Identifying Substrate Motifs of Protein Kinases by a Random Library Approach†

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ABSTRACT: Protein phosphorylation is an important posttranslational modification process that plays a crucial role in signal transduction. There are many protein kinases involved in cell signaling. However, substrate motifs of many protein kinases in signal transduction are not well-known. Traditional methodologies for identifying these motifs are often difficult and inefficient. In the present study, we developed a novel approach for discovering linear substrate motifs of protein kinases. This method is based on the screening of random synthetic combinatorial peptide libraries on beads where each bead expresses only one peptide entity [Lam et al. (1991) *Nature 354*, 82−84]. We chose cyclic AMP-dependent protein kinase (cAPK) as a model system in the present study since it is a well-studied enzyme. Random pentapeptide and heptapeptide libraries were screened with the addition of [γ-^{32}P]ATP and cAPK. ^{32}P-Labeled peptide−beads were then isolated for microsequencing. The identified peptide motif for cAPK was RRXS and is identical to that reported in the literature. Kinetic studies of the best three peptides indicate that they are efficient substrates for cAPK discovered from random synthetic combinatorial peptide libraries. Our results also suggest that this method is potentially useful for identifying substrate motifs of various protein kinases with high sensitivity and specificity. In addition, this method can also be used as a general method for identifying linear substrate motifs for various posttranslational modifications.

Phosphorylation is one of the major posttranslational modifications which regulates many cellular processes such as cell cycle, growth, and differentiation (Moodie et al., 1993; Davis, 1993; Wu et al., 1993; Gille et al., 1992; Eck et al., 1993; Zhou et al., 1993; Edelman et al., 1987; Hunter, 1987). Protein kinases which phosphorylate proteins play an important role in regulating cellular functions, especially signal transduction. For example, Ras signaling pathways involve a protein kinase cascade which transduces signals from the cell surface to the nucleus (Moodie et al., 1993; Egan et al., 1993). Protein tyrosine kinases (PTKs)[1] (e.g., Src family tyrosine kinases) are also very important in signal transduction (Cheng et al., 1992; Donella-Deana et al., 1992). Therefore, identification of the substrates or substrate motifs of various protein kinases is extremely important for the understanding of cellular functions. Traditionally, natural substrate motifs of protein kinases are discovered by sequencing phosphorylated cellular proteins. These proteins have to be identified, purified, sequenced, and analyzed for determination of the phosphorylation sites. Usually, an enormous amount of work is needed before a substrate motif for a certain protein kinase can be identified. In the present study, we developed a fast, efficient, and specific method to discover linear substrate motifs for protein kinases.

There are two major classes of protein kinases: serine/threonine kinase and tyrosine kinase. Cyclic AMP-dependent protein kinase (cAPK) is a serine/threonine kinase which phosphorylates many proteins through serine and/or threonine residues. The structure and function of cAPK have been well studied (Bramson et al., 1984; Krebs et al., 1985; Beebe & Corbin, 1986). In most eukaryotes, cyclic AMP (cAMP) acts by binding to the regulatory (R) subunit of cAPK. This results in dissociation of the R subunit from the catalytic (C) subunit, thereby activating the C subunit which in turn phosphorylates specific target proteins with ATP as a phosphoryl donor. Two major forms of mammalian cAPK (types I and II) have been characterized on the basis of DEAE-chromatography, cAMP analog specificity, and their ability to undergo autophosphorylation. The C subunits of the two forms are essentially identical in terms of their gross physical and enzymatic properties (Zoller et al., 1979). The structural and functional differences of these two forms are conferred by R subunits in terms of tissue distribution, amino acid sequence, interactions with C subunits, cAMP analog-binding properties, and the ability to be autophosphorylated (Jahnsen et al., 1985; Edelman et al., 1987).

The protein phosphorylation and protein kinase recognition sites have been extensively studied (Hanks et al., 1988; Taylor, 1989; Berry & Nishizuka, 1990; Pondaven et al., 1990; Kemp & Pearson, 1990; Heilmeyer, 1991). Many investigations indicate that protein kinases phosphorylate their target proteins at discrete sites. The primary sequence around the phosphorylation sites plays a crucial role in recognition of substrates by protein kinases (Kemp & Pearson, 1990). For example, RRXS* is for cAPK, RXXS*XR for protein kinase C, XS*RX for cyclic GMP

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[1] Abbreviations: cAPK, cyclic AMP-dependent protein kinase; cAMP, cyclic AMP; Fmoc, fluorenylmethyloxycarbonyl; MES, 2-(*N*-morpholino)ethanesulfonic acid; BSA, bovine serum albumin; PBS, phosphate-buffered salts; MAP, mitogen-activated protein; PTK, protein tyrosine kinase.

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(cGMP)-dependent protein kinase, KKRXXRXXS* for myosin light chain kinase, EXXS* for casein kinase I, and S*XXE for casein kinase II (the asterisk indicates phosphorylation residues). Susuki et al. (1990) reported the histone H1 kinase recognized a S*PKK motif. The basic amino acids (K or R) are often found around phosphoserine sites (Martinage et al., 1980; Von Holt et al., 1984). Aizawa et al. (1991) found the phosphorylation sites of a proline-rich region in the microtubule-binding domain of microtubule-associated proteins. However, recognition motifs are not known for most of the PTKs, which play an important role in signal transduction and cell proliferation.

Synthetic peptide substrates have proven extremely useful in the study of protein kinases. By using specific peptides as substrates, one may detect specific protein kinase activities even in crude cell extracts. The sensitivity, specificity, and availability of synthetic peptide substrates have made them particularly useful in protein kinase research. In the last few years, the random synthetic combinatorial peptide library method has been proven to be an extremely useful tool in the identification of peptide ligands for macromolecular targets (Geysen et al., 1986; Houghten et al., 1991; Lam et al., 1991). The synthetic peptide library method developed in our laboratory is called the Selectide Process (Lam et al., 1991). This method is based on a "one bead one peptide" concept where a random peptide library can be generated such that each resin−bead contains one peptide entity by a "split synthesis method". Using a colorimetric enzyme-linked detection method, the positive color beads that interact with the macromolecular targets are isolated, and each individual bead is then subjected to microsequencing for structure determination. In addition to using the colorimetric enzyme-linked method for detection, fluorescent tag or radioactive tag (Kassarjian et al., 1993) may also be used to probe the peptide library. We have successfully used this method to rapidly identify peptide ligands specific for antibody (Lam et al., 1991, 1992, 1993), streptavidin (Lam et al., 1991; Lam & Lebl, 1992), avidin (Lam & Lebl, 1992), gp2b-3a (Salmon et al., 1993), MHC-class I molecules (Smith et al., 1994), and specific proteases (Lam et al., unpublished results). In this paper, we report on the extension of this methodology in the characterization of substrate motifs for posttranslational modifications using cAPK as a model system.

MATERIALS AND METHODS

Synthesis of Random Libraries. The random synthetic peptide libraries (pentapeptide and heptapeptide, 19L) were synthesized by a "split synthesis approach" as previously described (Lam et al., 1991, 1992; Lam & Lebl, 1992; Furka et al., 1991). Poly(ethylene glycol)-grafted polystyrene beads or TentaGel beads with a substitution of 0.25 mmol/g and a diameter of 90 μm (Rapp Polymere, Trubingen, Germany) were chosen as solid phase support. It already has a hydrophilic poly(ethylene glycol) linker, and therefore additional linker is usually not needed for our study. Standard solid phase peptide synthesis method with fluorenylmethoxyloxycarbonyl (Fmoc) chemistry was used to synthesize the peptide libraries (Stewart & Young, 1984; Atherton & Sheppard, 1989). A total of 19 natural L-amino acids were used except for cysteine to avoid intramolecular cyclization or intermolecular disulfide formation. The resin was first divided into 19 aliquots and each reacted with a single Fmoc-amino acid. Coupling was initiated by the addition of a 3-fold molar excess of benzotriazolyl-*N*-oxytris(dimethylamino)phosphonium hexafluorophosphate (BOP), 1-hydroxybenzotriazole (HOBT), and diisopropylethylamine (DIEA). The coupling reactions were driven to completion with a 3-fold molar excess of Fmoc-amino acids and monitored by the standard ninhydrin test. Occasionally, double coupling was needed. Subsequently, the aliquots were washed, then mixed thoroughly, washed, deprotected by 20% piperidine, washed, and divided into 19 aliquots again for the next cycle of coupling. After five or seven cycles of "split synthesis" were completed, the side-chain protecting groups were removed by mixture K (82.5% trifluoroacetic acid, 5% water, 5% anisole, 5% *p*-cresol, and 2.5% ethanedithiol). The resin beads were then washed and stored in 0.01% HCl.

Peptide Synthesis and Purification. All peptides were synthesized by standard solid phase peptide synthesis techniques using Fmoc chemistry (Stewart & Young, 1984; Atherton & Sheppard, 1989). Rink resin (Advanced ChemTech) with a substitution of 0.4 mmol/g was used to obtain peptides with carboxyl amide. The Fmoc released was determined by spectrophotometry at 302 nm in order to examine if deprotection was completed. Three-fold molar excess of Fmoc-protected amino acids was used in coupling. The completed peptides were cleaved from the resin by mixture K for 120 min at room temperature. The cleaved peptides were precipitated by ethyl ether, washed, and lyophilized. The crude peptides were then purified by reverse phase HPLC (protein and peptide C_{18} column, Vydac). The purity of the peptides was assessed by analytical reverse phase HPLC (protein and peptide C_{18} column, Vydac) and cation exchange column (Mono S, FPLC, Pharmacia) and was determined to be >98% pure. Mass spectrometry analysis was employed to confirm the molecular weights of the desired peptides.

Phosphorylation of Random Peptide Libraries. The random peptide libraries (pentapeptide and heptapeptide, 19L) were used to screen for substrates of cAPK. [γ-^{32}P]-ATP was used as a tracer to detect phosphorylated peptides on beads. The peptide phosphorylation assay was performed in the MES buffer (pH 6.8) containing 30 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 10 mM magnesium chloride, and 0.4 mg/mL bovine serum albumin (BSA). cAPK (catalytic subunit from bovine heart, containing 0.06% protein) was purchased from Sigma Chemical Co., St. Louis, MO; [γ-^{32}P]ATP (specific activity: 25 Ci/mmol) was purchased from ICN Biomedicals, Irvine, CA. The beads from either pentapeptide or heptapeptide library were washed 5 times with MES buffer before screening. The phosphorylation reaction was conducted in MES buffer containing 1.8 μg/mL protein kinase, 0.1 μM [γ-^{32}P]ATP, and a library of 500 000 peptide−beads. The final volumes of the assay were doubled volumes of settled beads. The reaction was initiated by the addition of the protein kinase. After the reaction mixture was incubated at room temperature for 1 h on a shaker, the beads were then washed 10 times with phosphate-buffered salts (PBS, pH 7.2, containing 0.68 M NaCl, 13 mM KCl, 40 mM Na_2HPO_4, 7 mM KH_2PO_4, and 0.05% Tween 20).

Detection and Isolation of ^{32}P-Labeled Beads. The thoroughly washed library beads were suspended in 1.5% agarose (SeaPlaque agarose; FMC BioProducts, Rockland,

ME) solution at 70−75 °C and carefully poured onto a glass plate (16 × 18 cm) and air-dried overnight at room temperature. Glogos II autoradiogram markers (Stratagene, La Jolla, CA) were taped on each corner of a dried agarose plate prior to exposure in order to be able to later align the immobilized beads with the autoradiogram. The immobilized beads on the plate were then exposed to X-ray film (Kodak X-OMAT LS) for 20−30 h at room temperature. The film was developed. The agarose gel with embedded beads corresponding to the dark spots on the X-ray film was excised by a razor blade and swollen in double-distilled water and then carefully transferred to a tube with 5 mL of water. The agarose gel with beads was dissolved in the water using a 70−75 °C water bath. The beads were washed 5 times with PBS in order to further decrease noncovalent labeling. These beads were then subjected to secondary screening. The beads were suspended in 1.5% agarose solution, plated, dried, and exposed to X-ray films as described before. During the secondary screening, the beads were greatly diluted, and individual beads were relatively far away from each other so that a single ^{32}P-labeled peptide−bead can be precisely localized and extracted. Occasionally, a tertiary screening may be necessary in order to precisely localize and isolate individual beads. The area of the gel corresponding to the dark spot on the film was then swollen with 20 μL of water, and the single positive bead was dislodged from the agarose gel using a 25-gauge needle. The bead was then removed with a micropipeter, washed with water, transferred to a glass fiber filter, and inserted into a protein sequencer (Model 477A, Applied Biosystems, Foster City, CA) for structure determination as described previously (Lam et al., 1991; Lam & Lebl, 1992). Approximately 20−80 pmol of each amino acid residue should be recovered from an individual bead.

Phosphorylation Kinetics. The three best peptides obtained from library screenings and Kemptide (LRRASLG) were synthesized on Rink resin. The peptides were then cleaved from the resin and purified by HPLC. The peptide phosphorylation assay was conducted at 25 °C in a final volume of 100 μL of MES buffer (pH 6.8) in 96-well plates. The concentration of cAPK was fixed at 0.6 μg/mL. The concentration range of the peptides used was from 0.2 to 25 μM; the ATP concentration range was from 1 to 100 μM. [γ-^{32}P]ATP (25 Ci/mmol) was used as a tracer to detect phosphorylation of peptides. Unlabeled ATP (magnesium salt, Sigma Chemical Co.) was added to [γ-^{32}P]ATP to obtain a final specific activity of 0.25 Ci/mmol. cAPK was added to the mixture of [γ-^{32}P]ATP, MgATP, and peptide in order to initiate the phosphorylation reaction. The time course of phosphorylation was examined. For determination of kinetic parameters, initial velocities of phosphorylation were measured by terminating the reaction at exactly 1 min by adding 100 μL of 150 mM phosphoric acid and 2 mM unlabeled ATP. Unlabeled ATP helps to reduce nonspecific binding of [γ-^{32}P]ATP to cation exchange paper. The stopped reaction mixtures (20 μL) were then spotted onto Whatman P81 cation exchange phosphocellulose paper (2 × 2 cm) and washed 4 times with 75 mM phosphoric acid (3 min each time) by gentle rocking to remove all free [γ-^{32}P]ATP. The paper was dried by a heat-gun, cut, and counted by a liquid scintillation analyzer (Model 1500; Packard Instrument Co., Downer Grove, IL).

Determination of Phosphorylation Sites. Phosphorylation of peptides SQRRFST and YRRTSLV was performed under similar conditions as described above. However, no [γ-^{32}P]-ATP was used in the assays. The reaction mixtures contained 800 μM peptides, 2 mM unlabeled ATP, and 1.8 μg/mL cAPK, and the total assay volume was 1 mL of MES buffer. The reaction mixtures were sterilized by a 0.45 μm filter and incubated at room temperature for 24 h. The cation exchange column filled with S-Sepharose fast flow (250 μL, Pharmacia, Sweden) was used to separate the phosphorylated peptides (positively charged) from the protein kinase and excess ATP. S-Sepharose was washed with PBS, 5 times, and then equilibrated with 75 mM H_3PO_4. The stopped reaction mixtures were loaded into the column and washed using 10 volumes (2.5 mL) of 75 mM H_3PO_4. The phosphopeptides were then eluted from the column by 100 mM NaCl solution (pH 9). The eluted phosphopeptides were analyzed by a mass spectrometer (LC/MS/MS system, Model API *III*; Perkin-Elmer Sciex Instruments, Thornhill, Ontario, Canada). The phosphopeptides were further purified by reverse phase HPLC (protein and peptide C_{18} column, Vydac). The purified phosphopeptides were subject to either trypsin digestion or acid hydrolysis. For trypsin digestion, the phosphopeptides were incubated with 20 μg of trypsin in 100 μL of 50 mM NH_4HCO_3 buffer (pH 8.0) at 37 °C for 3.5 h. Then 20 μg of trypsin was added, and the reaction mixtures were continued to incubate at 37 °C for 3.5 h. Trypsin was removed by an Ultrafree-MC filter (5000 NMWL; Millipore Corp., Bedford, MA). The digested phosphopeptides were analyzed by the mass spectrometer. For acid hydrolysis, the phosphopeptides were hydrolyzed at 110 °C for 3 h in 200 μL of 6 N HCl, lyophilized, and subjected to quantitative amino acid analysis by a Beckman 7300 dedicated amino acid analyzer (Beckman Instruments). In addition, the phosphopeptides were also sequenced using the protein sequencer (Model 477A, Applied Biosystems).

Data Analysis. The kinetic data were analyzed using a random Bi-Bi reaction model. The maximal velocities and Michaelis constants for peptides and ATP were estimated by a computer program called GraFit (Erithacus Software Ltd., Staines, U.K.). The standard errors were calculated by this program.

RESULTS

Screening of Random Peptide Libraries. Kemptide (LRRASLG) and its analog (LRRAALG) were synthesized on TentaGel beads and used to test the sensitivity and specificity of our method for identifying substrate motifs of protein kinases prior to library screening. Kemptide is the most widely used synthetic substrate for cAPK, and it can be efficiently phosphorylated with a K_m = 16 μM and a V_{max} = 20 μmol min^{-1} mg^{-1} (Kemp et al., 1977). Kemptide was used as a positive control in the current study. When the serine of Kemptide is replaced by alanine, this analog (LRRAALG) is no longer a substrate for cAPK and can be used as a negative control. Figure 1A,B shows cAPK-dependent phosphorylation of Kemptide−beads. The strongly labeled beads were found in the presence of cAPK and [γ-^{32}P]ATP (Figure 1A), but no labeling was found in the presence of [γ-^{32}P]ATP alone (Figure 1B). A total of 5 or 10 Kemptide−beads were mixed with approximately 1000 negative control beads prior to the addition of cAPK and [γ-^{32}P]ATP. The bead mixtures were then incubated with 1.8 μg/mL cAPK and 0.1 μM [γ-^{32}P]ATP at room temperature for 1 h. As shown in Figure 1C,D, 5 and 10 extremely

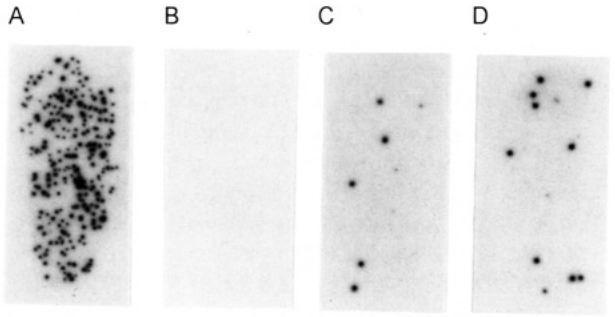

FIGURE 1: Autoradiographs of cAPK-dependent phosphorylation of Kemptide−beads. (A) Approximately 300 Kemptide−beads (LRRASLG-TentaGel) were incubated with 1.8 μg/mL cAPK and 0.1 μM [γ-^{32}P]ATP at room temperature for 1 h and washed. (B) Approximately 300 Kemptide−beads were incubated with 0.1 μM [γ-^{32}P]ATP at room temperature for 1 h in the absence of cAPK and washed. (C) Five Kemptide−beads were mixed with approximately 1000 Kemptide analog−beads (LRRAALG-TentaGel); then the mixture was incubated with 1.8 μg/mL cAPK and 0.1 μM [γ-^{32}P]ATP at room temperature for 1 h and washed. (D) Ten Kemptide−beads were mixed with approximately 1000 analog−beads of Kemptide, and the mixture was incubated as described in (C). In all cases, the beads were immobilized by 1.5% agarose and exposed to X-ray film.

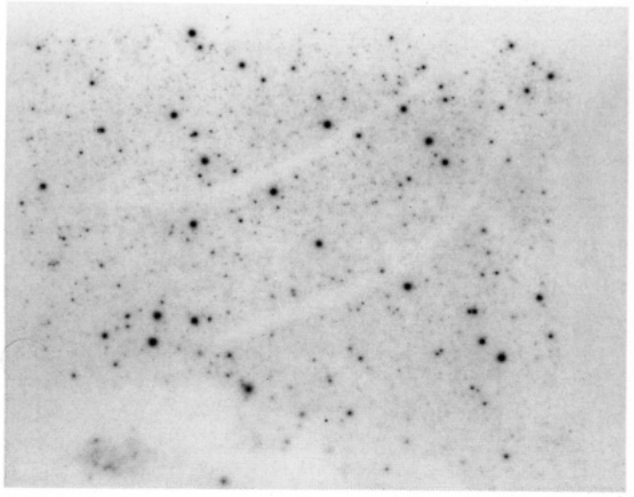

FIGURE 2: Screening of a random synthetic peptide library. The heptapeptide library containing approximately 500 000 beads was incubated with 1.8 μg/mL cAPK and 0.1 μM [γ-^{32}P]ATP at room temperature for 1 h and washed. The beads were immobilized in 1.5% agarose and exposed to X-ray film. Approximately 60 extremely dark spots were found on the autoradiogram. Some less dark spots were also observed.

dark spots (positive beads) were easily identified from the background of negative control beads on the autoradiograms. Several faint spots shown in Figure 1C,D might be artifacts from noncovalent binding of [γ-^{32}P]ATP to the beads or to other contaminated particulate impurities. These data suggest that this method can be used to detect the phosphorylation of solid phase bound peptides with high sensitivity and specificity.

The random pentapeptide and heptapeptide libraries, each having 500 000 beads in total, were screened with the method described above. Figure 2 shows the representative autoradiogram of the primary screening of a heptapeptide library of 500 000 beads, and approximately 60 deep dark spots which were significantly different from the background were detected on the autoradiogram. The three darkest spots were collected and subjected to secondary screening to isolate ^{32}P-labeled beads from surrounding unlabeled beads. After the secondary screening, three ^{32}P-labeled beads were detected on the autoradiogram (data not shown). These beads were relatively far away from other beads and easily localized and extracted for sequence determination. Similarly, two ^{32}P-labeled bead were isolated from the pentapeptide library (data not shown). However, for some unknown technical reasons, the sequence of one of the pentapeptide beads was not detectable. Table 1 lists the sequences of these four peptide−beads identified from the random synthetic peptide library (one from the pentapeptide library and three from the heptapeptide library). The results indicate all four peptides contain the motif RRXS, which is identical to that reported in the literature for cAPK (Kemp & Pearson, 1990).

Table 1: Peptide Substrates for cAPK Discovered by Screening Random Synthetic Combinatorial Peptide Libraries

library[a]	no. of positive beads[b]	sequence[c]
penta	55	**RR**YS**V**
hepta	60	SQ**RR**FS**T**
		Y**RR**TSL**V**
		II**RR**KS**E**

[a] Each library contains approximately 500 000 peptide−beads. [b] Only extremely dark spots are considered as positive beads in this case. [c] The two best beads from the penta library were selected for amino acid sequencing analysis (one of them was not sequenceable); the three best beads from the hepta library were selected for amino acid sequencing analysis.

Initial Velocity Kinetics of Phosphorylation. The three identified peptides and Kemptide were synthesized on Rink resin and cleaved as free peptides (carboxyl amide). The initial velocity of phosphorylation was measured in order to estimate kinetic constants of the peptides. The steady-state initial velocity (v) for bireactant, enzyme-catalyzed reactions in the absence of products is given by the expression (Cleland, 1970):

$$v = \frac{V_{max}AB}{K_{ab} + K_bA + K_aB + AB}$$

Here A and B are the substrate concentrations of peptides and ATP, respectively. V_{max}, K_a, K_b, and K_{ab} are kinetic parameters. V_{max} is the maximum initial velocity of the reaction. K_a and K_b are Michaelis constants for the peptide and ATP, respectively. K_{ab} is the complex constant. The initial velocity (v) is the average rate of the initial reaction in the linear range. Figure 3 illustrates the reaction time courses for the phosphorylation of peptides SQRRFST and YRRTSLV. The reaction rates were constant within the first 4 min. For determination of the parameters of initial velocity kinetics, the reactions were terminated at exactly 1 min to ensure the measurements of initial velocities for all the subsequent experiments. Figure 4A shows the initial velocities of phosphorylation of SQRRFST peptide at four peptide concentrations for each of four ATP concentrations. It is clear that V_{max} increased with ATP concentrations. The doube-reciprocal plots of the raw data are illustrated in Figure 4B. To determine kinetic parameters, the data were analyzed by GraFit using least-squares analysis. Several reaction models were tested to fit the data. It turned out that the best fitted mechanism was a Bi-Bi reaction model. All fitted lines in Figure 4A,B were given by this model. Table 2 lists the amino acid sequence and kinetic parameters of each peptide. In every case, the complex constant K_{ab} was not

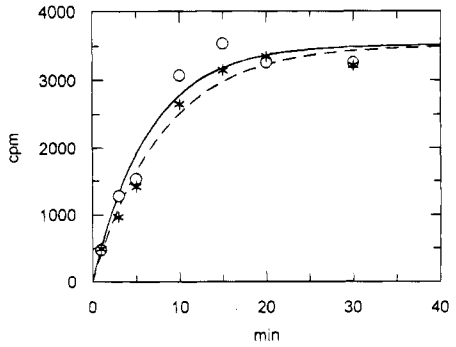

FIGURE 3: Time course. Phosphorylation assays were performed in the presence of 0.6 μg/mL cAPK, 5 μM [γ-^{32}P]ATP, and 1 μM SQRRFST (○) or YRRTSLV (*). The reaction was terminated at 1, 3, 5, 10, 15, 20, and 30 min by addition of an equal volume of a mixture of 150 mM H_3PO_4 and 2 mM unlabeled ATP. The solid line is the fitted line for SQRRFST; the dashed line is the fitted line for YRRTSLV. y-axis: cpm.

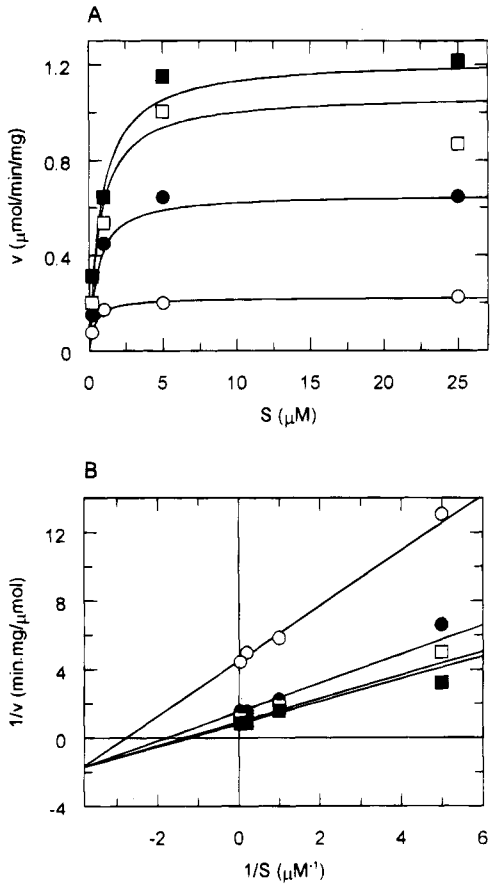

FIGURE 4: Initial velocities of phosphorylation of SQRRFST and double-reciprocal plots. (A) All phosphorylation reactions were carried out at 25 °C, and the concentration of cAPK was fixed at 0.6 μg/mL. The reactions were terminated at 1 min after adding the enzyme. Steady-state initial velocities (v) were determined as the average velocities during this period. The concentrations of peptide (S) were 0.2, 1, 5, and 25 μM. (B) Double-reciprocal plots of steady-state initial velocity data in (A). In both (A) and (B), the concentrations of ATP were 1 (○), 5 (●), 25 (□), and 100 μM (■). All lines are least-squares fits of the data according to the equation described in the text.

zero. This finding is consistent with a common Bi-Bi reaction mechanism involving the binding of both peptide and ATP to the enzyme so that a ternary complex is present at catalysis. Further, it indicates that the ping-pong mechanism is not consistent with the present data. In a ping-pong mechanism, there is no such ternary complex formed since the phosphate is transferred to the enzyme from ATP and ADP is released prior to the binding of the peptide. In such a mechanism, K_{ab} will be zero. Interestingly, the RRYSV peptide screened from a random library has a lower K_a (0.67 μM) and a higher V_{max}/K_a (2.52 L min^{-1} mg^{-1}) for cAPK than Kemptide (K_a = 1.33 μM and V_{max}/K_a = 1.83 L min^{-1} mg^{-1}) which is derived from the phosphorylation site of the natural protein substrate.

Determination of Phosphorylation Sites. All four peptides identified in the present paper contain a motif of RRXS which is specific for cAPK. However, two of the peptides (SQRRFST and YRRTSLV) also have additional threonine and/or serine residues which may be the potential phosphorylation sites by cAPK since it is a serine/threonine protein kinase. There is a threonine residue in both SQRRFST and YRRTSLV. In addition, SQRRFST contains a second serine at the amino terminus. It is important to determine which amino acid residue(s) in these two peptides was (were) phosphorylated by cAPK. The phosphorylation reactions were carried out using the method described above, and only unlabeled ATP was added to the reaction mixtures. After 24-h incubation, the phosphorylated peptides were purified by either cation exchange columns or reverse phase HPLC and subject to mass spectrometry, amino acid analysis, and microsequencing analysis. Figure 5 shows the mass spectrometry analyses for SQRRFST and YRRTSLV. Prior to the phosphorylation of SQRRFST, only a single peak was observed at 880.5 daltons which matches exactly the molecular mass of this peptide (Figure 5A). After the phosphorylation, the major peak was shifted to 960.5 daltons (Figure 5B). The difference between these two peaks was 80 daltons which corresponded to the molecular mass of one phosphate. The peak at 982.5 daltons matched the molecular mass of the phosphopeptide plus sodium ion since NaCl solution was used to elute the phosphopeptide from the column. In addition, several small peaks were found after the phosphorylation (Figure 5B). The minor peaks at 1096.6 and 1118.6 daltons did not match the molecular mass of the parent phosphopeptide or phosphopeptide·Na containing two or three phosphates. These two peaks were probably contaminants from the phosphorylation reaction mixture. Similar results were obtained for the phosphorylation of YRRTSLV (Figure 5C,D). These data suggest that only one phosphate was transferred to either SQRRFST or YRRTSLV. Mass spectrometry of trypsin-digested phosphopeptides and microsequencing analysis indicated that for SQRRFST the serine residue at the N-terminus was not phosphorylated and for YRRTSLV the threonine residue was not phosphorylated (data not shown). Further, quantitative amino acid analysis demonstrated that the phosphorylated residue was serine rather than threonine (data not shown). Combining the data from mass spectrometry, microsequencing, and amino acid analysis, it was strongly suggested that serine in the RRXS motif was indeed the phosphorylation site for these two peptides.

DISCUSSION

Posttranslational modification is a common mechanism in regulating protein functions. Approximately 200 derivatized amino acids are found to be naturally present in proteins (Yan et al., 1989; Han & Martinage, 1992a,b; Krishna et al., 1993). The important posttranslational modification processes include phosphorylation, acetylation, sulfation,

Table 2: Comparison of Kinetic Parameters of Peptide Substrates Identified from Random Peptide Libraries with a Known Substrate, Kemptide (LRRASLG)[a]

sequence	V_{max} (μmol min^{-1} mg^{-1})	K_a(peptide) (μM)	K_b(ATP) (μM)	K_{ab} (μM^2)	V_{max}/K_a (L min^{-1} mg^{-1})
RRYSV	1.69 ± 0.09	0.67 ± 0.16	3.60 ± 0.57	0.45 ± 0.68	2.52
SQRRFST	1.31 ± 0.06	0.83 ± 0.16	5.99 ± 0.92	1.38 ± 1.65	1.58
YRRTSLV	2.82 ± 0.07	1.42 ± 0.11	6.20 ± 0.42	0.41 ± 0.57	1.99
LRRASLG	2.44 ± 0.08	1.33 ± 0.16	7.29 ± 0.89	0.88 ± 1.52	1.83

[a] The three best peptides identified were selected for kinetic studies.

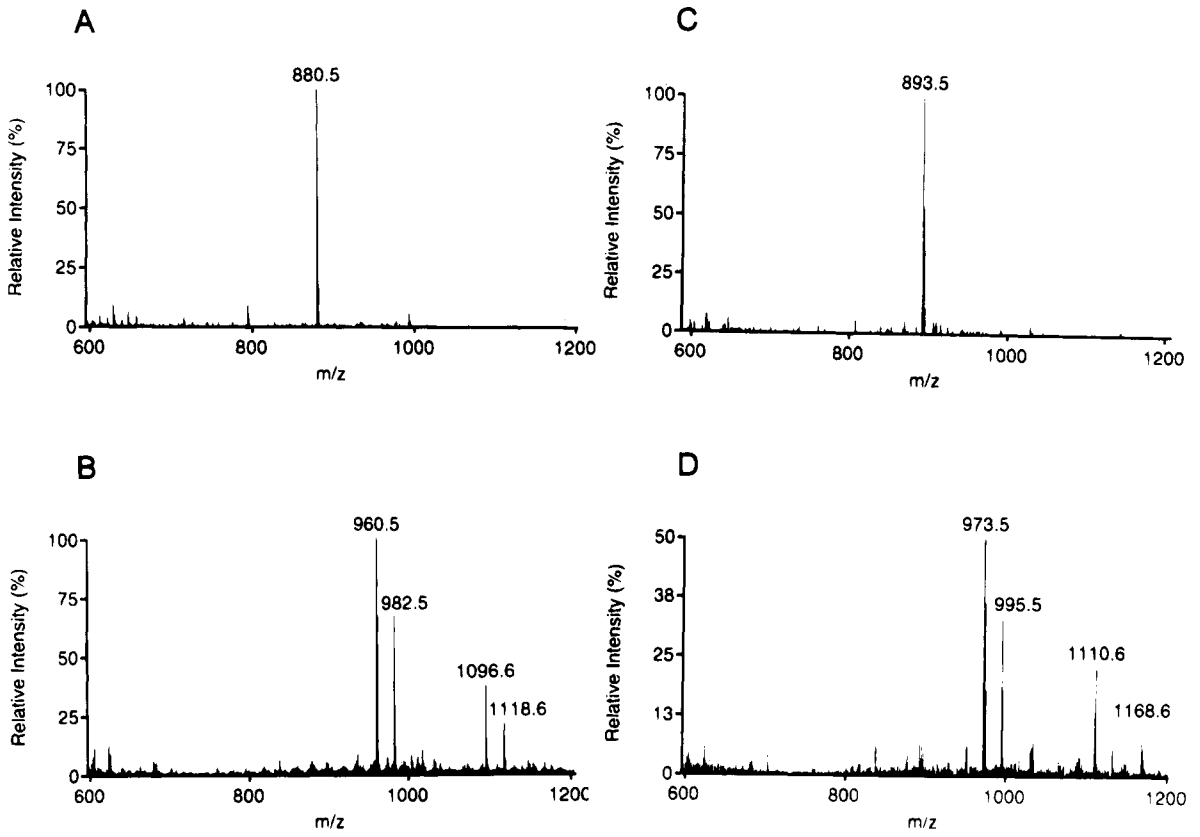

FIGURE 5: Mass spectrometry of the peptides. (A) A single peak at 880.5 daltons was found for SQRRFST prior to phosphorylation. This peak is consistent with the molecular mass of SQRRFST. (B) After the phosphorylation of SQRRFST, the major peak was shifted to 960.5 daltons. The difference between this peak and the peak for SQRRFST is 80 daltons, which is the molecular mass of the covalently bound phosphate. The peak at 982.5 daltons corresponds to phospho-SQRRFST·Na. Several minor peaks were also found by mass spectrometry (see the explanation in the text). (C) A single peak was found at 893.5 daltons for YRRTSLV prior to phosphorylation. This corresponds to the molecular mass of this peptide. (D) After the phosphorylation of YRRTSLV, the major peak was shifted to 973.5 daltons. The difference between the two major peaks in (C) and (D) is 80 daltons, which matches the molecular mass of one phosphate. The peak at 995.5 daltons corresponds to phospho-YRRTSLV·Na. Several minor peaks were also observed in (D) (see details in the text). *x*-axis: ratio of mass (m) to charge (z). In the above cases, $z = 1$. Thus, m/z is consistent with the molecular mass of the tested compounds. *y*-axis: relative intensities (%) of different components in a specific compound.

methylation, hydroxylation, ADP-ribosylation, amidation, carboxylation, adenylation, glycosylation, ubiquitination, and prenylation. Each posttranslational modification plays an important role in biological systems. The protein recognition sites for these modifications are often located in some specific regions containing relatively short, critical amino acid sequences. For example, GX-Hyl-GXR is a sequence motif for the hydroxylysine-linked carbohydrate units, and NXS-(T) is a sequence motif for N-glycosylation of asparagine. These motifs are often derived from sequencing naturally occurring modification sites in proteins. It usually takes a long time to identify substrate motifs which are critical to such modifications. Consistent motifs may not appear until many natural protein substrates have been sequenced.

In the present study, we developed a method which can quickly and specifically identify linear recognition motifs for phosphorylation by protein kinases. By applying it to the cAPK system, we demonstrated that this method is very specific and highly sensitive for identifying cAPK substrate motifs. Kemptide (LRRASLG) and its nonsubstrate analog (LRRAALG) were used as positive and negative controls to determine the specificity and sensitivity of this method. Figure 1C,D shows Kemptide−beads can be easily identified from the background of approximately 1000 nonsubstrate analog−beads. Since Kemptide is highly positively charged with two arginines, labeling of Kemptide−beads by [γ-^{32}P]-ATP may result from noncovalent interactions (e.g., charge effect) between [γ-^{32}P]ATP and Kemptide−beads. However, Figure 1A,B argues against this and demonstrates that labeling of Kemptide−beads was indeed cAPK-dependent. Significant labeling was found only in the presence of cAPK. We screened random pentapeptide and heptapeptide libraries, each containing 500 000 beads, by this approach. Since only 19 amino acids were used in each coupling, the number of

permutations for a pentapeptide library is 19^5 or 2.48×10^6, and for a heptapeptide library, it is 19^7 or 8.94×10^8. Approximately 500 000 beads were used in each screening; therefore, only a small fraction of the total number of possible permutations was screened. From our experience with other targets such as monoclonal antibodies (Lam et al., 1991, 1992, 1993, 1994), MHC-class I molecules (Smith et al., 1994), streptavidin (Lam et al., 1991; Lam & Lebl, 1992), and avidin (Lam & Lebl, 1992), the number of contact residues usually ranged between 3 and 5 and with various spacing in between. For cAPK, there are only three contact residues (RRXS). Therefore, 500 000 beads are more than enough beads necessary for the identification of the RRXS motifs, as clearly demonstrated in this paper. In fact, this motif was identified after sequencing only 5 of the 115 ^{32}P-labeled peptide beads (55 from the pentapeptide library and 60 from the heptapeptide library) (Table 1). This motif has been shown as an efficient motif required for cAPK-catalyzed phosphorylation in various studies (Kemp & Pearson, 1990; Kemp et al., 1991). As shown in Table 1, about 55 and 60 extremely dark spots were found on the autoradiograms of pentapeptide and heptapeptide libraries, respectively. All peptide−beads corresponding to these extremely dark spots probably have the motif of RRXS. As shown in Figure 2, in addition to 60 extremely dark spots, there were some less dark spots found on the autoradiogram by comparing to the background. These are probably due to less efficient motifs for cAPK such as RXS, RXXS, and KRXXS (Kemp & Pearson, 1990). These results suggest that our method can be used for screening linear substrate motifs for cAPK with high specificity and efficiency. However, it should also be considered that factors other than the primary sequence may also play an important role in the determination of substrate specificity for protein kinases, e.g., secondary and tertiary structures of protein substrates.

Kemptide is an excellent synthetic peptide substrate for cAPK. It is derived from the phosphorylation site of pyruvate kinase (Hjelmquist et al., 1974) and has values of V_{max} and K_m approaching the natural substrate (Zetterqvist et al., 1976; Kemp et al., 1977). Kemptide has been reported with a K_m of 16 μM for cAPK isolated from beef skeletal muscle (Kemp et al., 1977). Pommerantz et al. indicated that Kemptide has a K_m of 63 μM for cAPK purified from calf thymus. In the present study, the K_m of Kemptide with carboxyl amide is 1.32 μM for cAPK purified from bovine heart (purchased from Sigma Chemical Co.). Table 2 lists kinetic parameters of the peptides determined from screening the random libraries. By comparison to the kinetic parameters of Kemptide, RRYSV has a better affinity for cAPK than Kemptide in terms of K_a and V_{max}/K_a. Pseudosubstrates, on the basis of the primary sequences of the substrate motifs, have already been developed as inhibitors for different protein kinases (Kemp et al., 1991). Therefore, identification of efficient substrates from screening of random synthetic libraries may provide important information for the design of inhibitors for various protein kinases with high potency and specificity.

Kemptide was not identified in our screening. It could be either that it was not present in the 500 000 beads that had been screened or that it might be present in the positive beads that we did not sequence. The sampling statistics are based on Poisson distribution. Theoretically, if we want to look for better substrates for cAPK, we could synthesize a secondary library with the following structure: XXXR-RXSX. This library can then be screened under a more stringent condition, for example, by down-substituting the amount of peptides on the surface while retaining enough peptides in the interior of the bead for microsequencing. This differential loading between the surface and the interior of the bead can be accomplished by the shaving technique that was described by our group (Vagner et al., 1994). This sequential screening approach has already been proven in the anti-insulin monoclonal antibody system (Lam et al., 1994).

cAPK is a serine/threonine kinase. For peptides SQR-RFST and YRRTSLV, there are several potential phosphorylation sites since the former peptide has two serines and one threonine and the latter has one serine and one threonine. One may argue that the high affinities of these peptides may be due to existing multiple phosphorylation sites. It is necessary to determine which amino acids are the phosphorylated residues. Mass spectrometry, amino acid sequencing, and amino acid analysis were employed in the current study to detect the phosphorylated amino acid residue(s). Mass spectrometry analyses indicate that only one phosphate has been transferred to each of the above peptides during phosphorylation by cAPK (Figure 5). Due to technical difficulties with the double arginine residues in a peptide, we were unable to obtain complete fragments of the phosphopeptides from mass spectrometry analysis. The data from fragmentation analysis only indicated that the serine at the N-terminus of SQRRFST was not the phosphorylation site (data not shown). Mass spectrometry of trypsin-digested SQRRFST suggested that the SQR fragment did not contain a phosphate (data not shown). Amino acid sequencing of YRRTSLV has shown that tyrosine and threonine residues were not phosphorylated. Therefore, the only possible phosphorylation sites of these two peptides were the serine residues with the RRXS motif. This was confirmed by amino acid analysis. Quantitative amino acid analysis of the phosphopeptide hydrolysates showed that the phosphorylation site was indeed the serine residue and no phosphothreonine was detected (data not shown). Combining all these data together, we can confidently say that the serine residue at the sixth position from the N-terminus of SQR-RFST and the serine residue at the fifth position from the N-terminus of YRRTSLV were the phosphorylation sites. These results were consistent with the data reported in the literature that the serine residue within the RRXS motif is the phosphorylation site by cAPK (Kemp & Pearson, 1990).

In a very recent study, synthetic peptides were employed to probe protein kinase substrate specificity by Till et al. (1994). They used Kemptide, with the known recognition motif of RRXS for cAPK, as a parent compound, and amino acids were randomized only at 1 residue position (LXRASLG, X indicates the randomized position), resulting in 19 variants of Kemptide. Phosphorylation specificities of the 19 variants were determined by phosphopeptide-selective mass spectrometry. However, only very limited peptides can be screened by their method. In contrast, our method described in this paper allows us to screen random synthetic peptide libraries containing literally millions of different peptide entities. By applying our method, one can potentially identify new substrate motifs for various protein kinases.

Since unnatural amino acids can easily be incorporated into synthetic libraries, our method can also be used to screen

for protein kinase substrates containing unnatural amino acids (e.g., D-amino acids). The peptides consisting of unnatural amino acids have a number of advantages over the peptides consisting of 20 natural amino acids. For instance, unnatural amino acid-containing peptides resist proteolysis by enzymes. Furthermore, it is theoretically feasible that we may identify novel nonpeptide substrates for protein kinases by screening small organic or nonpeptide libraries (Lebl et al., 1994; Nikolaiev et al., 1993).

Protein kinases play an important role in signal transduction pathways. For example, mitogen-activated protein (MAP) kinases are important intermediates in signal transduction pathways initiated by many types of cell-surface receptors (Egan et al., 1993). Ras signaling pathways involve a protein kinase cascade which includes Raf-1, MAP kinase kinase, and MAP kinase (Moodie et al., 1993). This cascade transduces signals from Ras to the nucleus. Raf-1 and MAP kinase are serine/threonine kinases. However, specific substrate motifs of these kinases are not well-known. Previous studies indicate that a proline-rich motif (e.g., P-X-S/T-P) may be the phosphorylation site for MAP kinases (Gonzalez et al., 1991; Clark et al., 1991). This motif was identified by protein sequence analysis in the absence of information about the complete primary structure of the substrates. Protein tyrosine kinases have received a lot of attention in the last decade as one of the major enzymes in signal transduction pathways involved in both growth factors and oncogenes (Fantl et al., 1993; Heidecker et al., 1992; Davies et al., 1992). The sites of tyrosine phosphorylation by protein kinases (e.g., $p60^{c\text{-}src}$) have recently been reviewed (Pearson et al., 1991). Unlike cAPK where the substrate motifs are known, no specific motifs have been identified for various tyrosine kinases. Synthetic peptides based on the primary sequence of the limited number of known protein substrates had been synthesized and found to be rather inefficient substrates for tyrosine kinases (Kemp & Pearson, 1991). By applying the method described in this paper, one can potentially screen synthetic libraries containing millions of peptides for recognition motifs of various protein kinases. Preliminary data in our laboratory already demonstrated that novel, efficient, and specific substrates for tyrosine kinases, such as $p60^{c\text{-}src}$, can be rapidly identified using this approach. Besides protein phosphorylation, this method can potentially be used for the identification of other posttranslational modification sites provided that an appropriate radiolabeled donor is available. Even if radiolabeled donors are not available, one may still be able to detect the covalently modified sites by antibodies that recognize these modified sites.

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