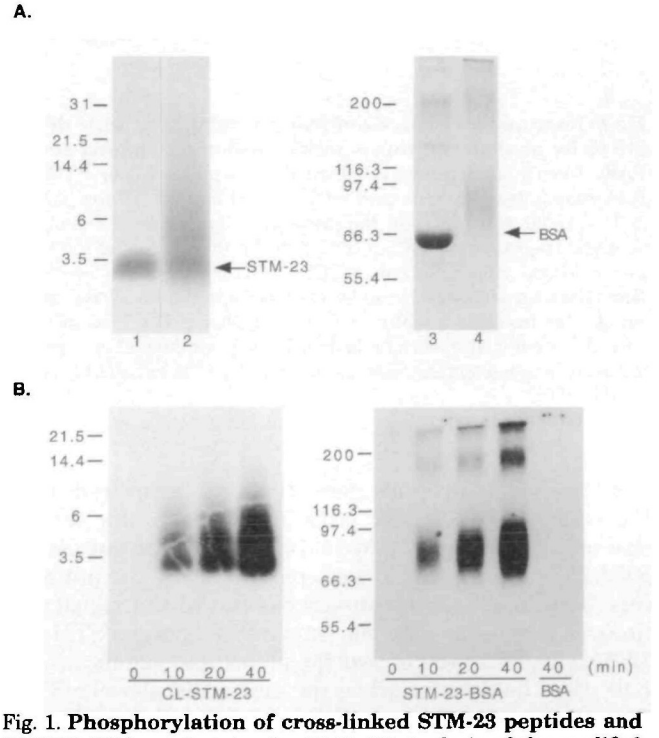


Fig. 1. **Phosphorylation of cross-linked STM-23 peptides and STM-23–BSA conjugates by PAK.** (A) Analysis of the modified peptide derivatives by SDS-PAGE. STM-23 peptide (lane 1), CL-STM-23 peptides (lane 2), BSA (lane 3), and STM-23-BSA conjugates (lane 4) (20 μg each) were resolved by 16.5% tricine-SDS-PAGE (lanes 1 and 2) or 8% SDS-PAGE (lanes 3 and 4) followed by Coomassie Blue staining. (B) Time course phosphorylation of the modified peptide derivatives by PAK. CL-STM-23 peptides or STM-23–BSA conjugates (20 μg) were phosphorylated in the presence of [γ-^{32}P]ATP by the catalytic fragment of PAK2 at 30°C for various times as described in "MATERIALS AND METHODS." The reaction products were resolved by SDS-PAGE, and ^{32}P-incoporation into the peptide derivatives was detected by autoradiography. BSA (20 μg) was also subjected to phosphorylation by PAK2 for 40 min, and no ^{32}P-incoporation into BSA was observed. The position of marker proteins and their molecular masses (in kDa) are shown to the left of each panel.

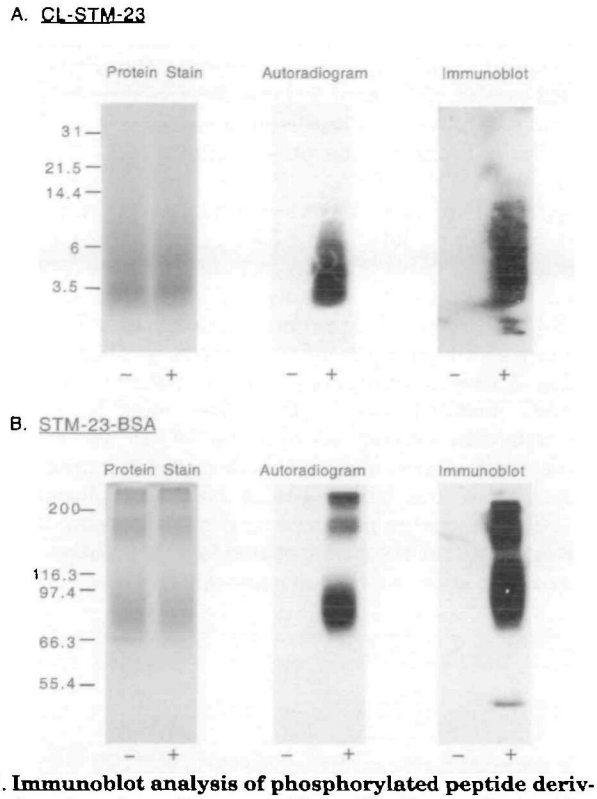


Fig. 2. **Immunoblot analysis of phosphorylated peptide derivatives by phospho-epitope-specific antibodies against active PAK.** Twenty micrograms of CL-STM-23 peptides (A) or STM-23-BSA conjugates (B) were incubated with 0.2 mM ATP (or [γ-^{32}P]-ATP) and 20 mM $MgCl_2$ in the presence (+) or absence (−) of the catalytic fragment of PAK2 at 30°C for 30 min. Aliquots of the reaction products were resolved in SDS-PAGE followed by Coomassie Blue staining for proteins or by electrotransfer onto PVDF membranes for autoradiography or immunoblotting. For immunoblotting, the membranes were probed with the phospho-epitope-specific antibody against active PAK as described in "MATERIALS AND METHODS."

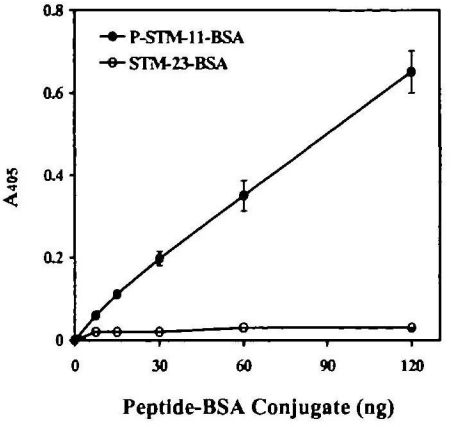


Fig. 3. **Detection of the phospho-STM peptide derivative by ELISA using the phospho-epitope-specific antibody against active PAK.** Various amounts of STM-23–BSA or phospho-STM-11–BSA conjugates were coated onto a microtiter plate. After washing and blocking procedures, the plate was incubated with the phospho-epitope-specific antibody against active PAK followed by ELISA signal detection as described in "MATERIALS AND METHODS."

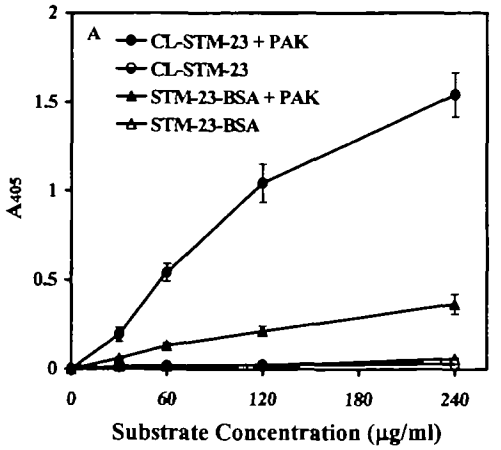
and the modified peptide derivatives was measured using the conventional ELISA procedure. The results showed that only the phospho-STM-11–BSA conjugates, but not the STM-23-BSA conjugates, were recognized by the antibody (Fig. 3). In addition, the intensity of the ELISA signal was linearly proportional to the amount of phospho-STM-11–BSA conjugates used to coat the plates, and significant signals could be detected when the amount of phospho-STM-11–BSA conjugates was as low as 7.5 ng (Fig. 3). The results taken together demonstrate that the phospho-epitope-specific antibody can be used to specifically and sensitively detect the phosphorylated form of peptide derivatives that are immobilized on solid supports.

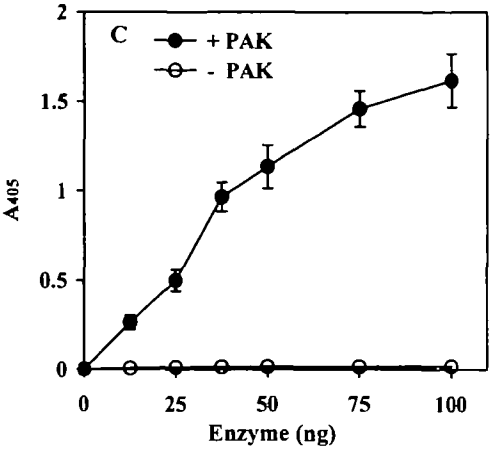
ELISA for PAK Activity—We then examine the ELISA conditions for the measurement of the PAK-catalyzed phosphorylation of the two peptide derivatives coated on plates.

ELISA plate coating condition: As shown in Fig. 4A, the ELISA signal increased proportionally to the concentration of the two modified peptide derivatives. Under our assay conditions, a very clear and strong ELISA signal could be detected using CL-STM-23 as the coating substrate in the concentration range of 60–240 μg/ml; in contrast, a weak, 5-fold lower ELISA signal was observed under the same conditions when STM-23–BSA was the coating substrate (Fig. 4A). It was noted that almost no background ELISA signal could be detected when the kinase was omitted from the phosphorylation reaction, and with no coating substrate, kinase/ATP·Mg^{2+} alone produced no ELISA signal even after a 1-h incubation (Fig. 4A). The results demonstrate that PAK2 can phosphorylate the two modified peptide derivatives immobilized on plates, and that the phosphorylation level of the immobilized substrates can be determined by the ELISA procedure described here. The observation also reveals the high signal-to-noise properties of this method, which is an essential requirement for feasible ELISA. In further experiments, 240 μg/ml was chosen as the substrate concentration of both CL-STM-23 and STM-23–BSA used for plate coating.

Linearity of the kinase reaction: The PAK activity was determined at different reaction times with a fixed amount of enzyme. It was found that the level of phosphorylation of CL-STM-23 was significant and increased linearly up to 40 min, whereas phosphorylation of STM-23–BSA was quite low within 40 min (Fig. 4B). This observation, together with the result from Fig. 4A, indicates that CL-STM-23 is a much better substrate than STM-23–BSA in PAK ELISA. Therefore, only CL-STM-23 was used as the coating substrate in further studies. The effect of kinase dose on the phosphorylation level of CL-STM-23 was also examined. As shown in Fig. 4C, the signal-to-enzyme dose was linear from 12.5 to 50 ng of kinase per assay. Kinase doses within this range were used for further experiments.

Apparent K_m of ATP: The ATP concentration that provides maximum phosphorylation of CL-STM-23 by the active PAK2 occurred between 60 and 200 μM (Fig. 5). The Michaelis constant (K_m) value of ATP was determined to be ~20 μM for this kinase. We have also tested whether another nucleotide substrate, GTP, can act as the phosphate donor in the PAK-catalyzed phosphorylation reaction. However, no detectable phosphorylation of CL-STM-23 was observed at GTP concentrations from 1 to 1,000 μM (data not shown). We chose 200 μM as the optimal ATP





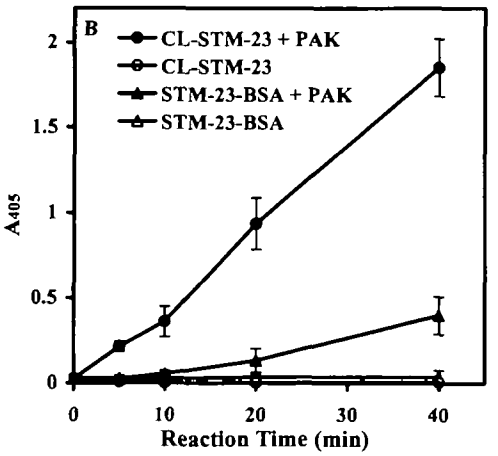


Fig. 4. **ELISA conditions for PAK activity assay.** (A) ELISA signals obtained from different dosages of coating substrates. Various amounts of CL-STM-23 or STM-23–BSA were coated onto microtiter plates. After washing and blocking procedures, 100 ng of purified catalytic fragment of PAK2 in 50 μl of kinase reaction mixture was added to the wells, and the reaction was allowed to proceed for 30 min. Phosphorylation of the two substrates was then detected by the ELISA procedure as described. (B) ELISA signals obtained after different reaction times. CL-STM-23 or STM-23–BSA (240 μg/ml) was coated onto microtiter plates. After washing and blocking procedures, 100 ng of purified catalytic fragment of PAK2 in 50 μl of kinase reaction mixture was added to the wells, and the kinase reaction was continued for various times as indicated. Phosphorylation of the two substrates was then detected by the ELISA procedure as described. (C) ELISA signals obtained from different dosages of kinase. CL-STM-23 (240 μg/ml) was coated onto microtiter plates. After washing and blocking procedures, various amounts of purified catalytic fragment of PAK2, as indicated, in 50 μl of kinase reaction mixtures were added to the wells, and the reaction was allowed to proceed for 30 min. Phosphorylation of CL-STM-23 was detected by the ELISA procedure as described.

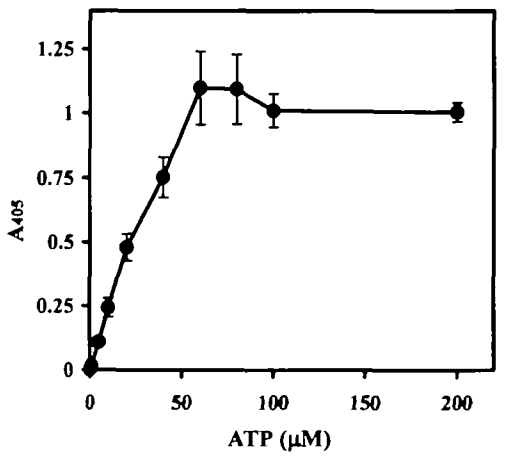


Fig. 5. **Effect of ATP concentration on the PAK activity determined by ELISA.** CL-STM-23 (240 μg/ml) was coated onto microtiter plates. After washing and blocking procedures, 50 μl of kinase reaction mixtures containing 50 ng of purified catalytic fragment of PAK2 and various concentrations of ATP (ranging from 1 to 500 μM) were added to the wells, and the kinase reaction was allowed to proceed for 30 min. Phosphorylation of CL-STM-23 was detected by the ELISA procedure as described.

concentration for PAK ELISA.

Inhibitor Analysis by PAK ELISA—The ELISA protocol described above was used to study the effects of various protein kinase inhibitors on PAK. We first tested staurosporine, an alkaloid isolated from microbes, because it is a potent and broad-spectrum inhibitor of protein kinases with IC_{50} values in the low nanomolar range (*28*). Kinetic studies using the PAK ELISA revealed a typical inhibition curve of staurosporine on the kinase with an IC_{50} value at ~20 nM (Fig. 6A). When compared with the inhibition curve determined by radioassay in solution phase (Fig. 6B), it was found that both curves are almost identical, indicating that the ELISA system is as sensitive as the solution phase radioassay of PAK activity and can be used to screen PAK inhibitors. In addition to staurosporine, several other inhibitors of protein kinases were also tested in the PAK ELISA; the IC_{50} value of each PAK2 inhibitor was determined and is listed in Table I. It was observed that besides staurosporine, of the compounds tested only two indolocarbazoles, K252a and KT5720, possess PAK inhibitory activity with IC_{50} of ~10–100 μM. Compounds including H7 and H89 (selective inhibitors of protein kinases C and A), olomoucine (selective inhibitor of cyclin-dependent kinases), PD98059 (selective inhibitor of MAPK kinases), SB203580 (selective inhibitor of p38 MAPK), and genistein (broad-spectrum protein tyrosine kinase inhibitor) were not able to inhibit PAK activity at concentrations below 1 mM under the same assay conditions.

Enhancement of the Sensitivity of PAK ELISA Using 3,6-

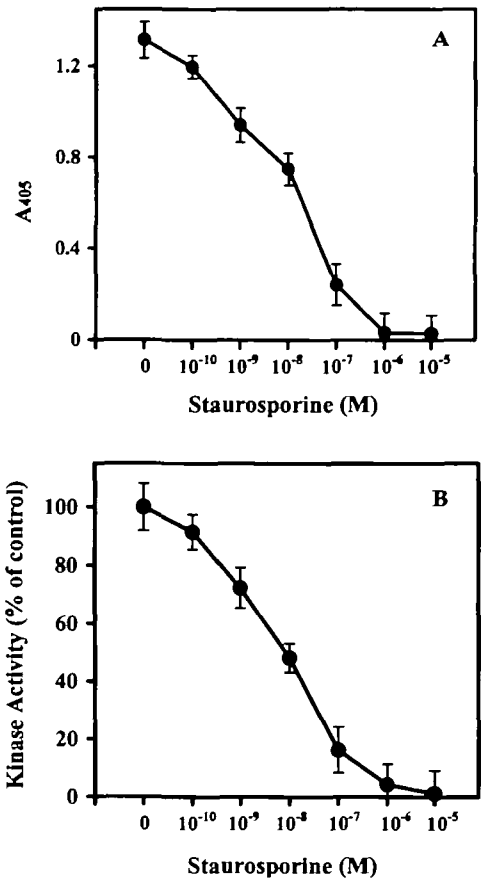


Fig. 6. **Dose effect of staurosporine on the PAK activity determined by radioassay and by ELISA.** (A) ELISA. CL-STM-23 (240 μg/ml) was coated onto microtiter plates. After washing and blocking procedures, 50 μl of kinase reaction mixtures containing 50 ng of purified catalytic fragment of PAK2 and various concentrations of staurosporine (ranging from 0.1 nM to 10 μM) were added to the wells, and the kinase reaction was allowed to proceed for 30 min. Phosphorylation of CL-STM-23 was detected by the ELISA procedure as described. (B) Radioassay. STM-23 peptide (0.25 mM) was phosphorylated with 50 ng of the purified catalytic fragment of PAK2 in 40 μl reaction mixtures containing 20 mM Tris-HCl (pH 7.0), 0.5 mM dithiothreitol, 0.2 mM [γ-^{32}P]ATP (500 cpm/pmol), 20 mM $MgCl_2$, and various concentrations of staurosporine (ranging from 0.1 nM to 10 μM) at 30°C for 10 min. Kinase activity expressed as ^{32}P-incorporation into STM-23 peptide was assayed as previously described (*6*), and the activity detected in the absence of staurosporine was taken as 100%.

TABLE I. **IC_{50} values of protein kinase inhibitors of PAK2.**

Inhibitor	IC_{50} value (μM)
Staurosporine	0.02
K252a	10
KT5720	100
H89	>1,000
H7	>1,000
Olomoucine	>1,000
PD98059	>1,000
SB203580	>1,000
Genistein	>1,000

CL-STM-23 was coated on 96-well microtiter plates at 37°C for 1 h. After washing and blocking procedures, the coated CL-STM-23 was phosphorylated by 50 ng of the catalytic fragment of PAK2 in 50 μl of kinase reaction mixture in the presence of various concentrations of inhibitors ranging from 0.1 nM to 1 mM at 37°C for 30 min. Phosphorylation of CL-STM-23 was then detected by ELISA. IC_{50} is defined as the drug concentration that causes 50% inhibition of kinase activity.

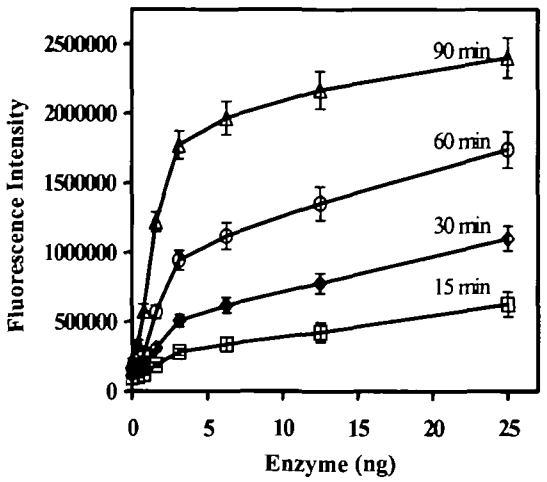


Fig. 7. **Fluorescence signal in PAK ELISA using fluorescein diphosphate as the detecting substrate.** CL-STM-23 (240 μg/ml) was coated onto microtiter plates. After washing and blocking procedures, various amounts of the purified catalytic fragment of PAK2 in 50 μl of kinase reaction mixture were added to the wells, and the kinase reaction time was allowed to proceed for 1 h. Phosphorylation of CL-STM-23 was detected by the ELISA procedure as described except that the plates were incubated with the anti-phospho-STM-11-C antibody (1 μg/ml, 50 μl/well) at 37°C for 1 h and the detecting substrate pNPP was replaced with FDP (5 μM). The fluorescence signal was monitored 15, 30, 60, and 90 min after adding FDP to the wells in a microplate fluorescence reader (excitation 485 nm, emission 535 nm).

Fluorescein Diphosphate (FDP) as the Detecting Substrate—As shown in Fig. 4C, the detection limit of PAK enzyme using pNPP as the substrate for colorimetric detection was about 5–10 ng, at which dose the ELISA signal is below 0.2 A_{405} unit. Because sensitivity is one of the critical factors in developing a good ELISA procedure, we tried to enhance the sensitivity of PAK ELISA using another detecting substrate, FDP, which exhibits strong fluorogenic properties when dephosphorylated by a variety of phosphatases (*29*, *30*). We used a microplate fluorescence reader to measure the fluorescence signal derived from various amounts of PAK using the described ELISA procedure at different time intervals. It was found that FDP is a suitable fluorogenic substrate for alkaline phosphatase in ELISA, and that the intensity of the fluorescence signal depends on the dosage of PAK enzyme used in ELISA (Fig. 7). More importantly, a significant fluorescence signal could still be detected at enzyme doses below 5 ng; approximately 500,000 and 1,000,000 fluorescence units were generated at 1.6 ng of PAK within 60 and 90 min during the ELISA, respectively (Fig. 7). The results clearly show that both the detection limit and the signal magnitude in PAK ELISA are improved when FDP is used as the detecting substrate. We also examined the dose effect of staurosporine on PAK activity in ELISA using this fluorogenic detection method and found that the IC_{50} value determined by this method is ~20–30 nM (data not shown), which is close to that obtained by radioassay or by the colorimetric ELISA described above.

DISCUSSION

Because of the emerging importance of PAK family enzymes in modulating diverse cell functions, activity measurements of these kinases by radioassays (such as counting the radioactivity of ^{32}P-labeled substrates bound to phosphocellulose paper or scoring the band intensity of the SDS-gel-fractionated ^{32}P-labeled substrates on autoradiograms) using protein substrates have been developed in many laboratories. Although these radioassays are rapid and sensitive, the radioactivity from isotopes is a potential hazard to the investigator, and special equipment for protection against and detection of radioactivity is required. Furthermore, it is hard to scale up these radioassays to automation for high-throughput screening of potential inhibitors. The aim of the present study is to develop a microtiter plate-based, non-radioactive kinase activity assay for PAK. We have characterized the entire ELISA procedure from kinase substrate to detection method, and show that PAK activity can be quantitatively measured by this ELISA procedure. With FDP as the substrate for alkaline phosphatase in fluorimetric detection, the ELISA procedure can detect as little as 1 ng of PAK (Fig. 7). This result, together with the observation that the IC_{50} value of staurosporine determined by the PAK ELISA is very close to that obtained by radioassay (Fig. 6), indicates that the PAK ELISA developed in this report is highly sensitive and suitable for analyzing the potency of kinase inhibitors of PAK. In conjunction with a laboratory robotic workstation, this ELISA can be easily adapted to automation for screening PAK inhibitors in a high-throughput way.

A distinct feature of this assay is the application of a cross-linked peptide as the kinase substrate immobilized on the plate that contains the regulatory autophosphorylation site sequence of PAK. Small peptides are easy to obtain in large quantity and with high quality, and although they lack a defined tertiary structure, peptides with specific sequences can still serve as good substrates for protein kinases in many cases (*31*). Therefore, peptides can be good substrate candidates in ELISA for measuring protein kinase activity if they can be appropriately immobilized on microtiter plates without losing their ability to be phosphorylated. To retain the accessibility of a peptide substrate to protein kinase during ELISA, modifications of the microtiter plates or peptide itself have been described. Rijksen *et al.* (*32*) developed an ELISA for determining src-related tyrosine kinase activity by coupling a 15–amino acid peptide derived from p34cdc2 kinase to the wells of maleic anhydride–activated microtiter plates. Another report showed that peptides N-terminally labeled with biotin can be efficiently immobilized on strepavidin-coated microtiter plates and phosphorylated by protein kinases (*24*). We show here that conjugating a peptide to BSA or cross-linking the peptide itself is a feasible alternative to modify the peptide as a suitable substrate for protein kinase in ELISA. In the present study we demonstrate that STM-23 peptide coupled to BSA or cross-linked to itself can act as a substrate for PAK when immobilized on microtiter plates. Although both CL-STM-23 and STM-23–BSA contain 2–4 potential phosphorylation sites per molecule (because ~2–4 mol of STM-23 peptide were estimated to be incorporated in 1 mol of the major CL-STM-23 and STM-23–BSA products, respectively), it is apparent that the former has a much higher phosphorylation site-to-molecular weight ratio than the latter, as the molecular weight of BSA (~66,000) is significantly larger (>24-fold) than that of STM-23 peptide (2,682). This together with the fact that the wells of a microtiter plate have a limited binding capacity for proteins/peptides, makes it likely that the density of phosphorylation will be much higher in wells coated with CL-STM-23 than those coated with STM-23–BSA. In this context, it is expected that CL-STM-23 would be a much better substrate than STM-23–BSA in PAK ELISA. This notion is supported by the observation that the ELISA signals detected from plates coated with CL-STM-23 were 3–6-fold stronger than those obtained from plates coated with STM-23–BSA (Fig. 4, A and B). The successful employment of a cross-linked peptide substrate for protein kinase in ELISA demonstrated in this report may provide a convenient method to enhance the sensitivity of protein kinase ELISA at the substrate level, and this method may be applied to ELISA for other protein kinases besides PAK in the future.

Another feature of this assay is the employment of a phospho-epitope-specific antibody against the regulatory autophosphorylation site sequence of PAK. The production and specificity of this antibody in distinguishing phosphorylated/activated and non-phosphorylated/inactive forms of PAK has been previously documented (*6*). In this report we further demonstrate its utility as a powerful tool in ELISA to detect the phosphorylation of immobilized CL-STM-23 peptide catalyzed by PAK. It is apparent that the high signal-to-noise ratio of this ELISA method is due to the high specificity of this antibody in differentiating the phosphorylated and non-phosphorylated forms of STM-23 peptide derivatives (Fig. 2). As mentioned, the phosphorylation of one of the threonine residues (Thr^{402}) in STM-23 peptide catalyzed by PAK creates the recognition sequence motif for this phospho-specific antibody. Although STM-23 peptide has been found to be a rather specific substrate for PAK (*6*), the possibility that other kinases can also act on this peptide has not been excluded. Theoretically, the activity of any other kinases able to phosphorylate STM-23 peptide at the Thr^{402} residue can be measured by the sensitive ELISA developed here. A plausible application of this ELISA method to detect kinase activities other than PAK is now under investigation.

Autophosphorylation is a common characteristic of many protein kinases, and in many cases, autophosphorylation at specific site(s) is involved in regulating the kinase activity (*33–37*). Synthetic peptides containing the autophosphorylation site sequence of a kinase are usually good substrates for the kinase itself (*6*, *36*, *38–40*). The success in developing the PAK ELISA described here indicates a feasible strategy for further development of ELISA methods to measure the activity of other protein kinases whose autophosphorylation sites have been identified. This strategy will involve three main steps as follows. First, synthesis of a substrate peptide containing the autophosphorylation site sequence of the kinase and demonstration of that the site in the peptide phosphorylated by the kinase corresponds exactly to the kinase autophosphorylation site. Second, generation of a phospho-epitope-specific antibody against the autophosphorylation site sequence of the kinase. Third, modification of the peptide so that its ability to be phosphorylated is retained after being immobilized to microtiter

plates.

In conclusion, using a cross-linked peptide as the kinase substrate and a phospho-epitope-specific antibody as the detecting reagent, we have developed a highly sensitive ELISA method to determine the activity of PAK. The principle of this method may be applicable to the further development of ELISA for measuring the activity of other protein kinases that are capable of undergoing autophosphorylation at specific site(s).

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