Determination of Cyclic Nucleotide-Dependent Protein Kinase Substrate Specificity by the Use of Peptide Libraries on Cellulose Paper†

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ABSTRACT: An iterative approach to the a priori determination of the substrate specificity of cAMP- and cGMP-dependent protein kinases (PKA and PKG) by the use of peptide libraries on cellulose paper is described. The starting point of the investigation was an octamer library with the general structure Ac-XXX12XXX, where X represents mixtures of all 20 natural amino acids and 1 and 2 represent individual amino acid residues. The library thus contained all possible 2.56×10^{10} octamers, divided into 400 sublibraries with defined amino acids 1 and 2 each consisting of 6.4×10^7 sequences. After phosphorylation with the kinases in the presence of [γ-^{32}P]ATP, the sublibrarys Ac-XXXRRXXX and Ac-XXXRKXXX were identified as the best substrates for PKA and PKG, respectively. The second-generation libraries had the structures Ac-XXXRR12X and Ac-XXXRK12X for PKA and PKG and resulted in the most active sequence pools Ac-XXXRRASX and Ac-XXXRKKSX. After delineation of every position in the octameric sequence and extension of the investigation to decameric peptides, the best sequences, Ac-KRAERKASIY and Ac-TQKARKKSNA, were obtained for PKA and PKG, respectively. Promising octameric and decameric peptides were assembled 5 or 10 times each and assayed in order to determine the experimental scatter inherent in the approach. The kinetic data of several octameric and decameric sequences were determined in solution and compared to data for known substrates. The recognition motif of PKA was confirmed by this approach, and a novel substrate sequence for PKG was identified. The approach can be expected to be of generally applicable for the elucidation of protein kinase specificity with linear peptide substrates.

Protein phosphorylation by protein kinases is the most important regulatory mechanism of cellular function and signal transduction (Hunter, 1987). Among the superfamily of protein kinases the two cyclic nucleotide regulated protein kinases, cAMP-dependent protein kinase (PKA)[1] and cGMP-dependent protein kinase (PKG), form a closely related subfamily of serine/threonine kinases [for reviews, see Francis and Corbin (1994a), Hofmann et al. (1992), and Taylor et al. (1993)].

PKA and PKG share many structural features such as the N-terminal dimerization region (Landgraf & Hofmann, 1989; Landgraf et al., 1990; Zick & Taylor, 1986), a proteolytic sensitive hinge region which also contains the substrate recognition sequence, two in-tandem cyclic nucleotide binding sites which allosterically regulate the enzyme activity, and a catalytic subunit responsible for phosphorylating the substrate (Titani et al., 1982; Takio et al., 1984). Despite these similarities, the two enzymes display distinct differences which account for their unique properties. While the PKA holoenzyme complex dissociates upon activation into the regulatory subunit dimer and the active catalytic subunits, the PKG holoenzyme complex remains as an entity and does not dissociate (Francis & Corbin, 1994b; Hofmann et al., 1992).

An additional characteristic difference is the substrate specificity. In general, protein kinases exhibit specificities that are often primarily determined by the amino acids around the phosphorylation sites (Kemp & Pearson, 1991; Glass, 1983). While PKA displays a well-defined specificity with the consensus motif RRXS(A)X for substrate or inhibitory peptides (Glass et al., 1989), PKG seems to have a less well defined recognition sequence (Glass, 1990; Butt et al., 1994). However, identification of amino acids that contribute to substrate motifs is essential for developing specific peptide substrates and inhibitors. In this respect, many investigations with large numbers of individual peptides have been conducted in order to find high-affinity substrates as well as inhibitors. Peptide libraries offer the possibility of investigating the sequence dependence of the phosphorylation more thoroughly and systematically. Recently, two approaches have been described that utilized peptide libraries generated by the method of "split synthesis" on resins (Till et al. 1994; Wu et al. 1994).

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[1] Abbreviations: Standard one-letter and three-letter abbreviations of amino acids have been used. Ac, acetyl; Acm, acetamidomethyl; BSA, bovine serum albumin; DCM, dichloromethane; DMF, dimethylformamide; Fmoc, 1-fluorenylmethoxycarbonyl; HPLC, high-performance liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid; PKA, catalytic subunit C_α of the cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; RP, reversed phase; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid.

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By employing cAMP- and cGMP-dependent protein kinases as model enzymes, we decribe here a new method for the systematic investigation of the sequence-dependent specificity of protein kinases with peptide libraries on cellulose paper. We followed an iterative approach similar to the one used in the identification of peptide ligands (mimotopes) for antibodies described by Geysen et al. (1986). In principle, a library comprising all possible peptide sequences of a given length made up from the 20 natural amino acids is devided into a full set of pools (sublibraries). Each pool is characterized by one or more defined positions in the context of the randomized other positions. A full set of pools thus covers all combinations of amino acid residues at the defined positions. These pools are synthesized as individual spots on a cellulose paper support (Frank, 1992; Frank et al., 1995) and assayed for activity as an array of immobilized peptide substrates. Our starting array of 400 pools contained two defined positions and had the structure Ac-XXX12XXX, in which the Xs represent mixtures of all 20 natural amino acids and 1 and 2 stand for individual amino acids. The best two amino acids 1 and 2 from the first evalulation were used throughout the second-generation array, where two other positions were screened. This procedure was carried out until all positions had been evalulated, followed by a C- and N-terminal extension of the octameric sequences. The method allows the identification of the major determining amino acids of the substrate recognition motifs and the systematic evaluation of every position. In addition to the investigations with peptides immobilized on paper, the kinetics of the most active sequences were determined in solution.

EXPERIMENTAL PROCEDURES

Materials. [γ-^{32}P]ATP (6000 Ci/mmol) was from Amersham, Germany. Phosphocellulose paper (P-81) and phosphocellulose (P11) were from Whatman. Kemptide was purchased from Sigma. The semiautomatic robot for the synthesis of the peptides on paper was from ABIMED Anlysentechnik, Langenfeld, Germany. The resins used for preparative peptide synthesis were from Rapp Polymere, Tübingen, Germany. All chemicals were of analytical grade. Organic solvents used during peptide synthesis were of HPLC grade.

Generation of the Peptide Arrays on Paper. The synthesis strategy of the peptide arrays on paper was adopted from the method described previously (Frank, 1992, 1994). Briefly, an APS 222 robotic station was used for semiautomatic assembly of the arrays on Whatman 540 paper that had been esterified uniformly with β-alanine followed by a second β-alanine at the position of the spots. Four hundred twenty-five spots (ϕ 3 mm) were arranged at a distance of 4 mm in a 17 × 25 format (6.7 × 9.8 cm). After each amino acid coupling cycle, the paper was treated with a solution of bromophenol blue in DMF (0.1 mg/mL), which resulted in a blue staining of the spots (Krchnak et al., 1988). All 20 natural amino acids (except for Gly) were used in their L-configuration. Fmoc chemistry was employed, with activation by equimolar amounts of diisopropylcarbodiimide and hydroxybenzotriazole. Side-chain protections were as follows: Asp, Glu, Ser, Tyr, and Thr, *t*-Bu; Asn, Gln, and His, trityl; Arg, Pmc; Lys and Trp, Boc. Cysteine is considered problematic in libraries because of its tendency to oxidize. For this reason some groups omit it from their libraries, and others use it in a protected form throughout their assays. We chose the latter option and used cysteine with acetamidomethyl (Acm) protection, which is not cleaved by TFA but confers good solubility in aqueous buffers. The randomized positions (termed X) were generated by applying 0.1 μL of a 30 mM solution of an equimolar mixture of all 20 amino acids onto the paper, which corresponds to an amount of approximately 70% of the number of amino groups on the paper. The reaction time was 1 h. Spotting was repeated 3 times, after which the reaction was complete as judged by a color change of the spots from blue to yellow or green. As published elsewhere, this procedure assures a sufficiently equal representation of all amino acids at these positions, including the more slowly coupling residues such as valine or isoleucine (Frank, 1995; Kramer, 1993). After completion of the synthesis, the N-termini were acetylated with 2% acetic acid anhydride in DMF. The peptides were deprotected by two successive 1-h treatments with DCM/TFA, 1:1, containing 3% triisobutylsilane and 2% water.

Assaying the Peptide Arrays with PKA and PKG. The paper with the peptide array was moistened with ethanol, washed with 50 mL of incubation buffer A (50 mM MOPS, pH 6.9, 200 mM NaCl, 1 mM Mg-acetate, 0.4 mM EGTA, and 1 mg/mL BSA), and kept overnight in 200 mL of this buffer. The buffer was decanted, and the paper was preincubated at 30 °C with 8 mL of fresh buffer A. One hundred microliters of 10 mM ATP and 100 μCi of [γ-^{32}P]-ATP were added. The reaction was started by addition of PKA catalytic subunit (C_α) or cGMP activated PKG. Final enzyme concentrations were 12.5 and 4 nM for PKA and PKG, respectively. The mixture was incubated for 10 min at 30 °C with slight agitation. The buffer solution was decanted and the paper was washed at least 10 times with 100 mL of 1 M NaCl. One hundred milliliters of an 8 M guanidine hydrochloride solution containing 1% SDS and 0.5% β-mercaptoethanol was added, and the paper was sonicated for 1 h at 40 °C to remove background. The paper was washed several times with water and ethanol and subsequently dried. Radioactivity was determined with the PhosphorImager system (Molecular Dynamics). Quantifications were carried out by integrating uniformly sized circular areas in the center of the spots.

Preparative Peptide Synthesis. The solid-phase synthesis of the peptides was carried out on a Milligen 9050 automatic peptide synthesizer employing Fmoc chemistry with TBTU activation. Side-chain protection of the amino acids was as described for the synthesis on paper. Peptides were cleaved from the resin and deprotected by a 3-h treatment with TFA containing 3% triisobutylsilane and 2% water (10 mL/g of resin). After precipitation with *tert*-butyl methyl ether, the resulting crude peptides were purified by preparative HPLC (RP-8) with water/acetonitrile gradients containing 0.5% TFA and characterized by amino acid analysis and MALDI-MS.

Enzymes. The catalytic subunit (C_α) of PKA was prepared in accordance with a previously described method (Slice & Taylor, 1989; Yonemoto et al., 1991). In brief, the enzyme was expressed in *Escherichia coli* Bl21De3 cells transformed with a pet-3a vector containing the coding sequence of the catalytic subunit. After IPTG induction the cells were incubated at 27 °C for 4−6 h. The bacterial pellet was lysed with a French press, and the enzyme in the soluble fraction was purified over phosphocellulose resin (P11, Whatman) to homogeneity. Peak fractions contained the recombinant catalytic subunit with a typical specific activity of 20 μmol

min^{-1} mg^{-1}. PKG was obtained according to a methods described by Landgraf and Hofmann (1989) and Feil et al. (1993).

Phosphotransferase Activity. Kinase activity was measured for 1.5 min at 30 °C in a final volume of 100 μL containing 20 μL buffer B (250 mM MES, pH 6.9, 2 mM EGTA, 5 mM Mg-acetate, and 50 mM NaCl), 10 μL of BSA (10 mg/mL), 10 μL of DTT (100 mM), 10 μL of [γ-^{32}P]-ATP (1 mM; specific activity, 300−400 cpm/pmol), 10 μL of cGMP (1 mM) or water, 10 μL of appropriate peptide solution, and 20 μL of PKA or PKG (5 mM TES, pH 7.0, 0.2 mM EDTA, and 0.5 mg/mL BSA) to final concentrations of 1 and 2.6 nM, respectively. Fifty-microliter aliquots were spotted on 2 × 2 cm phosphocellulose paper strips (P-81 Whatman), extensively washed in 75 mM phosphoric acid, and the dried strips were subjected to scintillation counting according to Ruth et al. (1991). Peptide concentrations ranged from 100 nM to saturating levels, and all assays were run in duplicate. K_m and V_{max} values were derived from linear regression analysis (Eadie−Hofstee plots).

RESULTS

Development of Conditions for Peptide Phosphorylation on Cellulose Paper. In order to optimize the assay conditions, we first synthesized the array Ac-RAARR1S2 which contained the general substrate motif RRXSX of PKA (Kemp & Pearson, 1990). All 400 combinations of the 20 natural amino acids were used at positions 1 and 2. Initially the commonly used procedure for the investigation of peptide phosphorylation was employed (Hardie, 1993) in which peptides are incubated with the kinases in low-salt buffers followed by an immobilization on phosphocellulose paper and subsequent removal of excess radioactive ATP with 75 mM phosphoric acid. We found that under these conditions ATP bound ionically to sequences on the paper containing basic amino acids and could not be washed off efficiently. However, removal was successful by washing the paper repeatedly in high-salt buffer (1 M NaCl). We also found that the incubation buffer should contain at least 100 mM salt; otherwise, misleading results were obtained because the phosphorylation of peptides that bind ATP ionically was partially inhibited.

Peptide Phosphorylations on Cellulose Paper. After the assay conditions were optimized, an array of the general type Ac-XXX12XXX was used for both kinases, where X represents an equal distribution of all 20 natural amino acids (Cys was used in its Acm-protected form). The whole array represents a library of all 2.56×10^{10} possible octamers, and each sublibrary with defined amino acids 1 and 2 consists of 6.4×10^7 sequences. Incubation with PKA and PKG gave similar phosphorylation patterns with differences between the best dipeptide motifs (Figures 1 and 2A,B). PKA favored the substrates Ac-XXXRRXXX and Ac-XXXRKXXX, whereas PKG was more selective for Ac-XXXRKXXX. The results indicate that two neighboring basic amino acids are the most strongly determinant residues for both kinases.

Based on the results of the first array, the second generation consisted of the structures Ac-XXXRR12X and Ac-XXXRK12X for PKA and PKG, respectively. Both kinases phosphorylated mainly sequences with Ser or Thr at position 2, Ser being generally favored (Figure 2C,D).

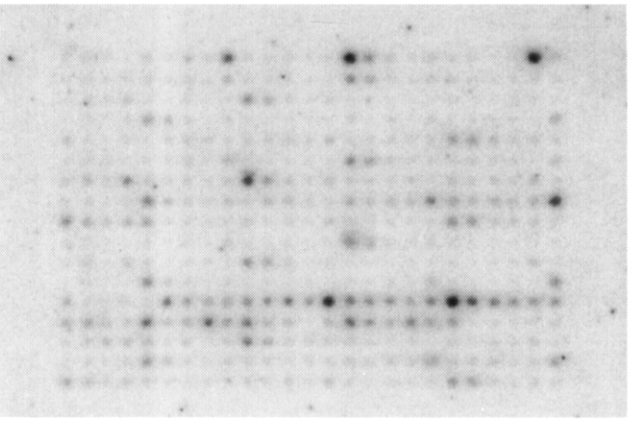

FIGURE 1: PhosphorImager scan of the paper with the array Ac-XXX12XXX after phosphorylation by PKA. Four hundred twenty-five spots are arranged in a 17 × 25 format. Rows 2−17 contain 400 sublibraries in an arrangement where 20 consecutive spots have a particular amino acid at position 1, and position 2 is one of the 20 amino acids, both positions being varied in alphabetical order (according to the single-letter code). For a preliminary estimation of the scattering, the first row contains 25 of the sequences that are used in rows 2−17.

This suggests that position 2 is the site of phosphorylation, which is obvious from a comparison with the general motif for PKA. PKA is less discriminative at position 1, where it shows a slight preference for Ala, whereas PKG clearly prefers Lys. In addition, the arrays Ac-X12RRXXX and Ac-X12RKXXX were used for screening the two positions N-terminal to the basic amino acids. There was no significant preference for any particular amino acid, although PKG had a slight tendency for basic residues (data not shown).

With the third-generation array we screened the structures Ac-X12RRASX and Ac-X12RKKSX for PKA and PKG, respectively (Figure 2E,F). As expected from the second-generation arrays and from previous investigations with soluble peptides (Glass, 1990; Mitchell et al., 1995) neither kinases discriminated strongly at both positions.

Next we used the array Ac-1AERKAS2 for PKA. The best substrate contained Arg at position 1 in combination with Ile at position 2 (Figure 3A), which is again in very good agreement with kinetic observations (Scott et al., 1986). The preference for Arg at position 1 is also consistent with the crystal structure of the PKA/inhibitor peptide complex (Knighton et al., 1991b). Proline at position 2 is particularly disfavored. For PKG we screened the array Ac-1KARKKS2 (Figure 3B). Here, Gln at position 1 and Asn at position 2 were favored. Again, Pro at position 2 was particularly disadvantageous.

In the fifth generation, the array for PKA had the structure Ac-1RAERKASI2 and extended the original octameric pattern by one residue N- and C-terminally. Lys at the N-terminus in combination with Tyr at the C-terminus was the favored combination (Figure 3C). The paper also contained the unextended octameric sequence Ac-RAERKASI (10 times) from the previous array. Several of the decapeptides were better substrates than the octapeptide, showing that the affinity has been improved by extending the peptide length. In addition, the peptides Ac-RAERRASI and Ac-RAERRASF were synthesized several times on the paper to reinvestigate the question of whether the middle section of the motif should be Arg-Arg or Arg-Lys and whether Ile or Phe would work best at the C-terminus (Figure 3C, lower part). The tendency from the first-generation array

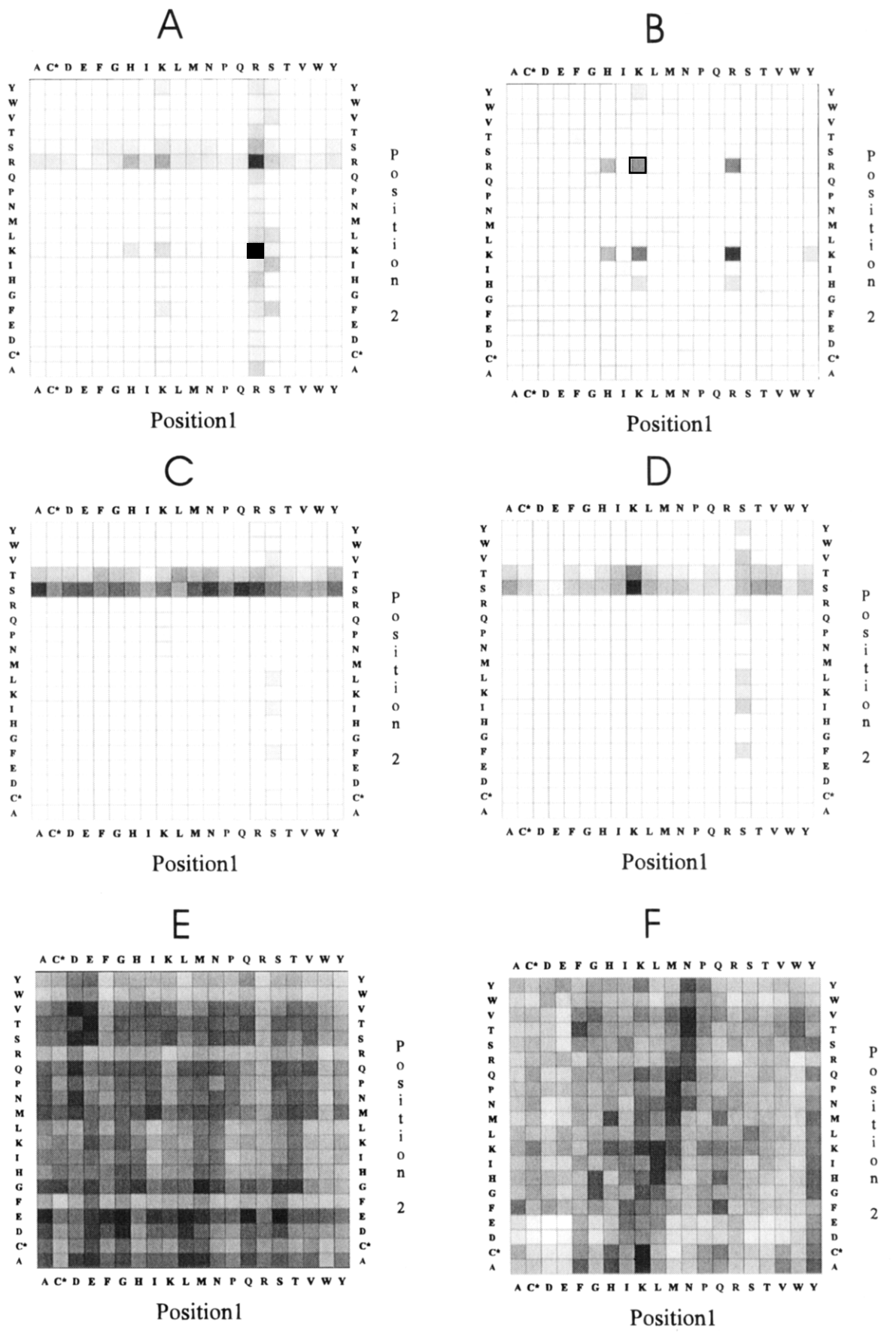

FIGURE 2: Quantified patterns of phosphorylation of the arrays Ac-XXX12XXX (A), Ac-XXXRR12X (C), and Ac-X12RRASX (E) by PKA and of the arrays Ac-XXX12XXX (B), Ac-XXXRK12X (D), and Ac-X12RKKSX (F) by PKG. The shading of each square corresponds in a linear dependence to the amount of phosphorylation of the corresponding spot on the paper.

that the middle motif Arg-Arg is superior to Arg-Lys was confirmed in this statistical comparison. The second conclusion was that the N-terminal Ile is preferred to Phe. The array Ac-1QKARKKSN2 screened with PKG revealed that

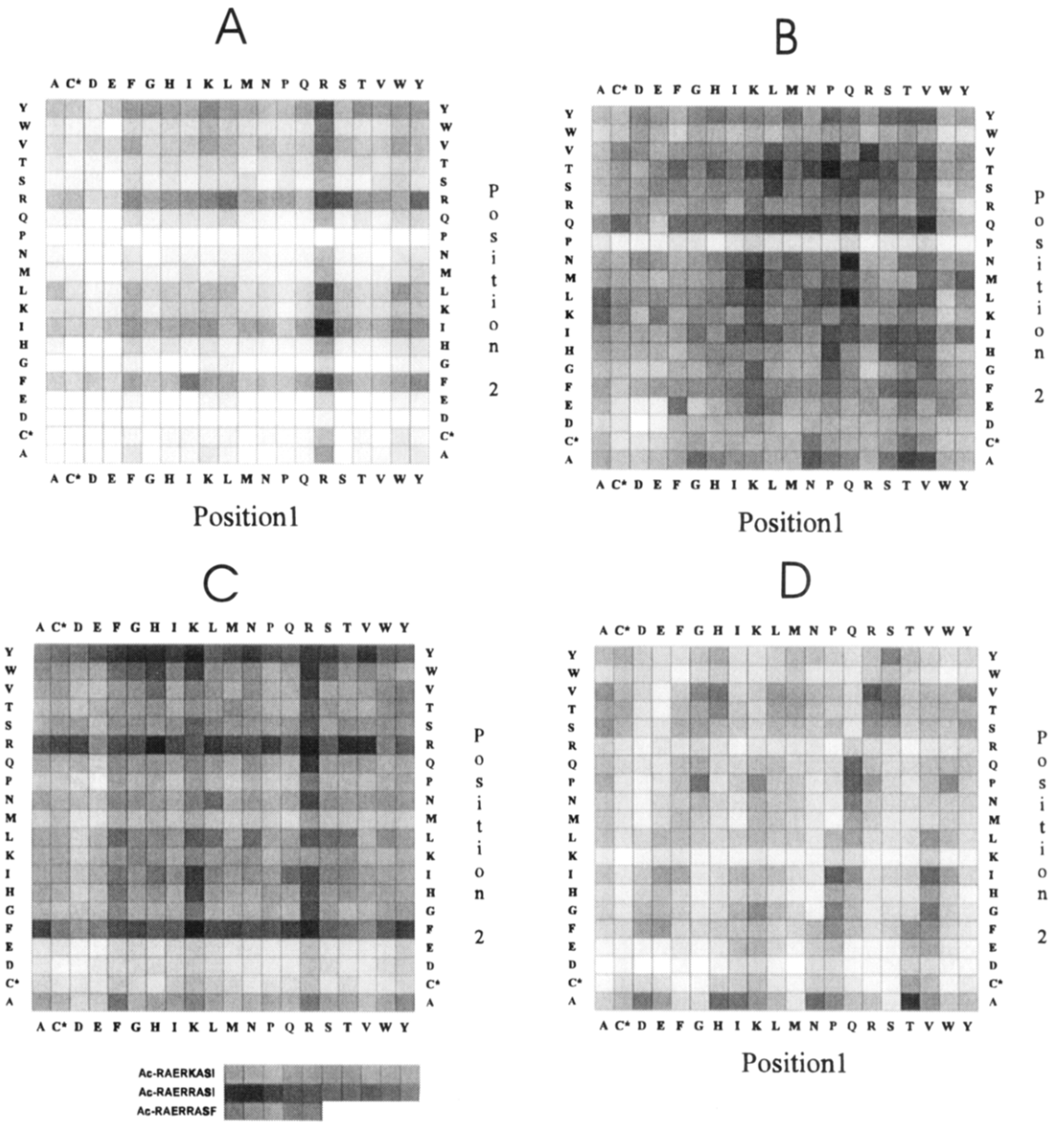

FIGURE 3: Quantified patterns of phosphorylation of the arrays Ac-1AERKAS2 (A) and Ac-1RAERKASI2 (C) by PKA and of the arrays Ac-1KARKKS2 (B) and Ac-1QKARKKSN2 (D) by PKG. The part below array C shows the phosphorylation of the three octameric peptides Ac-RAERKASI, Ac-RAERRASI, and Ac-RAERRASF by PKA.

Thr at position 1 and Ala at position 2 is the best substrate (Figure 3D).

In order to address the question of how reliably the phosphorylation patterns can be interpreted from an array where every sequence or sublibrary is present only once, five of the best octameric sequences from the fourth-generation PKG assay were assembled 10 times each on an extra paper (Figure 4). A scattering of up to 30% around the median value was found. The peptide density of the spots is more uniform (4.5 nmol) with a deviation of up to 10% (right lane in Figure 4). The assay conditions thus obviously add to the scattering. A comparison of short and long phosphorylation times (3 min versus 18 h) gave no differences in the deviation. In this statistical comparison, the sequence with Val at position 1 and Glu at position 2 (sequence 3, Figure 4) was on average better than the combination that was identified initially with the octameric array (sequence 1). A comparison of sequences 1 and 4 in Figure 4 showed that with these particular sequences Arg-Arg in the middle positions worked better than Arg-Lys, which had been found as the best combination on the very first array.

On the basis of the results for both kinases obtained so far, we reexamined the clustering of basic amino acids N-terminal of the phosphorylation site. Forty-three sequences were synthesized 5 or 10 times each as substrates for PKA. The comparison (Figure 5) suggested the following general rules for the specificity of the enzyme: in the general motif P^{-6}-P^{-5}-P^{-4}-P^{-3}-P^{-2}-P^{-1}-S-P^{+1}-P^{+2}, (i) P^{-3} and P^{-2} can be Arg-Arg or Arg-Lys but not Lys-Arg or Lys-Lys (with Lys-Lys being worse than Lys-Arg; Arg-Arg is in most cases better than Arg-Lys); (ii) P^{-1} can be Ala or Lys but not Arg; and (iii) at position P^{-4} Ala and Lys are better than Arg.

A similar investigation with PKG (Figure 6) resulted in the following rules: (i) at position P^{-3} Arg is much better than Lys; (ii) at P^{-2} Lys is in most cases better than Arg; and (iii) at P^{+1} Lys does not effect the activity very much, whereas at P^{+2} Lys is disadvantageous. In most cases AK was superior to KA at positions P^{-4} and P^{-5}. Particularly striking is the high activity of the sequence Ac-TQKARKK-

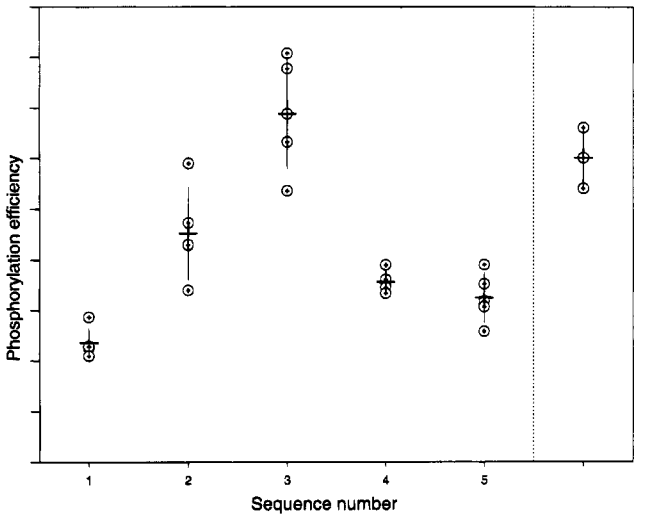

FIGURE 4: Phosphorylation of the following sequences (5 times each) by PKG: 1, Ac-QKARKKSN; 2, Ac-QKARKKSL; 3, Ac-VKARKKSQ; 4, Ac-QKARRKSN; 5, Ac-QKARRKSL. The right lane indicates the scattering of the peptide density between different spots (10%).

SLA (sequence 53, Figure 6). An exchange of Val or Arg at P^{-6} for Gln and of Gln at P^{+1} for Leu had a more dramatic effect than most of the variations of the basic amino acids. This result was unexpected from the systematic sreening of these positions, where both amino acids were inconspicuous. This finding indicates that the specificity at a certain position is influenced by the rest of the sequence.

Kinetic Constants. On the basis of the results from the fourth- and fifth-generation arrays, we synthezised the sequences shown in Table 1 and analyzed them in solution as substrates for the kinases over a wide range of peptide concentrations. Figure 7 shows the initial rate of phosphorylation for peptide 4-3 (Ac-RAERRASI-NH_2) and peptide 5-3 (TQAKRKKSLA-NH_2). Table 1 summarizes the kinetic constants along with kemptide (Whitehouse et al., 1983), *VASP* peptides (Butt et al., 1994), and histone H2B peptide (Glass & Krebs, 1979). Peptide 4-1 (Ac-QKARKKSN-NH_2) was obtained from a screening with PKG and had already proved selective for this enzyme. The K_m was low in comparison to those of peptides known so far and approximately 4-fold lower compared to that of PKA. Peptide 4-2 (Ac-QKARRKSN-NH_2) was used for a comparison of the motifs RKKSX and RRKSX for PKG. Table 1 shows that the substitution in peptide 4-2 does not affect the kinetic constants for PKG very much. Instead it becomes a better PKA substrate, resulting in a loss of specificity. Peptide 4-3 (Ac-RAERRASI-NH_2) was a result from the screening with PKA and confirms basically the well-known consensus sequence (Kemp & Pearson, 1990). From the crystal structure of the catalytic subunit of PKA (Knighton et al., 1991a,b; Zheng et al., 1993) the importance of Arg at position −6 of the peptide inhibitor is known which specifically interacts with residues of the lower lobe of the enzyme and could well explain the lower K_m of peptide 4-3 as compared to kemptide. The SPOT-screening method specifically picked up this Arg as the N-terminal residue. The sequence of peptide 5-3 was a result of the fifth-generation screening on the paper in combination with the statistical comparisons. The changes made between the fourth- and fifth-generation peptides have improved the K_m and the V_{max} by 5-fold (peptide 4-1 versus peptide 5-3). Peptide 5-3 proved to be a remarkably good substrate for PKG in terms of K_m (1.7 μM), catalytic rate (11 μmol/min/mg), and enzyme specificity (6.4). However, the same peptide proved to be a reasonable substrate for PKA as well. This observation was unexpected since the substrate recognition sequence in peptide 5-3 is clearly altered in comparison to peptide 4-3 which contains the well-defined PKA motif.

DISCUSSION

This paper describes the successful application of peptide libraries, displayed as arrays on cellulose paper, for the determination of the amino acids that are the major contributors to the substrate recognition motifs of cAMP- and cGMP-dependent protein kinases. These two kinases have been used as model enzymes for a number of reasons. First, PKA has a well-defined recognition motif. Thus, the literature data could serve as a measure of the performance of our approach and as a valuable guide during the development of the assay conditions. Second, the recognition motif for PKG is less unequivocal, although the two kinases are closely related to each other and share many similar features. Therefore, this approach was used to extend our understanding of the differences in substrate recognition between the two kinases. The recognition motif RRXS of PKA was confirmed by this approach and was used to ensure the reliability of the method. More important, a new substrate sequence (TQAKRKKSLA-NH_2) for PKG with the lowest known K_m value of 1.7 μM and a relatively high V_{max}/K_m ratio of 6.4 was identified. This hitherto unknown PKG substrate recognition motif, KRKKSL, seems to contain sequence parts from known PKG substrate peptides such as the histone H2B peptide RKRSRKE and the protein kinase inhibitor (PKI) peptide GRTGRRNSI (Mitchell et al., 1995). It appears that PKG requires more basic residues compared to PKA, particularly N-terminal of the phosphorylation site as was pointed out by Kennelly and Krebs (1991). Of the known *in vivo* substrates for PKG, histone H2B (chicken, rat) contains the KRKKS motif (Glass, 1990). In addition, the small molecular weight G-protein rap1b (human platelet), which is phosphorylated by PKA and PKG (Siess et al., 1990; Butt & Walter, 1994), contains the recognition sequence KARKKS, which scored extremely high in our screening system (Figure 6). Other *in vivo* and *in vitro* substrates seem to follow variations of the consensus sequence R/K_{2-3}-X/K-S*/T* (22 sites phosphorylated by PKG were examined). The motif KRKKS was also used to screen the SwissProt protein data bank. Several potential new candidates, i.e., prostaglandin receptor type E_3 (mouse) and somatostatin receptor type 2 (human) for *in vivo* phosphorylation by PKG, were identified and are currently being evaluated.

Recently, two other approaches have been decribed for the evaluation of peptides as protein kinase substrates (Wu et al., 1994; Till et al., 1994). Both are based on the generation of peptide libraries on beads by the concept of split synthesis, where each bead carries only one sequence. One study has used penta- and heptapeptide libraries for the determination of the substrate specificity of PKA (Wu et al., 1994). Identification of the beads with the most active substrates was carried out via the incorporation of radioactive phosphate. Since the sequence of the peptide on a particular bead is not known in itself, the peptides had to be analyzed by sequencing. A complete evaluation of all amino acids at the different positions of the library is impossible, and therefore, the approach has to concentrate on the most active

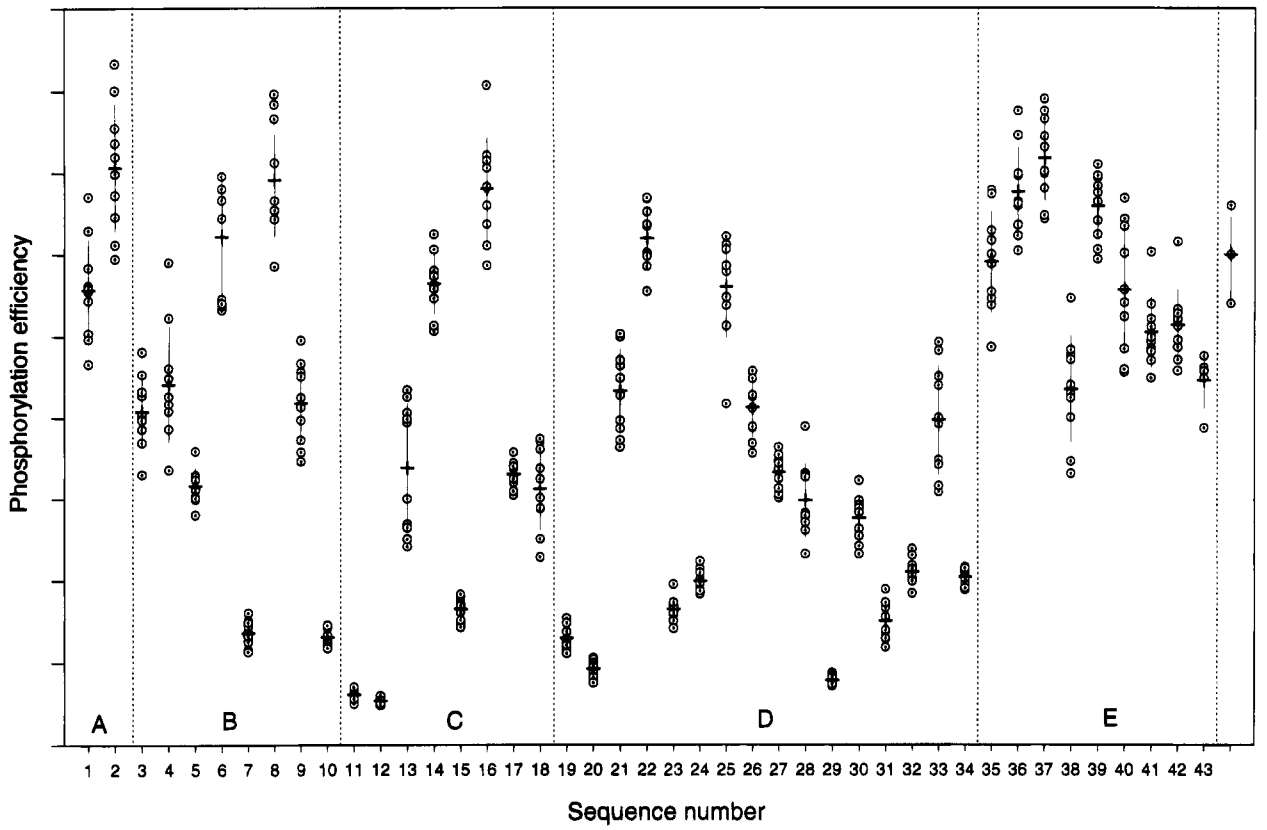

FIGURE 5: Phosphorylation of 43 different sequences (5 or 10 times each) by PKA (all peptides are acetylated at the N-terminus). (A–D) Sequences containing the following structures, where B can be arginine or lysine: A, ABBAS; B, ABBBAS; C, ABBBS; D, ABBBBS. (E) Different variations. Sequences: 1, ARAARKASIY; 2, ARAARRASIY; 3, ARARRRASIY; 4, ARARRKASIY; 5, ARARKRASIY; 6, ARAKRRASIY; 7, ARARKKASIY; 8, ARAKRKASIY; 9, ARAKKRASIY; 10, ARAKKKASIY; 11, ARAAKKKSIY; 12, ARAAKKRSIY; 13, ARAAKRKSIY; 14, ARAARKKSIY; 15, ARAAKRRSIY; 16, ARAARRKSIY; 17, ARAARKRSIY; 18, ARAARRRSIY; 19, ARAKKKKSIY; 20, ARAKKKRSIY; 21, ARAKKRKSIY; 22, ARAKRKKSIY; 23, ARARKKKSIY; 24, ARAKKRRSIY; 25, ARAKRRKSIY; 26, ARARRKKSIY; 27, ARAKRKRSIY; 28, ARARKRKSIY; 29, ARARKKRSIY; 30, ARAKRRRSIY; 31, ARARKRRSIY; 32, ARARRKRSIY; 33, ARARRRKSIY; 34, ARARRRRSIY; 35, KRAKRRASIY; 36, KRAARKASIY; 37, KREKRRASIY; 38, KREKRRASIR; 39, KREKRRASIF; 40, RREKRRASIF; 41, RRDKRRASIF; 42, RRKDRRASIF; 43, RRKERRASIF. The right lane indicates the scattering of the peptide density between different spots (10%).

members in a library. In the second study, derivatives of the heptapeptide kemptide degenerate at only one particular position were investigated as substrates for PKA, and another set of peptides with one degenerate position were used as substrates for the tyrosine kinase v-Abl (Till et al., 1994). Analysis of phosphorylated peptides was carried out by a coupled HPLC–ESMS system and a phosphate-selective stepped collision energy mass spectrometry method. The analysis was carried out with mixtures containing 38 different peptides. An extension to more complex pools is limited because of the degeneracy of the molecular masses if more than one amino acid position is unknown.

Our SPOT approach has the advantage that every amino acid at every position of the sequence can easily be evaluated and compared. This is important if, for example, the identification of the best substrate motif is not desired but rather determining the largest differences between enzyme activities is the major goal. Also, if more than one phosphorylatable amino acid is present in a sequence, each amino acid's individual function as a phosphate acceptor needs to be evaluated. For this purpose individual spots can easily be punched or cut out of the array and analyzed. Peptide quantities per spot are in the range of 5 nmol, which is sufficient for microsequencing or amino acid analysis.

In general, for a successful applicaton of methods based on short linear peptides, the site of phosphorylation (or generally the chemical modification) and at least some of the major contributors of the recognition motif must fit into the length of the peptide that is evaluated. It is possible that additional major contributors of the motifs of both kinases lie outside the range that has been investigated so far. It should also be kept in mind that only linear determinants are being identified. Interactions through secondary or tertiary structures that seem to be important for some classes of enzymes cannot been investigated with short peptides.

Because of the scattering that is inherent in this approach, a statistical comparison of the best sequences that have been identified in a first screening seems necessary in order to determine the best substrate, especially if only small differences among several sublibraries or sequences are present. The specificity at certain positions may be influenced to some extent by the amino acids around that site. This implies that an iterative procedure like the one presented here may not necessarily lead to the absolutely best peptide sequence, but to one that can be expected to contain the major contributors of a motif. It seems generally desirable to increase the number of sublibraries per surface area which would allow the presentation of more defined peptide pools to the enzymes. Simultaneous evaluation of three or more positions would reduce the problem of the sequence dependency of the specificity at a certain position.

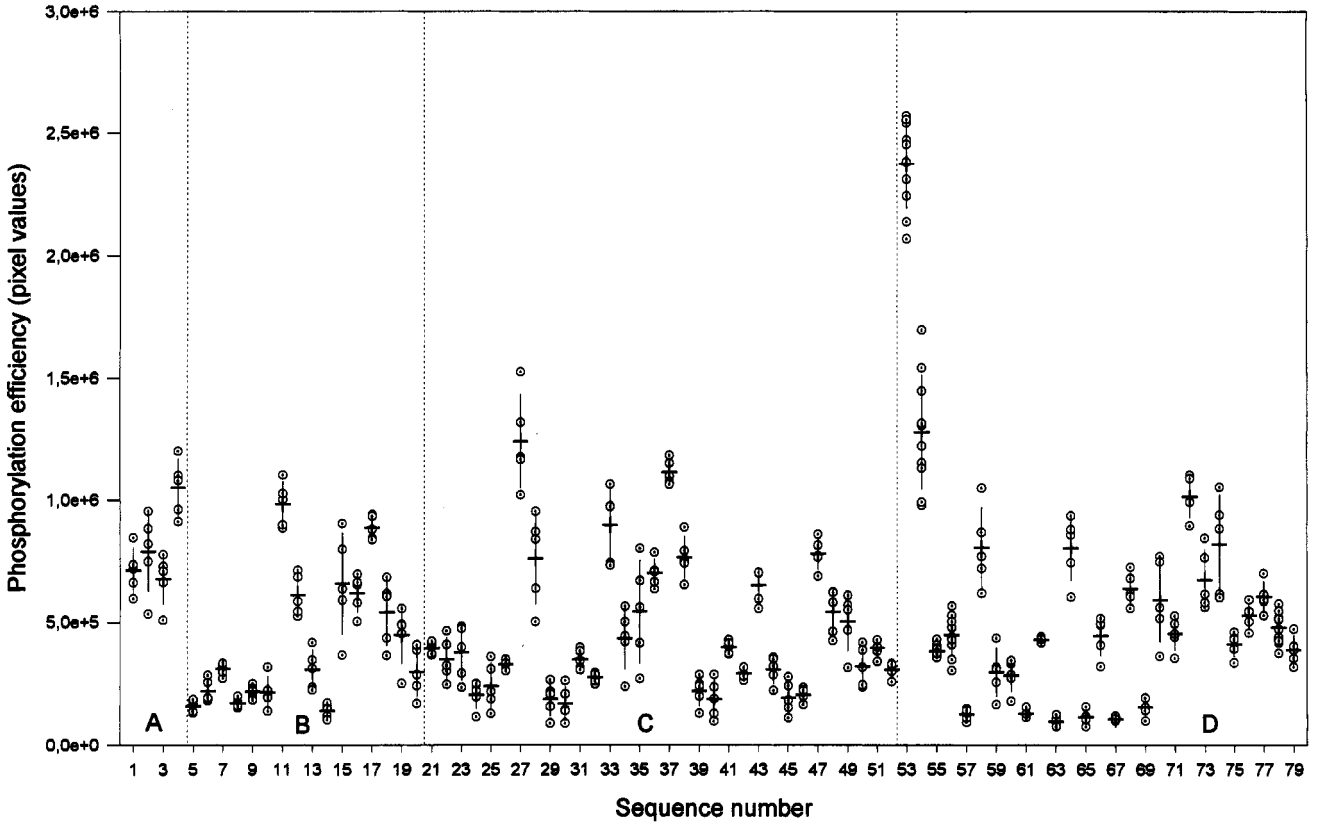

FIGURE 6: Phosphorylation of 79 different sequences (5 or 10 times each) by PKG (all peptides are acetylated at the N-terminus). (A–C) Sequences containing the following structures, where B can be arginine or lysine: A, ABBAS; B, ABBBS; C, ABBBBS. (D) Different variations. Sequences: 1, TVKARKASQA; 2, TRKARKASQA; 3, TVKARRASQA; 4, TRKARRASQA; 5, TVKAKKKSQA; 6, TRKAKKKSQA; 7, TVKAKKRSQA; 8, TRKAKKRSQA; 9, TVKAKRKSQA; 10, TRKAKRKSQA; 11, TVKARKKSQA; 12, TRKARKKSQA; 13, TVKAKRRSQA; 14, TRKAKRRSQA; 15, TVKARRKSQA; 16, TRKARRKSQA; 17, TVKARKRSQA; 18, TRKARKRSQA; 19, TVKARRRSQA; 20, TRKARRRSQA; 21, TVAKKKKSQA; 22, TRAKKKKSQA; 23, TVAKKKRSQA; 24, TRAKKKRSQA; 25, TVAKKRKSQA; 26, TRAKKRKSQA; 27, TVAKRKKSQA; 28, TRAKRKKSQA; 29, TVARKKKSQA; 30, TRARKKKSQA; 31, TVAKKRRSQA; 32, TRAKKRRSQA; 33, TVAKRRKSQA; 34, TRAKRRKSQA; 35, TVARRKKSQA; 36, TRARRKKSQA; 37, TVAKRKRSQA; 38, TRAKRKRSQA; 39, TVARKRKSQA; 40, TRARKRKSQA; 41, TVARKKRSQA; 42, TRARKKRSQA; 43, TRAKRRRSQA; 44, TRAKRRRSQA; 45, TVARKRRSQA; 46, TRARKRRSQA; 47, TVARRKRSQA; 48, TRARRKRSQA; 49, TVARRRKSQA; 50, TRARRRKSQA; 51, TVARRRRSQA; 52, TRARRRRSQA; 53, TQKARKKSLA; 54, TQKARRKSLA; 55, TVMPRKKSQA; 56, TVMPRRKSQA; 57, TVMPRRASQA; 58, TVLIRKKSQA; 59, TVLIRKKSQK; 60, TVLIRRKSQA; 61, TVLIRRKSQR; 62, TVLIRRASQA; 63, TVLIRRASKK; 64, TVYGRKKSQA; 65, TVYGRKKSKR; 66, TVYGRRKSQA; 67, TVYGRRKSRR; 68, TVYGRRASQA; 69, TVYGRRASRK; 70, QVKARKKSQP; 71, QVKARKKSKP; 72, PVKARKKSQI; 73, PVKARKKSKI; 74, RVKARKKSQV; 75, RVKARKKSRV; 76, NVKARKKSQA; 77, NVKARKKSRA; 78, QVKARRKSQP; 79, PVKARRKSQI.

Table 1: Kinetic Constants for the Phosphorylation of Synthetic Peptides and Other Peptide Substrates by PKA and PKG[a]

peptide	PKA K_m (μM)	PKA V_{max} (μmol/min/mg)	PKA V_{max}/K_m	PKG K_m (μM)	PKG V_{max} (μmol/min/mg)	PKG V_{max}/K_m	specificity index (V_{max}/K_m, PKG)/(V_{max}/K_m, PKA)	ref
4th generation								
4-1: Ac-QKARKKSN-NH_2	31.7 ± 0.8	2.36 ± 0.03	0.074	9.02 ± 2.32	2.19 ± 0.51	0.242	3.26	this study
4-2: Ac-QKARRKSN-NH_2	15.5 ± 0.8	5.6 ± 0.14	0.36	9.95 ± 2.1	2.44 ± 0.23	0.245	0.68	this study
4-3: Ac-RAERRASI-NH_2	1.99 ± 0.19	8.03 ± 0.24	4.04	13.95 ± 2.15	2.55 ± 0.19	0.183	0.045	this study
5th generation								
5-3: TQAKRKKSLA-NH_2	2.68 ± 0.36	7.92 ± 0.22	2.96	1.74 ± 0.06	11.11 ± 0.17	6.39	2.16	this study
kemptide: LRRASLG	4.28 ± 0.94	9.90 ± 0.64	2.31	120	4.5	0.037	0.016	Whitehouse et al. (1983); this study
H2B: RKRSRKE	113	1.18	0.0104	21.6	4.4	0.204	19.6	Glass and Krebs (1979)
VASP: LRKVSKQE	1395	2.6	0.0018	94	3.7	0.039	21	Butt et al. (1994)
VASP: IERRVSNAG	26	2.7	0.104	30	2.2	0.073	0.71	Butt et al. (1994)

[a] Values are given as means of three or four experiments ± SD.

The SPOT approach can be expected to be generally applicable to the elucidation of protein kinase specificity and to the investigation of other enzymatic transformations. Corresponding work is in progress in our laboratory for further improvements of the technique and applications of the method.

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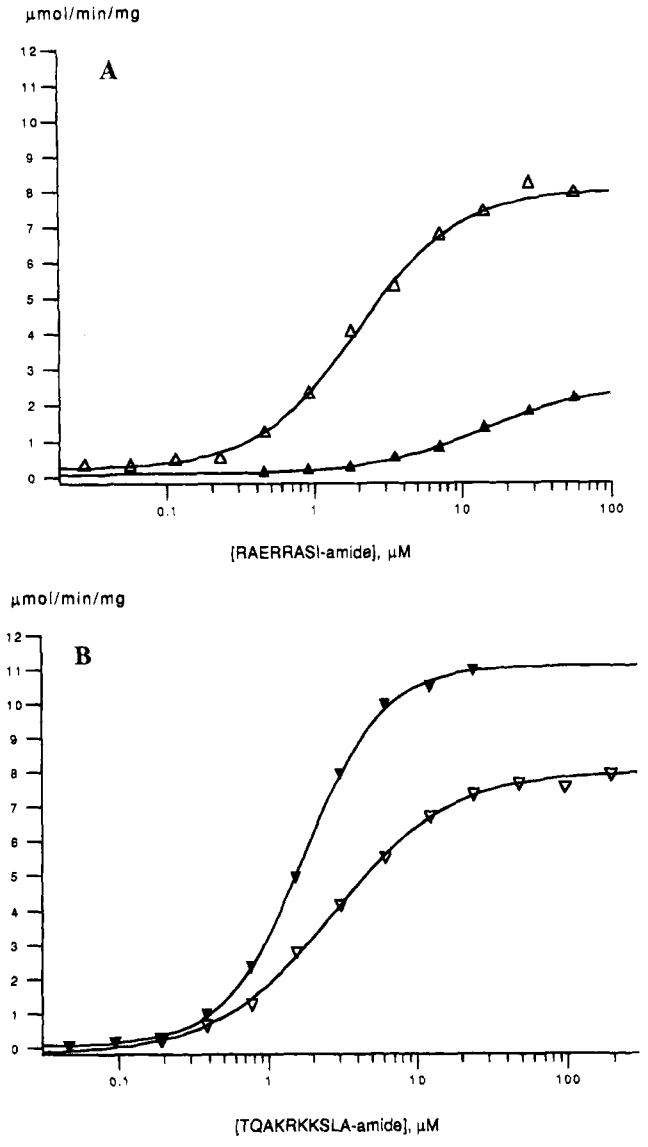

FIGURE 7: Protein kinase activity as a function of substrate concentration. (A) Peptide 4-3 (RAERRASI-NH_2, Table 1) was incubated with PKA (△) and PKG (▲), and (B) peptide 5-3 (TQAKRKKSLA-NH_2, Table 1) was incubated with PKA (▽) and PKG (▼), as described under Experimental Procedures. Initial velocities (μmol/min/mg) were plotted against substrate concentrations.

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