Determinants for Substrate Phosphorylation by p21-Activated Protein Kinase (γ-PAK)†

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ABSTRACT: γ-PAK, originally designated PAK I and subsequently identified as a member of the p21-activated protein kinase family, has been shown to have cytostatic properties and to be involved in maintaining cells in a nondividing state [Rooney, R. D., et al., (1996) *J. Biol. Chem. 271*, 21498−21504]. The determinants for phosphorylation of substrates by γ-PAK have been identified by examining the kinetics of phosphorylation of a series of synthetic peptides patterned after the sequence KKRKSGL, which is the site phosphorylated by γ-PAK in the Rous sarcoma virus nucleocapsid protein NC in vivo and in vitro. With these peptides, the recognition sequence for γ-PAK has been shown to contain two basic amino acids in the −2 and −3 positions, as represented by (K/R)RXS, in which the −2 position is an arginine, the −3 position is an arginine or a lysine, and X can be an acidic, basic, or neutral amino acid. A basic amino acid in the −1 or −4 position improves the rate of phosphorylation by increasing the V_{max} and decreasing the K_m. An acidic amino acid in the −1 position increases the rate (2.5-fold), as does an acidic residue in the −4 position, although to a lower extent (1.6-fold). Proline in the −1 or +1 position has a deleterious effect and inhibits phosphorylation by γ-PAK. The substrate requirements of protein kinases that recognize basic amino acids on the N-terminal side of the phosphorylatable residue such as cAMP-dependent protein kinase (PKA) and Ca^{2+}/phospholipid-dependent protein kinase (PKC) have been compared with γ-PAK using the same peptides. An acidic residue in the −1 position negatively affects PKA and PKC; thus, peptides containing the sequence KRES can be used to identify γ-PAK.

γ-PAK[1] (also known as PAK 2), a member of the p21-activated protein kinase (PAK) family (*1*−*7*) and formerly designated PAK I, is a serine/threonine kinase of 58 000 Da found in a number of tissues and species (*8*−*14*). γ-PAK was first identified in rabbit reticulocytes as an inactive holoenzyme that could be activated in vitro by limited proteolysis with trypsin, chymotrypsin, or a Ca^{2+}-stimulated protease, hence the initial nomenclature of protease-activated kinase (PAK) I (*8*, *9*). Limited proteolysis of the inactive holoenzyme with trypsin produces a catalytically active peptide of 37 000 Da that contains the catalytic domain and a part of the regulatory domain (*7*). The enzyme from rabbit reticulocytes has been shown to be highly homologous to γ-PAK from human (*15*) and rat (*16*) with some homology to STE 20 from yeast (*17*). Like other PAK enzymes (*18*, *19*), γ-PAK can bind small G proteins such as Cdc42 in the presence of GTP to stimulate autophosphorylation, resulting in activation of the protein kinase activity.

γ-PAK activity is elevated in serum-starved and quiescent cells and is drastically reduced in actively dividing cells.[2] In frog eggs, γ-PAK activity and protein are high in frog oocytes and are reduced following fertilization and at the 2-cell stage. In the 4- and 16−32-cell stages, γ-PAK reappears, but mainly as the inactive form (*13*). Injection of γ-PAK into one blastomere of 2-cell frog embryos results in cleavage arrest, while the noninjected blastomere continues to divide through mid- to late-cleavage. These observations suggest that γ-PAK is involved in the maintenance of cells in a nondividing state (*13*).

γ-PAK has been shown to phosphorylate a number of protein substrates such as histones 2B and 4 (*8*); myosin light chain from smooth and skeletal muscle (*10*, *11*); translation initiation factors eIF-3, eIF-4B, and eIF-4F (*9*, *20*); and avian and Rous sarcoma virus nuclear capsid protein NC (*21*−*23*). Phosphorylation of myosin light chain in smooth muscle by γ-PAK increases the actin-activated myosin ATPase activity to the same extent as that observed upon phosphorylation by the Ca^{2+} calmodulin-dependent myosin light chain kinase (*11*). In the Rous sarcoma virus nucleocapsid protein NC, phosphorylation by γ-PAK at serine 40 increases the affinity for single-strand RNA by up to 100-fold (*22*). Studies with site-specific mutants of NC indicate phosphorylation by γ-PAK can regulate binding to viral RNA (*23*). Thus, it appears from the diversity of substrates that γ-PAK may be involved in regulation of multiple pathways of cell metabolism.

One approach to elucidate the role of protein kinases in general, and γ-PAK in particular, is to identify their substrates. The search for possible substrates can be facilitated by a knowledge of the amino acids critical for efficient phosphorylation. It is generally accepted that the primary sequence around the phosphorylation site plays a crucial role in the recognition of substrates for a number of protein kinases (*24*). In these studies, determinants for

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[1] Abbreviations: PAK, p21 activated protein kinase; eIF, eukaryotic initiation factor; JNK, Jun N-terminal activating kinase; TFA, trifluoroacetic acid; DMF, dimethylformamide; GTPγS, guanosine, 5′-*O*-(3-thiotriphosphate); PKA, cAMP-dependent protein kinase; PKC, Ca^{2+}/phospholipid-dependent protein kinase; Fmoc, 9-fluorenylmethoxycarbonyl-; DIPCDI, 1,3-diisopropylcarbodiimide.

[2] R. D. Rooney and J. A. Traugh, manuscript in preparation.

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phosphorylation by γ-PAK have been characterized using synthetic peptides patterned after the phosphorylation site identified as Ser 40 in the sequence PKKRKSGL in the Rous Sarcoma virus nuclear capsid protein NC, which is similar to γ-PAK sites identified in other proteins (*21*). A model peptide KKRKSAA was synthesized and the amino acids around the phosphorylation site in the model peptide were systematically substituted with other amino acid residues to determine the minimum phosphorylation sequence for γ-PAK. The rates of phosphorylation of the peptides by γ-PAK were compared with those obtained with two other protein kinases that require basic residues around the phosphorylation site, protein kinase A (PKA) and protein kinase (PKC). On the basis of the substrate specificity requirements, a synthetic peptide that could be useful in characterizing γ-PAK in complex mixtures of kinases such as crude extracts of cells or tissues has been identified.

EXPERIMENTAL PROCEDURES

Materials. γ-PAK was purified from rabbit reticulocytes to apparent homogeneity by chromatography on DEAE-cellulose, SP-Sepharose, protamine agarose, and FPLC on Mono S and Mono Q. Histone I (type IIIS), mixed histone (type IIAS), trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone treated), soybean trypsin inhibitor, bovine serum albumin (fatty acid free), and myelin basic protein were purchased from Sigma. Purified histones 2B and 4 were purchased from Boehringer-Mannheim. Myosin light chain from rabbit skeletal muscle was a gift from Dr. James Stull, University of Texas Southwestern Medical Center, Dallas, TX; rat prolactin was provided by Dr. Ameae Walker, University of California, Riverside, CA; and the purified catalytic subunit of cAMP-dependent protein kinase from bovine heart was a gift from Dr. William H. Fletcher, J. L. Pettis Memorial V. A. Hospital, Loma Linda, CA. Protein kinase C was purified from bovine brain according to the procedure of Walton et al. (*25*), with modifications (*26*). The clone for GST-Cdc42 was generously provided by Dr. Channing Der, University of North Carolina, Chapel Hill, NC. Cellulose thin-layer chromatography sheets were from Kodak.

The 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids, 1-hydroxy-7-azabenzotriazole (HOAt), and Fmoc−PAL−PEG−PS [[5-(4′-Fmoc-aminomethyl-3′,5′-dimethoxyphenoxy)valeric acid]−poly(ethylene glycol)−polystyrene resin] were purchased from Millipore Corp. Acetonitrile (HPLC grade), methanol (HPLC grade), glacial acetic acid, *N*,*N*-dimethylformamide (DMF), and anhydrous ethyl ether were obtained from Fisher Scientific. Liquified phenol was supplied by Mallinckrodt. Triisopropylsilane, piperidine, 1,3-diisopropylcarbodiimide (DIPCDI), and redistilled trifluoroacetic acid (TFA) were purchased from Aldrich Chemical Co.

Peptide Synthesis. Peptides were synthesized with an amide C-terminal group using a Millipore Model 9050 Plus peptide synthesizer using Fmoc solid phase peptide synthesis chemistries (*27*). A theoretical yield of ≈0.08 mmol of each peptide was synthesized on a PAL−PEG−PS resin using 4-fold excesses of the appropriate Fmoc-labeled amino acids and the HOAt/DIPCDI activating reagents. All amino acid coupling reactions were allowed to proceed for 60 min in DMF. A mixture of 20% (v/v) piperidine in DMF was used to remove Fmoc protecting groups during synthesis.

To cleave the peptide from the resin and remove side-chain protecting groups, ≈0.25 g of peptide containing resin (0.04 mmol of peptide) was placed in a vial with 3.0 mL of 88% (v/v) TFA, 5% phenol, 2% triisopropylsilane, and 5% water (*28*). This mixture was gently agitated for 3−3.5 h, and the mixture was poured into a sintered glass funnel to separate the liquid containing the peptide from the resin. The resin was washed with 2−3 mL of TFA; the wash was combined with the cleavage liquid and rotoevaporated to remove the majority of the TFA. The remaining liquid was injected into ice-chilled ethyl ether to precipitate the peptide. The crude precipitated peptide was washed several times with cold ether and dried under vacuum.

Peptide Purification and Characterization. Crude peptide was dissolved in water to make solutions with a nominal peptide concentration between 10 and 20 mg/mL. The desired peptide product was isolated by reversed phase HPLC (Model 2700 pump, Bio-Rad Laboratories) using a 10 by 250 mm C_{18} column with a 50 mm guard column (Rainin Instrument Co.). The peptide was eluted under isocratic conditions with a water−acetonitrile mobile phase containing 0.1% TFA and ≈5% acetonitrile. The desired peptide fractions were collected, pooled, and lyophilized.

The purity of the isolated peptide was established on the basis of peak areas as determined by capillary zone electrophoresis. All separations were performed with a BioFocus 3000 CE system (Bio-Rad Laboratories) in a 50 cm length of 50 μm i.d., 360 μm o.d., fused silica capillary (Polymicro Technologies Inc.) with 46.4 cm from inlet to the detector. The peptide sample was prepared by dissolving lyophilized peptide in 10 mM sodium phosphate, pH 2.5, to provide a solution with a nominal peptide concentration of 1.0 mg/mL. This solution was introduced into the capillary containing 100 mM sodium phosphate, pH 2.5, with a 1.0 psi s pressure pulse. A 200 V cm^{-1} electric field was used to drive the separation, and the analytical signal was monitored with an on-column absorbance detector at 200 nm. Integration of the resulting electropherograms using Biofocus Integration Software version 5.0 (Bio-Rad Laboratories) provided relative peak areas. All peptides displayed >98% purity on this basis.

A portion of the peptide solution was diluted 20-fold with a solution of methanol/water/acetic acid (50:50:3) and analyzed by electrospray ionization mass spectrometry (Vestec Model 201). This technique was used to establish that the mass of the collected peptide corresponded within experimental error to the calculated value for the desired peptide.

Phosphorylation Assays. Phosphorylation of protein substrates was carried out in 35 μL reaction mixtures containing 20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 30 mM 2-mercaptoethanol, 0.2 mM ATP (specific activity = 1000 cpm/pmol), 2 μg of protein substrate, and 20 units of active γ-PAK (an enzyme unit is defined as the amount of enzyme that incorporates 1 pmol of phosphate into histone IIAS/min at 30 °C). Under these conditions, phosphate incorporation was linear and initial rates of phosphorylation were measured. After incubation at 30 °C for 15 min, reactions were terminated by the addition of 5 μL of 100 mM nonlabeled ATP and sample buffer for gel electrophoresis (*29*). Reaction mixtures were subjected to SDS−PAGE on 15% polyacrylamide gels followed by autoradiography. Phosphorylation was quantified by excising the protein band

and counting the incorporated ^{32}P in a liquid scintillation counter. The specific activity of γ-PAK was 400 pmol min^{-1} μg^{-1} as reported previously using 0.2 mM ATP and 1 mg/mL mixed histone as substrate (*13*).

Assays with peptides (1 mM) were carried out with γ-PAK (1.6−5.6 units) in 25 μL reaction mixtures incubated for 30 min at 30 °C under conditions described above, except that 0.4 mg/mL of bovine serum albumin was added and the specific activity of ATP was 300−2000 cpm/pmol. Reactions were terminated with 5 μL of 100 mM ATP, and the amount of ^{32}P incorporated was quantitated by precipitation of 20 μL aliquots on P81 phosphocellulose paper with 75 mM H_3PO_4 as described previously (*30*). Peptides containing only one basic amino acid were analyzed by thin-layer electrophoresis on cellulose sheets for 2 h at 600 V with pyridine/acetic acid/water (10:100:890), pH 3.5, as solvent, as described previously (*30*). Activation of γ-PAK by autophosphorylation in the presence of Cdc42(GTPγS) was performed as previously described (*7*). γ-PAK was also activated by limited digestion with trypsin as described previously (*13*).

K_m and V_{max} values were determined from Lineweaver−Burk plots of the rate of phosphorylation at four to six different peptide concentrations and at a fixed concentration of ATP (0.2 mM). Kinetic values reported are typical of two to three independent determinations, and the standard error for all reported kinetic constants is <20%. The K_m for ATP was determined to be 65−80 μM at concentrations of 1−3 mM peptide. This K_m value with the peptides is lower than the K_m value obtained with mixed histone as substrate (*8*). Since the concentration of ATP used in these studies was significantly greater than the K_m for ATP, the reported V_{max} values approximate the real V_{max} values.

Reactions with PKA (10.5 units) were carried out under the same conditions as with γ-PAK except that 0.14 mM ATP was used. Reactions with PKC (3.4 units) were performed as with PKA, with the addition of 0.4 mM $CaCl_2$, phosphatidylinositol (15 μg/mL), phosphatidylserine (15 μg/mL), and 1,2-diolein (3 μg/mL), as described (*20*). A unit of PKA or PKC is defined as the amount of enzyme that incorporates 1 pmol of phosphate/min at 30 °C into histone IIAS or histone 1, respectively.

RESULTS

Basic Amino Acids as Recognition Determinants for γ-PAK. The phosphorylation site of the Rous and avian sarcoma virus nucleocapsid protein NC in vivo was identified as Ser 40 in the sequence PKKRKSL, and was shown to be the site phosphorylated by γ-PAK in vitro (*21*). On the basis of this phosphorylation site, which was similar to sites identified in several other proteins (*21*), the peptide KKRKSAA was synthesized and tested as a substrate for γ-PAK. It proved to be an efficient substrate with K_m and V_{max} values of 0.61 mM and 302 pmol min^{-1} μg^{-1}, respectively. The observed K_m and V_{max} values were within the range of values noted for synthetic peptide substrates with other serine/threonine protein kinases including casein kinases I and II, PKA, PKC, S6 kinases, Cdc2, p44 MAP kinase, and myosin light chain kinase (*30*−*45*).

Additional peptides based on the above sequence with amino acid substitutions on the N-terminal and C-terminal sides of serine were synthesized to define the amino acids around the phosphorylation site essential for γ-PAK. Rates of phosphorylation with γ-PAK activated by limited digestion with trypsin were measured and compared to the rate of KKRKSAA (Table 1). Specific basic residues on the N-terminal side of the phosphorylated serine were shown to be essential for recognition by γ-PAK. Basic amino acids on the C-terminal side, as in AAAASKR, were not involved in γ-PAK recognition/phosphorylation. Since γ-PAK is also activated by Cdc42(GTPγS), it was important to determine whether the phosphorylation/recognition sequence was the same using both methods of activation. As shown in Table 1, similar relative rates were obtained with the peptides when γ-PAK was activated by autophosphorylation in the presence of Cdc42(GTPγS). Peptides that were good substrates for γ-PAK activated by trypsin were also good substrates for γ-PAK activated by Cdc42(GTPγS). Inactive γ-PAK showed <5% of the activity of the activated forms with all peptides; thus, there was no effect of peptide on activation of the inactive enzyme.

Table 1: Relative Rates of Phosphorylation of Synthetic Peptides by γ-PAK[a]

	proteolytically activated		Cdc42-activated
peptide	^{32}P incorporated (pmol min^{-1} μg^{-1})	rel rate (%)	rel rate (%)
AKRESAA	750	247	295
PKRASAA	636	209	184
KKRASAA	586	193	181
AKRKSAA	548	181	158
EKRASAA	484	160	151
AKRASAA	426	141	189
ARRASAA	391	129	134
AKRASEA	326	108	88
AKRGSAA	381	126	nd
AKRASGA	324	107	nd
KKRKSAA	302	100	100
AARASAA	169	56	56
AKRPSAA	16	6	11
ARRASAA	16	6	9
AKAASAA	5	2	3
AKRASPA	0	0	3
RKKKSAA	0	0	3
KKAASAA	0	0	3
AKKASAA	0	0	1
AAKKSAA	0	0	0
AAKRSAA	0	0	0
AAAASKR	0	0	0

[a] Rates of phosphorylation of peptides (1 mM) were measured with γ-PAK (5.6 units) in 0.025 mL reaction mixtures incubated for 30 min at 30 °C. ^{32}P incorporation into peptides was determined as described under Experimental Procedures. γ-PAK was activated by autophosphorylation in the presence of Cdc42(GTPγS) or by proteolysis as described under Experimental Procedures. nd, not determined.

The influence of basic residues at the N terminus on the rate of phosphorylation was evaluated further by systematically varying the amino acids around the phosphorylation site. Although the peptide KKRKSAA was a good substrate, replacement of the lysine residues in either the −1 or −4 position with alanine as in KKRASAA or AKRKSAA resulted in an increase in the rate of phosphorylation to 193 and 181%, respectively. Replacement of lysine at the −1 and −4 positions simultaneously, as in AKRASAA, also resulted in a faster rate (141%) when compared to KKRKSAA, but slower than the single amino acid substitution in either the −1 or −4 position. When lysine in the −3 position of AKRASAA was replaced by alanine, the resulting peptide AARASAA was phosphorylated at a significantly slower rate

Table 2: Kinetic Parameters for Phosphorylation of Synthetic Peptides by γ-PAK: Influence of Basic Amino Acids[a]

peptide	K_m (mM)	V_{max} (pmol/min)	V_{max}/K_m (pmol min^{-1} mM^{-1})
AKRKSAA	0.48	2.34	4.8
KKRASAA	0.37	1.59	4.3
ARRASAA	0.65	2.11	3.2
KKRKSAA	0.61	1.84	3.0
AKRASAA	0.46	1.04	2.2
AARASAA	1.88	2.27	1.2
ARKASAA	>5	nd	nd
AKAASAA	>5	nd	nd

[a] V_{max} and K_m values were calculated from double-reciprocal plots of the initial rates and peptide concentrations. Rates of phosphorylation were determined using γ-PAK (1.6 units) and various concentrations of peptide (0−1.0 mM). nd, not detected.

(40 and 56%) as compared to AKRASAA and KKRKSAA, respectively. When arginine in the −2 position was replaced with alanine, as in AKAASAA, the peptide could not be phosphorylated by γ-PAK, suggesting that an important part of the recognition sequence is the arginine in the −2 position. In other studies, arginine in the −3 position (ARAASVA) phosphorylated at a slower rate (40%) compared to ARRASVA (100%).

The importance of the type of basic residue in the −2 and −3 positions was examined further. ARRASAA was as good a substrate as AKRASAA. suggesting that the −3 position can be either a lysine or an arginine when arginine is in the −2 position. However, AKKASAA and ARKASAA were poor substrates, suggesting that lysine in the −2 position was not tolerated by PAK I even though the −3 position was occupied by a basic amino acid. Since AARASAA and ARAASVA reacted to a small but significant extent, arginine in the −2 or −3 position, but not lysine in the −3 position, may be a minimum recognition determinant for γ-PAK. However, two basic residues in the −2 and −3 positions, with an absolute requirement for arginine in the −2 position, are more efficient for phosphorylation and more specific for recognition by γ-PAK.

To analyze further the variations in phosphorylation rates, kinetic constants for peptides with basic residues on the N-terminal side were measured as shown in Table 2. Peptides containing the sequence X(K/R)RXSAA, with X as alanine or a basic amino acid, varied only slightly in the measured K_m and V_{max} values. The K_m values (0.37−0.65 mM) were within the range observed with other protein kinases for synthetic peptides (*30*−*45*); the V_{max} values varied from 169 to 750 pmol min^{-1} μg^{-1}. Peptides with a third basic amino acid in the −1 or −4 position had the highest overall rate as shown by the V_{max}/K_m value, ∼2-fold higher than that of AKRASAA. Four basic amino acids on the N-terminal side, as in KKRKSAA, had a reduced V_{max}/K_m as compared to AKRKSAA or KKRASAA.

Effects of Glutamic Acid Residue. Using AKRASAA as a model peptide, the amino acids at positions −4, −1, and +1 were varied by replacement with acidic amino acid residues, since acidic residues at these positions have been shown to inhibit recognition/phosphorylation by other protein kinases that recognize basic residues. AKRESAA had the highest rate of phosphorylation among all peptides examined (Table 1). Glutamate residues in the −1 or −4 position, as in AKRESAA or EKRASAA, increased the rate of phosphorylation relative to AKRASAA to 175 and 113%, respectively, while a glutamate residue in the +1 position decreased the rate to 71% (Table 1). Glutamate in the −1 or +1 position increased the K_m by ∼3-fold, while the K_m was increased by 2-fold when glutamate was in the −4 position when compared to AKRASAA (Table 3). However, V_{max} values were highest with AKRESAA, 6-fold higher than with AKRASAA, which accounts for an increase in the overall rate (V_{max}/K_m) of ∼2.2-fold. There was a >2-fold increase in the V_{max} when glutamate was in the −4 or +1 position. Compared to AKRASAA, the overall rate of phosphorylation (V_{max}/K_m) was 1.3-fold with glutamate in the −4 position and 0.7-fold when glutamate was in the +1 position.

Table 3: Kinetic Parameters for Phosphorylation of Synthetic Peptides by γ-PAK: Influence of Acidic Amino Acids

peptide	K_m (mM)	V_{max} (pmol/min)	V_{max}/K_m (pmol min^{-1} mM^{-1})
AKRESAA	1.35	6.62	4.9
EKRASAA	0.89	2.48	2.8
AKRASAA	0.46	1.04	2.2
AKRASEA	1.42	2.22	1.6

Table 4: Kinetic Parameters for Phosphorylation of Synthetic Peptides by γ-PAK: Influence of Proline and Glycine[a]

peptide	K_m (mM)	V_{max} (pmol/min)	V_{max}/K_m (pmol min^{-1} mM^{-1})
PKRASAA	0.24	1.10	4.6
AKRASAA	0.46	1.04	2.2
AKRPSAA	>5	nd	nd
AKRASPA	>5	nd	nd
AKRGSAA	3.52	6.44	1.8
AKRASGA	2.09	3.49	1.7

[a] nd, not detected.

Effects of Proline and Glycine. Proline in the −1 or +1 position as in AKRPSAA or AKRASPA virtually abolished phosphorylation by γ-PAK (Table 1). This is not surprising because proline in these positions is a phosphorylation determinant for the proline-directed protein kinases. Proline in the −4 position, as in PKRASAA, decreased the K_m by ≈2-fold as compared to AKRASAA but had no effect on the V_{max}, resulting in an overall effect of a 2-fold increase in the phosphorylation rate (Table 4).

Glycine in the −1 position, as in AKRGSAA, increased the K_m by 7-fold over that of the peptide containing alanine at that position. At the same time the V_{max} was increased by 6-fold, leading to a slight overall decrease in the phosphorylation rate. Glycine in the +1 position increased the K_m by 4-fold and the V_{max} by 3-fold, with a slight overall decrease in rate (Table 4).

Comparison of Rates of Phosphorylation with PKA and PKC. PKA and PKC are known to require basic amino acids on the N-terminal side of the phosphorylatable serine/threonine. To distinguish their substrate specificities from that of γ-PAK, the relative rates of phosphorylation were determined with the synthetic peptide substrates using the conditions for γ-PAK, with the addition of Ca^{2+} and phospholipids for PKC. The rates shown in Table 5 are calculated with equivalent units of enzyme; the rate of phosphorylation of the peptide AKRASAA is taken as 100% for all three enzymes. As would be expected, PKA preferentially phosphorylated the peptides that contained RR or RK at the −3 and −2 positions and with basic residues

Table 5: Comparative Rates of Phosphorylation of Synthetic Peptides by γ-PAK, PKC, and PKA[a]

	^{32}P incorporated					
	PAK I		PKC		PKA	
peptide	pmol/unit	%	pmol/unit	%	pmol/unit	%
AKRESAA	47.2	176	0.4	12	1.8	38
PKRASAA	40.5	151	8.7	264	6.9	144
EKRASAA	30.2	113	0.1	3	1.7	4
AKRASAA	26.8	100	3.0	100	4.8	100
ARRASAA	25.0	93	18.8	570	137.1	2856
KKRKSAA	19.2	72	11.1	370	19.8	412
AKRASEA	18.3	68	0.2	6	3.6	75
AKRPSAA	1.2	5	3.9	118	10.3	215
ARKASAA	0.9	3	5.7	173	107.5	2240
RKKKSAA	0.1	0	3.1	94	5.4	112
AAAASKR	0	0	13.3	403	nd	nd

[a] nd, not determined.

Table 6: Relative Rates of Phosphorylation and Phosphorylation Sequences of Proteins with γ-PAK[a]

substrate	^{32}P incorporated (pmol)	rel rate (%)	phosphorylation sequence
histone 2B	11.0	100	YNKRST^{88}TI
histone 4	8.3	74	GVKRIS^{47}GL
myelin basic protein	4.2	37	APKRGS^{55}GK
prolactin	1.8	17	CLRRDS^{205}HK
protamine	0	0	RRRRSS^{8}SR
histone 1	0	0	none

[a] Sequences are for bovine histone 2B (*46*), bovine histone 4 (*47*), bovine myelin basic protein (*48*), rat prolactin (*49*), protamine from salmon (*50*), and bovine histone 1 (*51*). Sites phosphorylated by γ-PAK have been confirmed with histone 4.

or proline at the −4 and −1 positions inhibitory. PKC recognized SKR > RKS > KKS. The most notable difference between γ-PAK and PKA or PKC is the effect of acidic residues on the susceptibility of the peptide to be phosphorylated. An acidic residue in the −1 or −4 position was beneficial for PAK I but was detrimental for both PKA and PKC (Table 5). An acidic residue in the +1 position was detrimental to PKC and PKA but only slightly reduced the rate for γ-PAK. In addition, γ-PAK could not tolerate an arginine in the −3 position when lysine was in the −2 position. On the other hand, PKC and PKA phosphorylated ARKASAA much more quickly than AKRASAA.

Substrate Specificity of γ-PAK with Proteins. A number of proteins were examined as substrates, and their relative rates of phosphorylation were compared (Table 6). Histone 4 and histone 2B had the highest rates of phosphorylation, whereas histone 1 and protamine did not serve as substrates. Relative to histone 2B, myelin basic protein and prolactin had rates of 37 and 17%, respectively.

When the phosphorylation sequences of the substrates were examined (Table 6), all of the substrates phosphorylated by PAK I, including histone 4, histone 2B, myelin basic protein, and prolactin, contained two basic residues in the −2 and −3 positions, with R in the −2 position in every case, as represented by (K/R)RX(S/T). Those proteins containing KRXS were phosphorylated at higher rates than those containing RRXS. The site of phosphorylation has been determined in histone 4. Protamine and histone 1 were not substrates for γ-PAK, although protamine contains a potential phosphorylation site, RRSS.

DISCUSSION

The substrate determinants for phosphorylation by γ-PAK have been identified using a series of synthetic peptides patterned after the sequence KKRKSGL, which is the site phosphorylated in the nucleocapsid protein NC in vivo and in vitro by γ-PAK (*21*). The phosphorylation determinants have been shown in these studies to be two basic amino acids in the −2 and −3 positions, as represented by (K/R)RXS, where X can be an acidic, basic, or neutral amino acid. However, proline was shown to be inhibitory. When lysine is in the −2 position and arginine is in the −3 position, the peptide is not phosphorylated by γ-PAK, suggesting lysine in the −2 position is detrimental for γ-PAK recognition. Peptides with only a lysine at the −3 position (AKAASAA) did not serve as substrate for γ-PAK. However, an arginine in the −2 or −3 position can serve as a minimum recognition determinant since both AARASAA and ARAASVA were phosphorylated, although at a much slower rate (40%) compared to AKRASAA. The K_m of γ-PAK for synthetic peptides containing the phosphorylation sequence (K/R)RXS is 0.4−0.6 mM. An additional basic residue in the −4 or −1 position, such as KKRASAA or AKRKSAA, slightly improves the K_m or the V_{max}, resulting in higher V_{max}/K_m values. A fourth basic amino acid, as in KKRKSAA, is no better than the peptides with three basic residues. Interestingly, an acidic residue in the −1 or −4 position improves the rate of phosphorylation.

This phosphorylation sequence is similar to, but different from, those established for PKA and PKC. Lysine at −3 inhibits PKA and also PKC, although to a lesser extent, while both protein kinases can phosphorylate the peptide with lysine at −2 and arginine at −3 at a favorable rate. In contrast to γ-PAK, PKA and PKC cannot tolerate acidic residues in either the −1 or −4 position. Thus, peptides containing acidic amino acids in the −1 or −4 position such as AKRESAA or EKRASAA can be used to assay specifically for γ-PAK in crude extracts of cells or tissues. It is unlikely that calmodulin-dependent protein kinases and p90 rsk, which also recognize basic amino acids, will phosphorylate the above peptides. A survey of known protein substrates for calmodulin-dependent protein kinases and insulin-stimulated protein kinase 1 (ISPK-1), the mammalian homologue of p90 rsk, shows that they contain arginine in the −3 position; no substrates containing lysine in the −3 position or acidic residues in the −1 or −4 position have been identified (*52*, *53*).

Proline in the −1 or +1 position has a deleterious effect on γ-PAK recognition. The detrimental effect of proline is interesting since proline in the −1 or +1 position is critical for substrate phosphorylation by the proline-directed protein kinases, such as MAPK and cdc2, which are involved in signal transduction pathways that stimulate cell growth. Since γ-PAK has been shown to have cytostatic properties and appears to be involved in maintaining cells in a nondividing state (*12*, *13*), the hypothesis that sites phosphorylated by γ-PAK cannot be phosphorylated by the proline-directed protein kinases and vice versa is consistent with these observations.

Identification of potential substrates for γ-PAK is facilitated by a knowledge of the recognition/phosphorylation requirements. The phosphorylation sequence, (K/R)RXS, is found in substrates phosphorylated by γ-PAK, such as the

Rous sarcoma virus nucleocapsid protein NC, histone 4, histone 2B, myelin basic protein, and prolactin. Proteins that are not substrates for γ-PAK, such as casein, histone 1, cdc42, and rac-1 (data not shown), do not contain a potential phosphorylation site. Protamine contains RRXS as a possible phosphorylation site, but it is not a good substrate for γ-PAK. It is possible that the unique structure of protamine resulting from an abundance of arginine residues may block the predicted phosphorylation site from phosphorylation by γ-PAK and that secondary/tertiary structure may exert some influence on substrate specificity.

The identification of KRXS as a major and relatively specific phosphorylation sequence recognized by PAK I allows the identification of potential substrates for γ-PAK and targets research toward an examination of the functional role of γ-PAK in cytostasis.

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