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# Determination of the Specific Substrate Sequence Motifs of Protein Kinase C Isozymes\*

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Protein kinase C (PKC) family members play significant roles in a variety of intracellular signal transduction processes, but information about the substrate specificities of each PKC family member is quite limited. In this study, we have determined the optimal peptide substrate sequence for each of nine human PKC isozymes ( $\alpha$ ,  $\beta$ I,  $\beta$ II,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\mu$ , and  $\zeta$ ) by using an oriented peptide library. All PKC isozymes preferentially phosphorylated peptides with hydrophobic amino acids at position +1 carboxyl-terminal of the phosphorylated Ser and basic residues at position -3. All isozymes, except PKC $\mu$ , selected peptides with basic amino acids at positions -6, -4, and -2. PKC $\alpha$ , - $\beta$ I, - $\beta$ II, - $\gamma$ , and - $\eta$  selected peptides with basic amino acid at positions +2, +3, and +4, but PKC $\delta$ , - $\epsilon$ , - $\zeta$ , and - $\mu$  preferred peptides with hydrophobic amino acid at these positions. At position -5, the selectivity was quite different among the various isozymes; PKC $\alpha$ , - $\gamma$ , and - $\delta$  selected peptides with Arg at this position while other PKC isozymes selected hydrophobic amino acids such as Phe, Leu, or Val. Interestingly, PKC $\mu$  showed extreme selectivity for peptides with Leu at this position. The predicted optimal sequences from position -3 to +2 for PKC $\alpha$ , - $\beta$ I, - $\beta$ II, - $\gamma$ , - $\delta$ , and - $\eta$  were very similar to the endogenous pseudosubstrate sequences of these PKC isozymes, indicating that these core regions may be important to the binding of corresponding substrate peptides. Synthetic peptides based on the predicted optimal sequences for PKC $\alpha$ , - $\beta$ I, - $\delta$ , - $\zeta$ , and - $\mu$  were prepared and used for the determination of  $K_m$  and  $V_{max}$  for these isozymes. As judged by  $V_{max}/K_m$  values, these peptides were in general better substrates of the corresponding isozymes than those of the other PKC isozymes, supporting the idea that individual PKC isozymes have distinct optimal substrates. The structural basis for the selectivity of PKC isozymes is discussed based on residues predicted to form the catalytic cleft.

Protein kinase C (PKC)<sup>1</sup> family members play crucial roles in

the signal transduction of a variety of extracellular stimuli, such as hormones and growth factors (1). To date, twelve isozymes of PKC have been identified in mammalian tissues and subdivided into conventional PKC (cPKC) members comprising  $\alpha$ ,  $\beta$ I,  $\beta$ II, and  $\gamma$  isoforms (activated by calcium, acidic phospholipid, and diacylglycerol (DAG)), novel PKCs (nPKC) comprising  $\delta$ ,  $\epsilon$ ,  $\eta$ , and  $\theta$  (activated by DAG and acidic phospholipid but insensitive to calcium), and atypical PKCs (aPKC)  $\nu$  and  $\zeta$  (mechanism of regulation not clear) (1–6). Another subgroup of PKCs may be defined by PKC $\mu$ , which has a potential signal peptide and transmembrane domain (7). Since these PKC isozymes differ in their expression in different tissues and in their mode of activation (1), each isozyme may play some specific role in signal transduction processes. Recent investigations using various approaches such as overexpression and down-regulation of specific isozymes support this idea (1, 5).

A large number of proteins have been shown to be phosphorylated by PKC *in vivo* and *in vitro*, such as growth factor receptors, ion channels, ion pumps, transcription factors, and translation factors (1, 8). Based on the sequences of the phosphorylated sites and the use of synthetic peptides based on these sites, a consensus phosphorylation site motif for PKC was determined to be RXXS/TXRX, where X indicates any amino acid (8). However, the optimal substrates have not been investigated by peptide library approaches, and relatively little information is available about differences in substrate selectivity between individual PKC isoforms. Histone H1S, myelin basic protein, protamine, and protamine sulfate, which contain the above consensus phosphorylation site motif, are known to be efficient substrates for cPKCs, but poor substrates of the nPKC group (1). Recently, elongation factor eEF-1 $\alpha$  was shown to be phosphorylated with much greater efficiency by nPKC $\delta$  than by cPKCs, nPKC $\epsilon$  or - $\eta$ , or aPKC $\zeta$  (9). Heterogeneous ribonucleoprotein A1 is efficiently phosphorylated by PKC $\zeta$  but not by cPKCs or PKC $\epsilon$  (10). These findings suggest that the substrate specificity of each PKC isozyme is quite different.

We have developed a new technique for determining the substrate specificity of protein kinases, using an oriented library of more than 2.5 billion peptide substrates (11–13). In this approach, the consensus sequence of optimal substrates is determined by sequencing the mixture of products generated during a brief reaction with the kinase of interest. This technique predicts an optimal sequence and provides information about the relative importance of each position for selectivity. Here we have used this approach to determine optimal peptide

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<sup>1</sup> The abbreviations used are: PKC, protein kinase C; cPKC, conventional PKC; nPKC, novel PKC; aPKC, atypical PKC; DAG, diacylglycerol; eEF-1 $\alpha$ , elongation factor-1 $\alpha$ ; Sf9 cells, *Spondoptera frugiperda* cells; PS, phosphatidylserine; PKA, protein kinase A; PKI, Walsh inhibitor pseudosubstrate; DTT, dithiothreitol; MES, 4-morpholineethanesulfonic acid.

substrates for human PKC $\alpha$ , - $\beta$ I, - $\beta$ II, - $\gamma$ , - $\delta$ , - $\epsilon$ , - $\eta$ , - $\zeta$ , and - $\mu$  and found that each PKC isozyme has a unique optimal substrate sequence. Furthermore, we prepared synthetic peptides based on the predicted optimal sequences for PKC $\alpha$ , - $\beta$ I, - $\delta$ , - $\zeta$ , and - $\mu$  and showed that these peptides are high affinity and selective substrates for the respective PKC isozymes.

#### EXPERIMENTAL PROCEDURES

**Materials**—DAG was purchased from Boehringer Mannheim. PS was purchased from Avanti Polar Lipids. Isozyme-selective antipeptide antibodies for PKC $\alpha$ , - $\beta$ I, - $\beta$ II, - $\gamma$ , - $\delta$ , - $\epsilon$ , - $\eta$ , and - $\zeta$  were purchased from Santa Cruz Biotechnology, Inc. PKC $\epsilon$  substrate peptide (PKRKQGSVRRRV) was purchased from Upstate Biotechnology Inc. Anti *c-myc* antibody was purchased from Oncogene Sciences. P81 phosphocellulose paper was purchased from Whatman. [ $\gamma$ - $^{32}$ P]ATP (3000Ci/mmol) was obtained from DuPont NEN. Liquiscint was purchased from National Diagnostics. A ferric iminodiacetic acid (IDA) beads was purchased from Pierce. All other chemicals were obtained from Sigma. Synthesis of the degenerate peptide library was accomplished according to the standard 1-benzotriazolyl-oxy-tris-dimethyl-amino-phosphonium hexafluorophosphate (BOP/HOBt) *N*-hydroxy-benzotriazole coupling protocols using Peptide BioSynthesizer (Minipore Model) as described previously (12).

**Expression and Preparation of PKC Family Members**—The full-length cDNAs for human PKC $\alpha$ , - $\beta$ I, - $\beta$ II, - $\gamma$ , - $\delta$ , - $\epsilon$ , - $\eta$ , and - $\zeta$  were subcloned into baculovirus transfer vectors as described previously (6). Recombinant baculoviruses were produced by co-infecting *Spondoptera frugiperda* cells (SF9 cells) with the purified baculovirus transfer vectors and purified genomic AcNPV viral DNA using established protocols (14). Recombinant baculovirus encoding *c-myc* tagged PKC $\mu$  and anti-PKC $\mu$  monoclonal antibodies was prepared as described previously (7, 15). Baculoviruses expressing each of the nine different PKC isozymes were confirmed using PKC activity screens and immunoblotting with isozyme-selective antipeptide antibodies. Purification of PKC $\alpha$ , - $\beta$ I, - $\beta$ II, - $\gamma$ , - $\epsilon$ , and - $\delta$  were performed by several steps of column chromatography as described previously (16, 17). PKC $\eta$ , - $\zeta$ , and - $\mu$  were isolated by immunoprecipitation using isozyme-selective antipeptide antibodies (for PKC $\eta$  and - $\zeta$ ) and anti-*c-myc* antibody (for PKC $\mu$ ), respectively. 1 unit of PKC $\alpha$ , - $\beta$ I, - $\beta$ II, - $\gamma$ , - $\delta$ , - $\epsilon$ , - $\eta$ , or - $\zeta$  is defined as the amount of kinase required to incorporate 1 nmol of phosphate into the  $\epsilon$ -pseudosubstrate peptide per min. 1 unit of PKC $\mu$  is defined as the amount of kinase required to incorporate 1 nmol of phosphate into the  $\mu$ -peptide (see Fig. 2) per min.

**Kinase Reaction and Phosphopeptide Separation**—Each PKC isozyme (0.2–0.5 units) was added to 300  $\mu$ l of solution containing 1 mg of degenerate peptide mixture, 100  $\mu$ M ATP with a trace of [ $\gamma$ - $^{32}$ P]ATP (roughly  $6 \times 10^5$  cpm), 1 mM DTT, 10 mM MgCl $_2$ , 50 mM Tris-HCl (pH 7.5), 20  $\mu$ g/ml phosphatidylserine (PS), 10  $\mu$ M DAG, 200  $\mu$ M CaCl $_2$  (for PKC $\alpha$ , - $\beta$ I, - $\beta$ II, and - $\gamma$ ), and 0.5 mM EGTA (for PKC $\delta$ , - $\epsilon$ , - $\eta$ , - $\zeta$ , and - $\mu$ ). Reactions were started by addition of PKC and incubated at 30 °C. Reaction conditions were adjusted to allow phosphorylation of about 1% of the total peptide mixture. After reaction, peptide separation was performed as described previously (12). Briefly, the peptide supernatant was removed and diluted with 300  $\mu$ l of 30% acetic acid. This mixture was then added to a 1-ml DEAE column previously equilibrated with 30% acetic acid, and the column was eluted with 30% acetic acid. After the 600- $\mu$ l void volume, the next 1 ml contained both phosphorylated and non-phosphorylated peptides but was free of [ $\gamma$ - $^{32}$ P]ATP. A 0.5-ml column of ferric iminodiacetic acid beads was charged with 2.5 ml of 20 mM ferric chloride, washed with 4 ml of water, then washed with 3 ml of 500 mM NH $_4$ HCO $_3$  (pH 8.0), washed again with 3 ml of water, and then equilibrated with 3 ml of buffer A (50 mM MES, 1 M NaCl (pH 5.5)). The dried sample of peptide/phosphopeptide mixture was dissolved in 200  $\mu$ l of buffer A and loaded onto the ferric column. The column was then eluted with 2.5 ml of buffer A followed by 2.5 ml of buffer B (2 mM MES (pH 6.0)). The phosphopeptides were then eluted with 2 ml of 500 mM NH $_4$ HCO $_3$  (pH 8.0). Control experiments, in which the peptides were subjected to a mock phosphorylation, were conducted. The same column protocol was used and the fractions in which phosphopeptides usually elute were collected.

**Sequencing and Data Analysis**—Typically, 1–2 nmol of phosphopeptide mixture was added to the sequencer. The data analysis was performed as described previously (12). Briefly, the abundance of each amino acid at a given cycle in the sequence of the phosphopeptide mixture from the mock phosphorylation experiments was subtracted from the kinase experiments to correct for the background. To calculate the relative preference for amino acids at each degenerate position, the

corrected data were then compared with the starting mixture to calculate the ratios of abundance of amino acids. The sum of the abundance of each amino acid at a given cycle was normalized to 14, 15, or 16 (the number of amino acids present at the degenerate positions) so that each amino acid would have a value of 1 in the absence of selectivity at a particular position.

**PKC Assay**—PKC activity was assayed *in vitro* essentially as described previously using the standard PKC vesicle assay (16, 17). The reaction mixture (30  $\mu$ l) contained 100  $\mu$ M ATP with [ $\gamma$ - $^{32}$ P]ATP (5  $\mu$ Ci), 1 mM DTT, 5 mM MgCl $_2$ , 25 mM Tris-HCl (pH 7.5), 20  $\mu$ g/ml PS, 10  $\mu$ M DAG, 200  $\mu$ M CaCl $_2$  (for PKC $\alpha$ , - $\beta$ I, - $\beta$ II, and - $\gamma$ ), 0.5 mM EGTA (for PKC $\delta$ , - $\epsilon$ , - $\eta$ , - $\zeta$ , and - $\mu$ ) and indicated amount of synthetic substrate peptide. Reactions were started by addition of PKC (0.002–0.005 units) and incubated at 30 °C for 10 min. Reaction mixtures were spotted onto P81 phosphocellulose paper and washed 4 times in 500 ml of 1% phosphoric acid. Incorporation of  $^{32}$ P was determined by liquid scintillation counting. For each experimental condition, values for control reactions lacking substrate peptide were subtracted as blanks. In all assays to determine  $K_m$  and  $V_{max}$ , reaction rates were linear with respect to time for all conditions of peptide, and less than 10% of the peptide substrate was phosphorylated.

#### RESULTS

**Identification of Optimal Substrate Sequence for Nine PKC Isozymes**—In order to determine optimal substrate sequences for each of nine human PKC isozymes ( $\alpha$ ,  $\beta$ I,  $\beta$ II,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\mu$ , and  $\zeta$ ), we used a degenerate peptide library, comprising peptides of sequence: MAXXXXRXSXXXXXAKKK (RS-peptide library), where X indicates all amino acids except Trp, Cys, Ser, or Thr. Trp and Cys were omitted to avoid problems with sequencing and oxidation, whereas Ser and Thr were omitted to ensure that the only potential site of phosphorylation was the Ser at position 10. The Met-Ala sequence at the amino terminus was included to verify that peptides from this mixture are being sequenced and to quantify the peptides present. Ala at position 16 provides an estimate of how much peptide loss has occurred during sequencing. The poly(Lys) tail prevents wash-out during sequencing and improves the solubility of the mixture. Arg was “locked-in” at position 7 since previous studies had shown the importance of Arg at the p–3 position for PKC substrates (8). The library was sequenced, and all 16 amino acids were present at similar amounts at all 11 degenerate positions (data not shown). Another Ser-kinase substrate library (12), comprising peptides of sequence MAXXXSXXXX-AKKK, was also used to investigate the 9 PKC isozymes, and was poorly phosphorylated compared with the RS-peptide library confirming the importance of Arg at the p–3 position.

The RS-peptide library was incubated with each PKC isozyme under conditions in which approximately 1% of the total peptide mixture was phosphorylated. The phosphopeptide products were separated from non-phosphorylated peptides using the ferric-iminodiacetic acid column, and the mixture was sequenced. In Fig. 1, the relative abundance of amino acids at each of the 11 positions of degeneracy are presented from experiments using PKC $\alpha$  and - $\mu$ . These two enzymes clearly selected for distinct peptide substrates. PKC $\alpha$  preferred peptides with Arg at p–5 and p–4, while PKC $\mu$  selected peptides with Leu and Val, respectively, at p–5 and p–4. In fact, PKC $\mu$  had an extremely strong selectivity for peptides with Leu at position p–5. More than 40% of the phosphopeptide products of PKC $\mu$  had Leu at this position. At the p–2 position, both PKCs selected peptides with hydrophilic residues, with Gln and Lys preferred. PKC $\alpha$  selected for peptides with Gly at p–1, while PKC $\mu$  selected against peptides with Gly at p–1. Both PKCs preferred peptides with hydrophobic amino acids at p+1, though PKC $\alpha$  selected Phe while PKC $\mu$  selected Val. At p+2, p+3, and p+4 positions, PKC $\alpha$  strongly selected for peptides with the basic amino acids Arg or Lys. In contrast, PKC $\mu$  preferred peptides with hydrophobic amino acids in both positions.

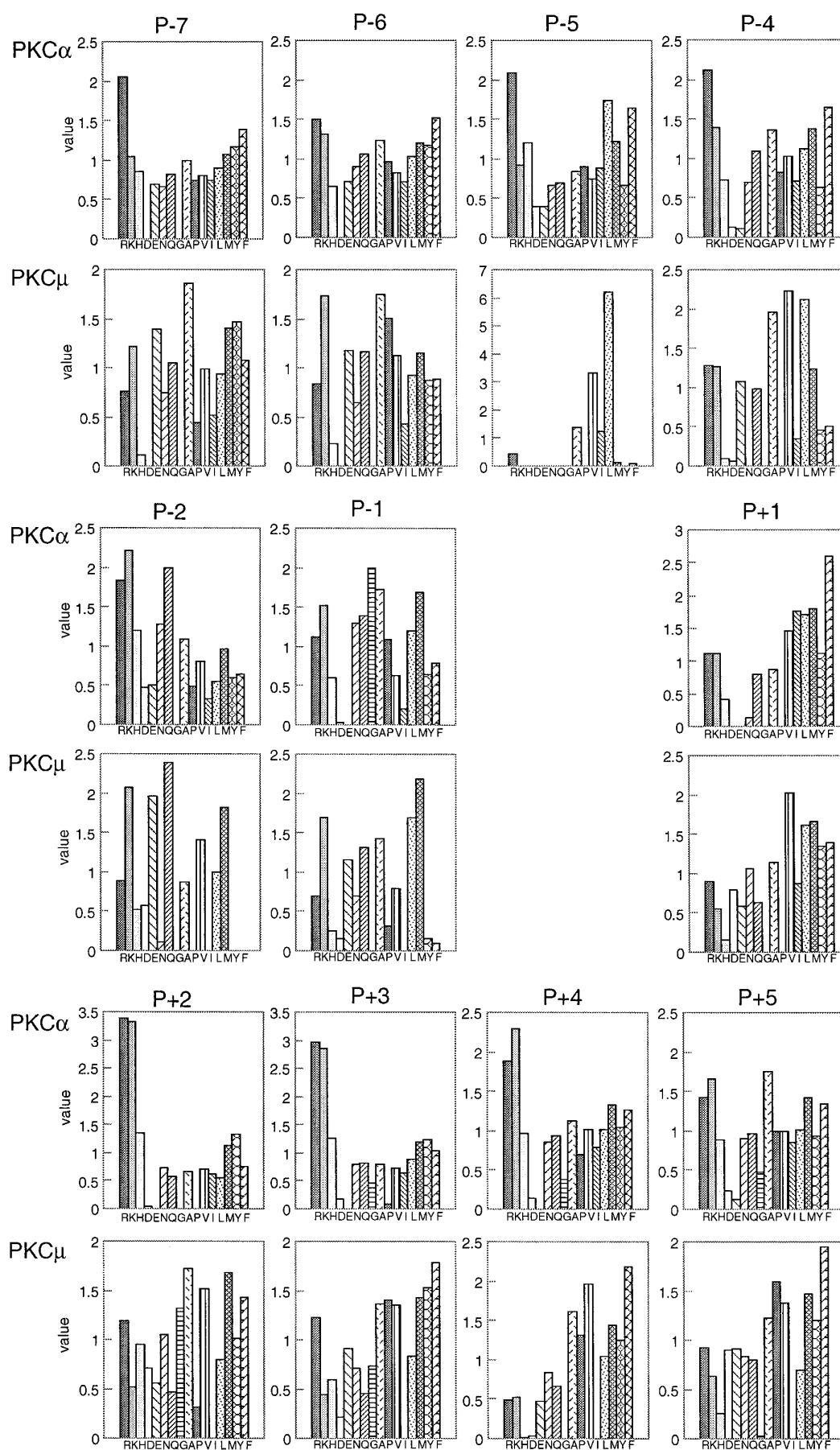


FIG. 1. Comparison of the substrate specificities of PKC $\alpha$  and PKC $\mu$ . Human PKC $\alpha$  and  $\mu$  were expressed in Sf9 cells using baculovirus. A degenerated substrate library with the sequence Met-Ala-X-X-X-X-Arg-X-X-Ser-X-X-X-X-Ala-Lys-Lys-Lys (where X indicates any amino acid



TABLE I  
Substrate specificities of protein kinase C isozymes

Values in parentheses indicate the relative selectivities for the amino acids; amino acids with values less than 1.5 are omitted. Bold letters indicate amino acids that are strongly selected; X indicates no selectivity. The one-letter amino acid code is used. All human PKC isozymes were expressed in Sf9 cells using baculovirus. A kinase substrate library with the sequence Met-Ala-X-X-X-Arg-X-X-Ser-X-X-X-X-Ala-Lys-Lys-Lys (where X indicates any amino acid apart from Trp, Cys, Ser or Thr) was presented to each PKC isozyme. The kinase reaction was performed as described in the legend for Fig. 1. Each PKC isozyme was evaluated at least twice; average values are shown.

| PKC isozymes   | Position                 |                          |                                    |                                    |          |                                                              |                                    |          |                                            |                                              |                                      |                                            |                              |
|----------------|--------------------------|--------------------------|------------------------------------|------------------------------------|----------|--------------------------------------------------------------|------------------------------------|----------|--------------------------------------------|----------------------------------------------|--------------------------------------|--------------------------------------------|------------------------------|
|                | -7                       | -6                       | -5                                 | -4                                 | -3       | -2                                                           | -1                                 | 0        | +1                                         | +2                                           | +3                                   | +4                                         | +5                           |
| PKC $\alpha$   | <b>R</b> (2.1)           | <b>R</b> /F(1.5)         | <b>R</b> (2.1)<br>L/F(1.7)         | <b>R</b> (2.1)<br>F(1.6)           | <b>R</b> | <b>K</b> (2.2)<br><b>Q</b> (2.0)<br>R(1.8)                   | <b>G</b> (2.0)<br>A/M(1.7)         | <b>S</b> | <b>F</b> (2.6)<br>M/L(1.8)<br>V(1.5)       | <b>R</b> /K(3.4)                             | <b>R</b> /K(3.0)                     | <b>K</b> (2.3)<br>R(1.9)                   | A(1.8)<br>K(1.7)             |
| PKC $\beta$ I  | R/K/F(1.5)               | K(1.8)<br>R(1.6)         | <b>L</b> /R(2.1)<br><b>F</b> (2.0) | <b>K</b> /R(2.3)<br>F(1.5)         | <b>R</b> | <b>K</b> (2.7)<br><b>Q</b> (2.2)<br>R/A(1.7)                 | <b>G</b> (2.0)<br>A(1.9)<br>K(1.8) | <b>S</b> | <b>F</b> (3.1)<br>M(1.8)<br>I/V(1.6)       | <b>K</b> (2.5)<br>R(1.9)<br>F(1.8)           | <b>K</b> (2.0)<br>F/R(1.7)           | F(1.8)<br>V(1.6)                           | <b>A</b> (2.3)<br>F(1.8)     |
| PKC $\beta$ II | Y(1.8)                   | K(1.6)                   | L(1.9)<br>F(1.8)                   | K(1.9)<br>R(1.6)                   | <b>R</b> | <b>K</b> (2.7)<br><b>Q</b> (2.0)<br>E(1.5)                   | <b>G</b> (2.1)<br>K(1.8)<br>M(1.7) | <b>S</b> | <b>F</b> (3.0)<br><b>M</b> (2.1)<br>L(1.8) | <b>K</b> (3.0)<br><b>R</b> (2.0)<br>F/M(1.5) | <b>K</b> (2.6)<br>Y(1.7)<br>F/R(1.5) | K(1.9)<br>F(1.5)                           | A(1.7)<br>F/K(1.6)<br>M(1.5) |
| PKC $\gamma$   | R(1.9)                   | R(1.9)<br>K(1.6)         | <b>R</b> (2.7)<br>F(1.5)           | <b>R</b> (3.5)<br>K(1.6)           | <b>R</b> | <b>K</b> (3.5)<br>R(1.9)<br>Q(1.8)                           | <b>G</b> (2.7)<br>K(2.4)<br>A(1.6) | <b>S</b> | <b>F</b> (2.1)<br>K(1.9)<br>M(1.5)         | <b>K</b> (2.8)<br><b>R</b> (2.3)<br>F(1.8)   | <b>R</b> (3.4)<br><b>K</b> (2.3)     | <b>K</b> (2.0)<br>R(1.9)                   | A(2.1)<br>K(1.6)<br>R(1.5)   |
| PKC $\delta$   | A(1.6)                   | A/R(1.7)<br>K(1.6)       | <b>R</b> (2.1)                     | K/A(1.7)                           | <b>R</b> | <b>K</b> (2.1)<br>E(1.9)<br>Q(1.5)                           | <b>G</b> (2.6)<br>A(1.7)<br>K(1.5) | <b>S</b> | <b>F</b> (2.4)<br>M/V(1.8)                 | F(1.8)<br>K/M(1.6)                           | Y/F(1.5)                             | G(1.9)<br>F(1.6)<br>V(1.5)                 | <b>G</b> (2.2)<br>F(1.8)     |
| PKC $\epsilon$ | Y(1.9)<br>E(1.6)         | Y/K(1.5)                 | X                                  | K(1.7)<br>A/Q(1.5)                 | <b>R</b> | <b>K</b> (3.1)<br><b>E</b> (2.7)<br>R(2.1)<br>D(1.9)         | M(1.7)<br>K(1.6)                   | <b>S</b> | F(1.9)<br>I/M(1.7)<br>V/L(1.6)             | F/A(1.7)                                     | E(1.7)<br>Y/D(1.6)<br>F(1.5)         | F/E(1.5)                                   | D/E/F(1.5)                   |
| PKC $\eta$     | <b>A</b> (2.1)<br>R(1.8) | <b>R</b> (2.0)<br>A(1.6) | L/R(1.9)                           | R(1.9)<br>K(1.5)                   | <b>R</b> | <b>R</b> (2.2)<br><b>K</b> (2.0)<br>Q(1.9)                   | <b>R</b> (2.3)<br>G(1.8)           | <b>S</b> | <b>F</b> (2.4)<br>R(1.9)<br>M(1.5)         | <b>R</b> (2.0)<br>Y(1.9)<br>F(1.8)           | <b>R</b> (2.1)<br>Y(1.6)             | X                                          | R(1.6)                       |
| PKC $\zeta$    | R(1.8)                   | R/K(1.6)                 | <b>F</b> (2.5)<br>L/R(1.6)         | K(1.8)<br>F(1.6)                   | <b>R</b> | <b>Q</b> /K(1.8)<br>Y(1.6)                                   | G(1.7)<br>K/M/Y(1.5)               | <b>S</b> | <b>F</b> (2.6)<br><b>M</b> (2.0)           | <b>F</b> (2.1)<br><b>M</b> (2.0)<br>A(1.5)   | Y/F(1.8)<br>K(1.7)                   | F(1.9)<br>Y/K(1.6)                         | F(1.7)<br>K/Y(1.5)           |
| PKC $\mu$      | A(1.9)<br>Y(1.5)         | A/K(1.7)<br>P(1.5)       | <b>L</b> (6.2)<br><b>V</b> (3.3)   | <b>V</b> (2.2)<br>L(2.1)<br>A(2.0) | <b>R</b> | <b>Q</b> (2.4)<br><b>K</b> (2.1)<br><b>E</b> (2.0)<br>M(1.8) | <b>M</b> (2.2)<br>L/K(1.7)         | <b>S</b> | V(2.0)<br>M(1.7)<br>L(1.6)                 | A/M(1.7)<br>V(1.5)                           | F(1.8)<br>Y(1.5)                     | <b>F</b> (2.2)<br><b>V</b> (2.0)<br>A(1.6) | F(1.9)<br>P(1.6)<br>M(1.5)   |

The results obtained for other PKC isozymes are summarized in Table I. All PKC isozymes selected for peptides with hydrophobic amino acid at p+1 position, and all isozymes except PKC $\mu$  selected for peptides with basic amino acid at the p-6, p-4 and p-2 positions. Interestingly, all PKC isozymes selected for peptide substrates with Gln or Glu at the p-2 position, although in most cases Lys at this position was optimal. PKC $\alpha$ , - $\beta$ I, - $\beta$ II, - $\gamma$ , and - $\eta$  selected for substrates with basic amino acid at positions p+2, p+3, and p+4, but PKC $\delta$ , - $\epsilon$ , - $\zeta$ , and - $\mu$  preferred substrates with hydrophobic amino acid in these regions. At p-5, the selectivity was quite different among these isozymes. PKC $\alpha$ , - $\gamma$ , and - $\delta$  selected peptides with Arg; in contrast PKC $\beta$ II, - $\zeta$ , and - $\mu$  selected for peptides with hydrophobic amino acids such as Phe, Leu, or Val. PKC $\beta$ I and - $\eta$  preferred substrates with either Leu or Arg.

**Comparison of the Predicted Optimal Substrate Sequences with Corresponding Pseudosubstrates and Other Known PKC Substrates**—In Table II, the predicted optimal sequence for each PKC isozyme was compared with the pseudosubstrate region of the respective isozyme by lining up the pseudosubstrate Ala with the Ser of the substrate. The amino acid at p-3 in all the pseudosubstrate sequences was Arg, consistent with our observation that peptides with Arg at p-3 are preferentially phosphorylated. Furthermore, hydrophobic amino acids

are present at the p+1 position in all the pseudosubstrate sequences except that of PKC $\zeta$ . The predicted optimal sequences from p-3 to p+2 for PKC $\alpha$ , - $\beta$ I, - $\beta$ II, - $\gamma$ , - $\delta$ , and - $\eta$  were in good agreement with pseudosubstrate sequences of the corresponding PKC isozymes, indicating that these core regions may be important to the binding of corresponding substrate peptides.

The optimal substrates predicted for the various PKC isozymes are in good agreement with known substrates. In Table III, the optimal substrate sequence of PKC $\alpha$  is compared with known PKC substrates, most of which were determined to be PKC substrates using cPKCs or partially purified PKC isozyme mixtures (probably mixtures of PKC $\alpha$ , - $\beta$ , and - $\gamma$ ). Most of these proteins have the motif R/K-X-R/K-R/K-X-S/T- $\Phi$ -R/K-R/K, where  $\Phi$  indicates hydrophobic amino acids (F, L, V). This motif is in agreement with the predicted optimal peptide from the peptide library experiment. The strongest selectivities from the library were for hydrophobic amino acids at p+1 and R/K at p+2, and almost every protein substrate has these characteristics. As mentioned above, the presence of Arg at the p-3 position is important for PKC substrates. However, some known substrates do not have Arg at this position (Table III). Using another library, comprising MAXXXXXXXXAKKK, we found that PKC $\alpha$ , - $\beta$ I, and - $\delta$  strongly selected for peptides with

apart from Trp, Cys, Ser, or Thr) was presented to PKC $\alpha$  and - $\mu$ . Each PKC isozyme was added to 300  $\mu$ l of solution containing 1 mg of degenerate peptide mixture, 100  $\mu$ M ATP with a trace of [ $\gamma$ - $^{32}$ P]ATP (roughly  $6 \times 10^5$  cpm), 1 mM DTT, 10 mM MgCl<sub>2</sub>, 50 mM Tris-HCl (pH 7.5), 20  $\mu$ g/ml PS, 10  $\mu$ M DAG, 200  $\mu$ M CaCl<sub>2</sub> (for PKC $\alpha$ ), and 0.5 mM EGTA (for PKC $\mu$ ). After 2 h at 30  $^{\circ}$ C, the phosphopeptides were separated on DEAE-Sephacel and a ferric chelation column. Reaction conditions were adjusted to allow phosphorylation of about 1% of the total peptide mixture. The phosphopeptides mixture was sequenced. Each panel indicates the relative abundance of the 15 (position -5 to -2 N-terminal to the phosphorylated Ser) or 16 (position -1 to +3 C-terminal to the Ser) amino acids at a given cycle of sequencing. The signal of Gly at position -7 to -2 and the signal of Ile for PKC $\mu$  at position +2 to +5 were not available because of a high background. The columns represent average values from two independent experiments. The one-letter amino acid code is used: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; V, Val; Y, Tyr.

TABLE II

Comparison of the optimal sequence of each PKC isozyme determined by the peptide library with the pseudosubstrate region of each isozyme

The optimal sequence of each PKC isozyme determined by the peptide library (optimal.) and the pseudosubstrate region of each isozyme (pseudo.) are presented by the one-letter codes, respectively. Bold letters indicate the phosphorylated Ser in the optimal sequence and the corresponding Ala in the pseudosubstrate region. Boxed amino acids emphasize positions that are similar between optimal sequence and pseudosubstrate region.

| PKC isozymes   | Position |          |          |          |          |          |          |          |          |          |          |          |          |
|----------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
|                | -7       | -6       | -5       | -4       | -3       | -2       | -1       | 0        | 1        | 2        | 3        | 4        | 5        |
| PKC $\alpha$   |          |          |          |          |          |          |          |          |          |          |          |          |          |
| optimal.       | R        | <b>R</b> | R        | R        | <b>R</b> | <b>K</b> | <b>G</b> | <b>S</b> | <b>F</b> | <b>R</b> | R        | <b>K</b> | A        |
| pseudo.        | N        | <b>R</b> | F        | A        | <b>R</b> | <b>K</b> | <b>G</b> | <b>A</b> | <b>L</b> | <b>R</b> | Q        | <b>K</b> | N        |
| PKC $\beta$ I  | <b>F</b> | <b>K</b> | <b>L</b> | <b>K</b> | <b>R</b> | <b>K</b> | <b>G</b> | <b>S</b> | <b>F</b> | <b>K</b> | <b>K</b> | <b>F</b> | <b>A</b> |
|                | <b>V</b> | <b>R</b> | <b>F</b> | <b>A</b> | <b>R</b> | <b>K</b> | <b>G</b> | <b>A</b> | <b>L</b> | <b>R</b> | <b>Q</b> | <b>K</b> | <b>N</b> |
| PKC $\beta$ II | <b>Y</b> | <b>K</b> | <b>L</b> | <b>K</b> | <b>R</b> | <b>K</b> | <b>G</b> | <b>S</b> | <b>F</b> | <b>K</b> | <b>K</b> | <b>K</b> | <b>A</b> |
|                | <b>V</b> | <b>R</b> | <b>F</b> | <b>A</b> | <b>R</b> | <b>K</b> | <b>G</b> | <b>A</b> | <b>L</b> | <b>R</b> | <b>Q</b> | <b>K</b> | <b>N</b> |
| PKC $\gamma$   | R        | R        | R        | R        | <b>R</b> | <b>K</b> | <b>G</b> | <b>S</b> | <b>F</b> | <b>K</b> | R        | <b>K</b> | <b>A</b> |
|                | P        | L        | F        | C        | <b>R</b> | <b>K</b> | <b>G</b> | <b>A</b> | <b>L</b> | <b>R</b> | Q        | <b>K</b> | <b>V</b> |
| PKC $\delta$   | <b>A</b> | R        | R        | K        | <b>R</b> | <b>K</b> | <b>G</b> | <b>S</b> | <b>F</b> | F        | Y        | G        | G        |
|                | <b>P</b> | T        | M        | N        | <b>R</b> | <b>R</b> | <b>G</b> | <b>A</b> | <b>I</b> | K        | Q        | A        | K        |
| PKC $\epsilon$ | Y        | <b>Y</b> | X        | <b>K</b> | <b>R</b> | K        | M        | <b>S</b> | <b>F</b> | F        | E        | F        | <b>F</b> |
|                | R        | <b>P</b> | R        | <b>K</b> | <b>R</b> | Q        | G        | <b>A</b> | <b>V</b> | R        | R        | R        | <b>V</b> |
| PKC $\eta$     | <b>A</b> | R        | <b>R</b> | <b>R</b> | <b>R</b> | R        | <b>R</b> | <b>S</b> | <b>F</b> | <b>R</b> | <b>R</b> | X        | R        |
|                | <b>F</b> | T        | <b>R</b> | <b>K</b> | <b>R</b> | Q        | <b>R</b> | <b>A</b> | <b>M</b> | <b>R</b> | <b>R</b> | R        | V        |
| PKC $\zeta$    | <b>R</b> | R        | <b>F</b> | K        | <b>R</b> | Q        | <b>G</b> | <b>S</b> | F        | F        | <b>Y</b> | F        | F        |
|                | <b>K</b> | S        | <b>I</b> | Y        | <b>R</b> | R        | <b>G</b> | <b>A</b> | R        | R        | <b>W</b> | R        | K        |

TABLE III

Comparison of the optimal sequence of each PKC isozyme determined by the peptide library with sequences at the same regions of known PKC substrates

Amino acids are represented by the one-letter code. Positions that showed great selectivity for each PKC are indicated by an asterisk.

| Peptide/protein                               | position |    |    |    |    |          |    |    |    |    |    |
|-----------------------------------------------|----------|----|----|----|----|----------|----|----|----|----|----|
|                                               | -5       | -4 | -3 | -2 | -1 | 0        | +1 | +2 | +3 | +4 | +5 |
| PKC $\alpha$ optimal seq.                     | R        | R  | R  | K  | G  | <b>S</b> | F  | R  | R  | K  | A  |
| MARCKS protein <sup>a</sup>                   | K        | K  | K  | R  | F  | <b>S</b> | F  | K  | K  | S  | F  |
| MARCKS protein <sup>a</sup>                   | F        | S  | F  | K  | K  | <b>S</b> | F  | K  | L  | S  | G  |
| MARCKS protein <sup>a</sup>                   | K        | L  | S  | G  | F  | <b>S</b> | F  | K  | K  | N  | K  |
| Neuromodulin <sup>a</sup>                     | A        | K  | I  | Q  | A  | <b>S</b> | F  | R  | G  | H  | I  |
| GABA type A receptor $\gamma 2L^b$            | L        | L  | R  | M  | F  | <b>S</b> | F  | K  | A  | P  | T  |
| EGF receptor <sup>a</sup>                     | I        | V  | R  | K  | A  | <b>T</b> | L  | R  | R  | L  | L  |
| Ribosomal protein S6 <sup>a</sup>             | R        | R  | R  | L  | S  | <b>S</b> | L  | R  | A  | S  | T  |
| PTP1B <sup>c</sup>                            | R        | V  | V  | G  | G  | <b>S</b> | L  | R  | G  | A  | Q  |
| Troponin I <sup>d</sup>                       | K        | F  | K  | R  | P  | <b>T</b> | L  | R  | R  | V  | R  |
| Insulin receptor tyrosine kinase <sup>e</sup> | N        | G  | R  | I  | L  | <b>T</b> | L  | P  | R  | S  | N  |
| P-glycoprotein <sup>f</sup>                   | R        | S  | T  | R  | R  | <b>S</b> | V  | R  | G  | S  | Q  |
| P-glycoprotein <sup>f</sup>                   | L        | I  | R  | K  | R  | <b>S</b> | T  | R  | R  | S  | V  |
| Kit/SCFR <sup>g</sup>                         | A        | D  | K  | R  | R  | <b>S</b> | V  | R  | I  | G  | S  |
| Annexin II <sup>a</sup>                       | P        | S  | A  | Y  | G  | <b>S</b> | V  | K  | P  | Y  | T  |
| PKC $\delta$ optimal seq.                     | R        | K  | R  | K  | G  | <b>S</b> | F  | F  | Y  | G  | G  |
| Elongation factor-1 $\alpha^h$                | R        | D  | M  | R  | Q  | <b>T</b> | V  | A  | V  | G  | V  |
| PKC $\mu$ optimal seq.                        | L        | V  | R  | Q  | M  | <b>S</b> | V  | A  | F  | F  | F  |
| GS-peptide <sup>i</sup>                       | L        | S  | R  | T  | L  | <b>S</b> | V  | A  | A  | L  | L  |

<sup>a</sup> From Ref. 8.

<sup>b</sup> From Ref. 26.

<sup>c</sup> From Ref. 27.

<sup>d</sup> From Ref. 28.

<sup>e</sup> From Ref. 29.

<sup>f</sup> From Ref. 30.

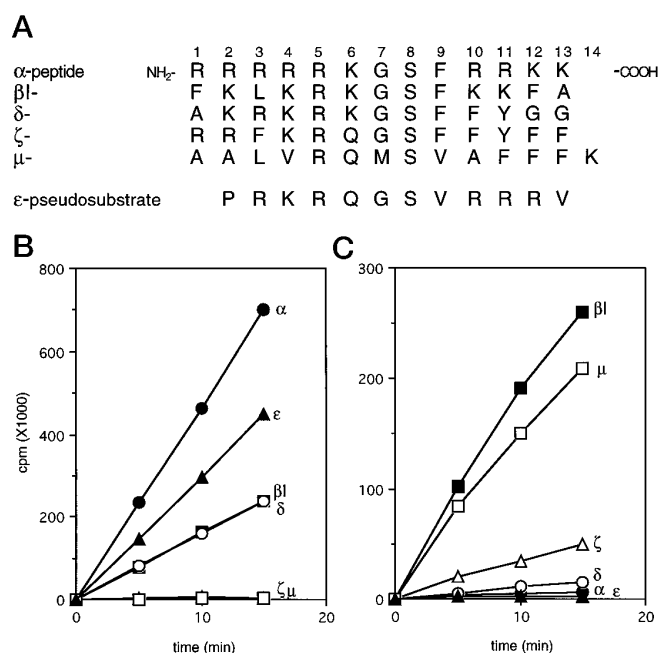
<sup>g</sup> From Ref. 31.

<sup>h</sup> From Ref. 9.

<sup>i</sup> From Ref. 15.

Arg at p-3 (selectivity values 6.0, 4.5, and 6.5, respectively), followed by His (1.9, 1.9, and 1.7) and Lys (1.3, 2.8, and 1.4) (data not shown). Thus, although Arg is preferred, substrates with His or Lys at p-3 are also selected. It is also expected that peptides lacking a basic amino acid at p-3 but with optimal amino acids at the other critical positions could still be reasonable substrates. Recently, a few proteins has been shown to be

isozyme-specific substrates. eEF-1 $\alpha$  is reported to be a specific substrate for PKC $\delta$  (9). The sequence of 426-436 from murine eEF-1 $\alpha$  containing Thr-431 was compared with the predicted optimal substrate of PKC $\delta$  (Table III). PKC $\delta$  strongly selects for substrates with Arg at p-5, basic at p-2, hydrophobic at p+1, and Gly at p+4. The site in eEF-1 $\alpha$  meets these criteria. The failure of cPKCs to phosphorylate this site could be ex-



**FIG. 2. Comparison of the phosphorylation efficiency of synthetic peptides by PKC $\alpha$  and PKC $\mu$ .** A, synthetic peptides derived from the predicted optimal sequence of PKC $\alpha$ ,  $\beta$ I,  $\delta$ ,  $\zeta$ , and  $\mu$  were defined as  $\alpha$ -,  $\beta$ I-,  $\delta$ -,  $\zeta$ -, and  $\mu$ -peptides, respectively. An additional Lys was added to the carboxyl terminus of the predicted optimal peptide for PKC $\mu$  to ensure binding to phosphocellulose during the kinase assay. PKC $\alpha$  (B) or  $\mu$  (C) was added to 30  $\mu$ l of solution containing 100  $\mu$ M of the indicated synthetic peptide, 100  $\mu$ M ATP with [ $\gamma$ - $^{32}$ P]ATP ( $1.1 \times 10^7$  cpm), 1 mM DTT, 5 mM MgCl<sub>2</sub>, 25 mM Tris-HCl (pH 7.5), 20  $\mu$ g/ml PS, 10  $\mu$ M DAG, 200  $\mu$ M CaCl<sub>2</sub> (for PKC $\alpha$ ), and 0.5 mM EGTA (for PKC $\mu$ ). After incubation for the indicated periods at 30  $^{\circ}$ C, an aliquot (5  $\mu$ l) of the reaction mixture was spotted onto P81 phosphocellulose paper and washed. Incorporation of  $^{32}$ P was determined by liquid scintillation counting.

plained by the lack of basic residues at p+2 and p+3. PKC $\mu$  is shown to phosphorylate glycogen synthase-derived peptide (15) much better than MARCKS, which is known to be a good substrate for other PKC isozymes (18, 19). The glycogen synthase-derived peptide sequence is in good agreement with the optimal peptide sequence that we predicted for PKC $\mu$ . Hydrophobic amino acids are found at positions -1 and +1 to +5, Arg is at -3, and most importantly, Leu is at position -5, the position at which PKC shows greatest selectivity (Table III). None of the PKC phosphorylation sites in the MARCKS protein have Leu at the p-5 position (Table III), explaining why MARCKS is not a good substrate for PKC $\mu$ .

**Determination of  $V_{max}$  and  $K_m$  Values of Synthetic Peptides Derived from the Predicted Optimal Substrate Sequences**—The predicted optimal peptide substrates for PKC $\alpha$ , PKC $\beta$ I, PKC $\delta$ , PKC $\zeta$ , and PKC $\mu$  were synthesized (Fig. 2A) and investigated as substrates of the various PKC isozymes. For comparison, a commercially-available PKC substrate peptide based on the pseudosubstrate region of PKC $\epsilon$  was used to assay the various PKC isozymes. A set of experiments using 100  $\mu$ M of the various peptides as substrates of PKC $\alpha$  and PKC $\mu$  are presented in Fig. 2, B and C. The  $V_{max}$  and  $K_m$  values determined for each of the peptides with each of the PKC isozymes are summarized in Table IV. It is clear from Fig. 2 and Table IV that different PKC family members selectively phosphorylate different subsets of the peptides. The optimal substrates for PKC $\alpha$ , PKC $\beta$ I, PKC $\delta$ , and PKC $\mu$  (as judged by  $V_{max}/K_m$  ratios) were the  $\alpha$ -peptide,  $\beta$ I-peptide,  $\delta$ -peptide, and  $\mu$ -peptide respectively, in agreement with the library predictions. Curiously, the  $\zeta$ -peptide was not the optimal peptide for PKC $\zeta$  as judged by  $V_{max}/K_m$  ratio,

although it was the lowest  $K_m$  substrate for PKC $\zeta$ . In fact, the predicted optimal peptide substrate for each PKC was the lowest  $K_m$  substrate for that PKC, except in the case of PKC $\alpha$  where the  $\beta$ I and  $\delta$ -peptides had slightly lower  $K_m$  values (2.7  $\mu$ M and 2.8  $\mu$ M, respectively) than the  $\alpha$ -peptide (3.8  $\mu$ M). These results are in agreement with previous results with the peptide library approach that have indicated that this technique selects substrates on the basis of low  $K_m$  and/or high  $V_{max}/K_m$  ratios rather than on the basis of  $V_{max}$  alone (11–13).

An interesting inference from the results in Table IV is that some sequences in peptides/proteins can act as generic substrates of almost all PKC isozymes, whereas other sequences are highly specific substrates for only one or two PKC isozymes. For example, in agreement with previous studies, the  $\epsilon$ -peptide is a good substrate for all the PKC isozymes investigated except PKC $\mu$ . The  $\beta$ I-peptide is a relatively good substrate for all the PKC isozymes, including PKC $\mu$ . In contrast, the  $\delta$ -peptide is relatively specific for PKC $\delta$ , and the  $\mu$ -peptide is very specific for PKC $\mu$ . In some cases, these results can be explained on the basis of a key residue at a specific position in the sequence. For example, as discussed above, PKC $\mu$  strongly selects for substrates with Leu at the p-5 position while other PKC isoforms are less sensitive to the amino acid at this location. Thus, the Leu at position p-5 in the  $\beta$ I-peptide allows it to be phosphorylated by PKC $\mu$  (only the  $\beta$ I-peptide and  $\mu$ -peptide have Leu at p-5). Although Leu is not the optimal residue at the p-5 position for PKC $\alpha$ , PKC $\delta$ , and PKC $\zeta$ , peptides with Leu at this position are clearly good substrates based on the peptide library results (Fig. 1 and Table I). Thus, including a Leu at this position broadens the number of kinases that could phosphorylate the substrate. The high specificity of the  $\mu$ -peptide for PKC $\mu$  can probably be explained by the lack of basic residues at p-2, p+2, and p+3 that are critical for substrates of the other PKC isoforms (Fig. 1, Table I).

## DISCUSSION

We have determined the optimal peptide substrates of nine human PKC isozymes using an oriented peptide library approach. The predicted optimal peptides are in good agreement with sequences at phosphorylation sites of known PKC substrates. Different PKC isozymes selected for different optimal peptide sequences based on residues both N-terminal and C-terminal of the site of phosphorylation. These differences can explain why distinct PKC isoforms phosphorylate distinct substrates *in vivo* and *in vitro*. The predicted optimal peptides for PKC $\alpha$ , PKC $\beta$ I, PKC $\delta$ , and PKC $\mu$  were synthesized and shown to be excellent substrates for the respective enzymes.

Although each PKC isozyme had a unique optimal peptide substrate, there were some features common to optimal substrates for all PKC family members and other features common to optimal substrates of subgroups of PKC family members. For example, all PKCs preferred substrates with a basic residue at position -3 and a hydrophobic residue (usually Phe) at position +1. The cPKC family members ( $\alpha$ ,  $\beta$ I,  $\beta$ II, and  $\gamma$ ) could be distinguished from other subfamilies in that they selected for substrates with basic residues at positions -6, -4, -2, +2, and +3. The nPKC family members ( $\delta$ ,  $\epsilon$ , and  $\eta$ ) and the aPKC $\zeta$  also selected for substrates with basic residues at -6, -4, and -2, but these kinases were not as selective for basic residues at +2 and +3. Instead, peptides with hydrophobic residues at these positions were usually selected. PKC $\mu$  was unique in that it selected for substrates with hydrophobic residues at -4, as well as at positions +2, +3, +4, and +5. However, the most critical residue for selectivity of PKC $\mu$  is a Leu at the -5 position.

The results we obtained for the specificities of the cPKC family members are in good agreement with previous studies. For example, substitution of the +1 Phe with Ile or the +2

TABLE IV

Determination of  $V_{\max}$  and  $K_m$  of synthetic peptides derived from the optimal sequences of PKC $\alpha$ , - $\beta$ I, - $\delta$ , - $\zeta$ , and - $\mu$  using these PKCs

Phosphorylation reaction was performed in the same condition as described in the legend for Fig. 2 in the presence of various amounts of synthetic peptides. Values are the average of three independent experiments. \* Values couldn't be determined because of the low phosphorylation efficiency.  $V_{\max}$  was expressed by the relative value; the highest value was calculated as 100 for each isozyme. The 100 of  $V_{\max}$  for PKC $\alpha$ , - $\beta$ I, - $\delta$ , - $\zeta$ , and - $\mu$  are corresponding to 1.6, 1.2, 2.5, 2.1, and 1.1 nmol/min/unit, respectively. Bold values indicate the best value (the smallest value for  $K_m$ , the largest value for  $V_{\max}$  and  $V_{\max}/K_m$ ) among the five synthetic peptides ( $\alpha$ -,  $\beta$ I-,  $\delta$ -,  $\zeta$ -, and  $\mu$ -peptides).

| PKC isozymes     | Peptides    |             |             |           |            |            |
|------------------|-------------|-------------|-------------|-----------|------------|------------|
|                  | $\alpha$    | $\beta$ I   | $\delta$    | $\zeta$   | $\mu$      | $\epsilon$ |
| PKC $\alpha$     |             |             |             |           |            |            |
| $K_m$ ( $\mu$ M) | 3.8         | <b>2.7</b>  | 2.8         | *         | *          | 2.6        |
| $V_{\max}$       | <b>100</b>  | 33          | 48          | *         | *          | 62         |
| $V_{\max}/K_m$   | <b>26.4</b> | 12.0        | 17.1        | *         | *          | 24.1       |
| PKC $\beta$ I    |             |             |             |           |            |            |
|                  | 8.2         | <b>2.8</b>  | 4.8         | *         | *          | 3.8        |
|                  | <b>100</b>  | 63          | 76          | *         | *          | 83         |
|                  | 12.2        | <b>22.9</b> | 16          | *         | *          | 22.0       |
| PKC $\delta$     |             |             |             |           |            |            |
|                  | 3.9         | 1.6         | <b>0.98</b> | 1.6       | *          | 2.1        |
|                  | <b>100</b>  | 24          | 39          | 9.6       | *          | 36         |
|                  | 25.9        | 14.6        | <b>39.9</b> | 6.1       | *          | 17.5       |
| PKC $\zeta$      |             |             |             |           |            |            |
|                  | 23          | 15          | 26          | <b>14</b> | 69.8       | 13         |
|                  | <b>100</b>  | 45          | 8.4         | 10        | 4.7        | 51         |
|                  | <b>4.3</b>  | 3.0         | 0.34        | 0.72      | 0.07       | 4.0        |
| PKC $\mu$        |             |             |             |           |            |            |
|                  | *           | 27          | 106         | 10        | <b>9.1</b> | *          |
|                  | *           | <b>100</b>  | 6.4         | 15        | 60         | *          |
|                  | *           | 3.8         | 0.06        | 1.5       | <b>6.6</b> | *          |

Arg with Ile in the neurogranin peptide substrate (AAKIQAS\*FRGHMARKK, asterisk indicates phosphorylation site) reduced phosphorylation by cPKCs (20). This result is consistent with our finding that Phe and Arg are optimal at the +1 and +2 positions for substrates of cPKCs. In another study, amino acid substitutions in the glycogen synthase-derived peptide (GGPLARALS\*VAAG, asterisk indicates phosphorylation site) have shown the importance of having basic residues at positions -4, -3, -2, +2, and +3 for phosphorylation by the cPKCs,  $\alpha$  and  $\gamma$  (21). These results are also in agreement with the predictions of the peptide library (Table I).

As discussed under "Results," the optimal peptides for the various PKC isozymes are similar but not identical to the pseudosubstrate regions of the respective enzymes (Table II). Peptide substrates based on the pseudosubstrate regions of PKC $\alpha$ ,  $\beta$ I, - $\gamma$ , and - $\epsilon$  were previously used to investigate the specificity of these enzymes (22). All four peptides had similar  $V_{\max}$  values with the four enzymes and had the lowest  $K_m$  when used as substrates for PKC $\beta$ I. This result was not surprising considering how similar these sequences are. In contrast, the optimal peptides predicted by the library are more divergent than the pseudosubstrate sequences and, with the exception of the  $\zeta$ -peptide, these peptides are preferential substrates of the kinases for which they were designed (Table IV).

A few isozyme-specific PKC substrates have been previously reported. A synthetic peptide based on region 422–443 of eEF-1a (RFAVRDMDRQT\*VAVGVKAVDKK) was reported to be phosphorylated at Thr-431 by PKC $\delta$  but not by other PKC isoforms (9). Conversion of Met-428 (the p-3 position) to Arg somewhat increased the ability of this peptide to be phosphorylated by cPKCs, consistent with known selectivity of these enzymes. Conversion of Ala-433 and Val-434 (the p+2 and p+3 positions) to Lys made this peptide a good substrate for all PKC isozymes. These results are consistent with our finding that the cPKCs prefer substrates with basic residues at p+2 and p+3, whereas PKC $\delta$  will utilize substrates with either hydrophobic or basic residues at these positions but with a slight preference for hydrophobic residues (Table I).

Prior to this study, very little was known about the substrate specificity of PKC $\mu$ . The peptide library results show that the two most critical residues for substrates of PKC $\mu$  are Leu at position p-5 and an aliphatic residue (preferentially Val) at

position p-4 (Table I). PKC $\mu$  also differed from the other PKCs in that it selected for peptides with Val rather than Phe at the p+1 position. A recent study (15) showed that PKC $\mu$  was very poor at phosphorylating known PKC substrates but phosphorylated the glycogen synthase-derived peptide (LSRTLS\*VAALL). This peptide has Leu at p-5, Arg at p-3, Val at p+1, and hydrophobic residues at p+2 through p+5 and thus is predicted to be a good PKC $\mu$  substrate based on the peptide library results (Table I). Syntide 2 (PLARTLS\*VAGLPQKK), a synthetic peptide derived from glycogen synthase, is also an efficient substrate of human PKC $\mu$  (15) and of the mouse homologue called PKD (23). This peptide also has the critical Leu at p-5 along with Arg at p-3, Val at p+1, and hydrophobic residues C-terminal of the phosphorylation site. Since PKC $\mu$  (and PKD) have very different substrate specificities than the other PKCs and are reported to be activated by phorbol esters (15, 23), these enzymes are likely to mediate novel phorbol ester signaling pathways distinct from those mediated by other PKCs.

The synthetic peptides we designed based on the predicted optimal substrates could be quite useful for further studies. For example, the  $\beta$ I-peptide is optimal for PKC $\beta$ I but is a useful general substrate for all PKC isoforms (including PKC $\mu$ ). The  $\alpha$ -peptide (like the  $\epsilon$ -pseudosubstrate peptide) is a general substrate for all PKCs except PKC $\mu$ . The  $\mu$ -peptide is extremely specific for PKC $\mu$ , and the  $\delta$ -peptide is relatively specific for PKC $\delta$ . The  $\zeta$ -peptide is phosphorylated by PKC $\zeta$ , PKC $\delta$ , and PKC $\mu$  but not by the cPKCs, so it would be useful for assaying the nPKCs and aPKCs without interference from cPKCs.

Finally, the crystal structure of protein kinase A (PKA) bound to the Walsh inhibitor (PKI) (24, 25) has provided a basis for explaining how protein kinases select for specific substrates. Recently, we proposed a model to explain substrate specificity of protein-Ser/Thr kinases based on the PKA/PKI structure and the alignments of various protein kinase sequences with that of PKA (13). Crystal structures of several protein-Ser/Thr kinases and protein-Tyr kinases indicate that these structures are highly conserved in the catalytic core, supporting the idea that homologous regions of sequences predicted to be in the catalytic cleft of diverse enzymes will be at analogous locations in the folded structures. The model we proposed assumes that all peptide substrates fit into the cata-



TABLE V

Alignment of residues of each PKC isozyme that are predicted to contact with side chains of optimal substrate sequences

Substrate position indicates the N- or C-terminal position of Ala present in Walsh inhibitor pseudosubstrate (PKI, TGRNNAIHD) or of phosphorylated Ser present in the obtained optimal substrate sequence of each PKC isozyme (see Fig. 1). Residue number indicates the residue present in the indicated subdomain of PKA. Residues present in each PKC isozyme, corresponding to the indicated residue number of PKA, are shown in the same column. Amino acids are represented by the one-letter code for the residue of each kinase or three-letter code for the residue of PKI or each optimal substrate sequence.

|                | Pocket      |     |      |     | Substrate position −5 | Pocket      |     |     | Substrate position −4 | Pocket      |     |     | Substrate position −3 |
|----------------|-------------|-----|------|-----|-----------------------|-------------|-----|-----|-----------------------|-------------|-----|-----|-----------------------|
|                | Subdomain   |     |      |     |                       | Subdomain   |     |     |                       | Subdomain   |     |     |                       |
|                | V           | VI  | VIII |     |                       | V           | V   |     |                       | VI          |     |     |                       |
|                | Residue no. |     |      |     |                       | Residue no. |     |     |                       | Residue no. |     |     |                       |
|                | 129         | 133 | 170  | 203 |                       | 127         | 133 | 328 |                       | 127         | 170 | 330 |                       |
| PKA            | F           | R   | E    | E   | THR                   | E           | R   | D   | GLY                   | E           | E   | Y   | ARG                   |
| PKC $\alpha$   | M           | Q   | D    | D   | ARG                   | D           | Q   | D   | ARG                   | D           | D   | F   | ARG                   |
| PKC $\beta$ I  | M           | Q   | D    | D   | LEU/ARG               | D           | Q   | D   | LYS/ARG               | D           | D   | E   | ARG                   |
| PKC $\beta$ II | M           | Q   | D    | D   | LEU                   | D           | Q   | D   | LYS                   | D           | D   | F   | ARG                   |
| PKC $\gamma$   | M           | Q   | D    | D   | ARG                   | D           | Q   | D   | ARG                   | D           | D   | F   | ARG                   |
| PKC $\delta$   | M           | Q   | D    | D   | ARG                   | D           | Q   | D   | LYS/ALA               | D           | D   | E   | ARG                   |
| PKC $\epsilon$ | M           | Q   | D    | D   | X                     | D           | Q   | D   | LYS                   | D           | D   | D   | ARG                   |
| PKC $\eta$     | M           | Q   | D    | D   | LEU/ARG               | D           | Q   | D   | ARG                   | D           | D   | D   | ARG                   |
| PKC $\zeta$    | M           | Q   | D    | N   | PHE                   | D           | Q   | D   | LYS                   | D           | D   | Q   | ARG                   |
| PKC $\mu$      | M           | S   | E    | A   | LEU                   | M           | S   | E   | VAL                   | M           | E   | Y   | ARG                   |

|                | Pocket      |      |     |     |     |     | Substrate position −2 | Pocket      |    | Substrate position −1 |
|----------------|-------------|------|-----|-----|-----|-----|-----------------------|-------------|----|-----------------------|
|                | Subdomain   |      |     |     |     |     |                       | Subdomain   |    |                       |
|                | VI          | VIII |     |     | IX  |     |                       | I           |    |                       |
|                | Residue no. |      |     |     |     |     |                       | Residue no. |    |                       |
|                | 170         | 201  | 203 | 204 | 230 | 236 |                       | 52          | 53 |                       |
| PKA            | E           | T    | E   | Y   | E   | P   | ARG                   | G           | S  | ASN                   |
| PKC $\alpha$   | D           | T    | D   | Y   | E   | P   | LYS                   | G           | S  | GLY                   |
| PKC $\beta$ I  | D           | T    | D   | Y   | E   | A   | LYS                   | G           | S  | GLY                   |
| PKC $\beta$ II | D           | T    | D   | Y   | E   | A   | LYS                   | G           | S  | GLY                   |
| PKC $\gamma$   | D           | T    | D   | Y   | E   | P   | LYS                   | G           | S  | GLY                   |
| PKC $\delta$   | D           | T    | D   | Y   | E   | S   | LYS                   | G           | S  | GLY                   |
| PKC $\epsilon$ | D           | T    | D   | Y   | E   | P   | LYS                   | G           | S  | MET                   |
| PKC $\eta$     | D           | T    | D   | Y   | E   | A   | ARG                   | G           | S  | ARG                   |
| PKC $\zeta$    | D           | T    | N   | Y   | E   | S   | GLN/LYS               | G           | S  | GLY                   |
| PKC $\mu$      | E           | T    | A   | Y   | V   | F   | GLN                   | G           | Q  | MET                   |

|                | Pocket      |      |     |     |     | Substrate position +1 | Pocket      |        |    |    | Substrate position +2 | Pocket      |    |    |     | Substrate position +3 |      |
|----------------|-------------|------|-----|-----|-----|-----------------------|-------------|--------|----|----|-----------------------|-------------|----|----|-----|-----------------------|------|
|                | Subdomain   |      |     |     |     |                       | Subdomain   |        |    |    |                       | Subdomain   |    |    |     |                       |      |
|                | VII         | VIII |     |     | X   |                       | I           | II–III |    |    |                       | II–III      |    |    |     |                       | VIII |
|                | Residue no. |      |     |     |     |                       | Residue no. |        |    |    |                       | Residue no. |    |    |     |                       |      |
|                | 187         | 198  | 202 | 205 | 247 |                       | 53          | 54     | 82 | 84 |                       | 81          | 82 | 83 | 198 |                       |      |
| PKA            | F           | L    | P   | L   | Y   | ILE                   | S           | F      | L  | Q  | HIS                   | K           | L  | K  | L   | ASP                   |      |
| PKC $\alpha$   | M           | F    | P   | I   | F   | PHE                   | S           | F      | D  | D  | ARG/LYS               | Q           | D  | D  | F   | ARG/LYS               |      |
| PKC $\beta$ I  | M           | T    | P   | I   | F   | PHE                   | S           | F      | D  | D  | LYS                   | Q           | D  | D  | T   | LYS                   |      |
| PKC $\beta$ II | M           | T    | P   | I   | F   | PHE                   | S           | F      | D  | D  | LYS                   | Q           | D  | D  | T   | LYS                   |      |
| PKC $\gamma$   | M           | F    | P   | I   | F   | PHE                   | S           | F      | D  | D  | LYS                   | Q           | D  | D  | F   | ARG                   |      |
| PKC $\delta$   | M           | F    | P   | S   | F   | PHE                   | S           | F      | D  | D  | PHE                   | I           | D  | D  | F   | TYR/PHE               |      |
| PKC $\epsilon$ | M           | F    | P   | L   | F   | PHE                   | S           | F      | D  | D  | PHE/ALA               | Q           | D  | D  | F   | GLU/TYR/ASP           |      |
| PKC $\eta$     | M           | F    | P   | L   | F   | PHE                   | S           | F      | D  | D  | ARG                   | L           | D  | D  | F   | ARG                   |      |
| PKC $\zeta$    | M           | F    | P   | I   | F   | PHE                   | S           | Y      | D  | D  | PHE/MET               | D           | D  | E  | F   | TYR/PHE               |      |
| PKC $\mu$      | F           | V    | P   | L   | Q   | VAL                   | Q           | F      | K  | E  | ALA/MET               | T           | K  | Q  | V   | PHE                   |      |

lytic cleft in an extended structure similar to that of PKI. Thus, the residues from the kinase that contact the side chains of substrate residues p-5 to p+3 can be predicted from the alignments with PKA.

Table V presents in single letter codes the residues from PKA that make contact with the p-5 to p+3 positions of PKI along with the residues at the analogous positions of the PKC isozymes. The optimal amino acid at each position, as determined with the peptide library, is also presented. It is clear that, as with PKA, the p-3 pockets of all the PKC isozymes are very acidic, and the p+1 pocket is very hydrophobic. This explains why a basic residue is selected at the p-3 position and a hydrophobic residue is selected at P+1 for all these enzymes. The p+1 pocket of PKC $\mu$  is more similar to the p+1 pocket of PKA than it is to the p+1 pockets of the other PKC isozymes. This may explain why this enzyme selects for peptides with Val

at the p+1 pocket (similar to the Ile selected at p+1 by PKA), while the other PKCs select for peptides with Phe at p+1.

The -5, -4, and -2 pockets of most of the PKC isozymes are quite acidic, consistent with basic residues being selected at these positions. The major exception is PKC $\mu$ , which has fewer acidic residues in these pockets. For example, all the PKCs except PKC $\zeta$  and PKC $\mu$  have an Asp at position 203 of the p-5 pocket. The Ala rather than Asp at this position may explain why PKC $\mu$  is unique in its strong selection for peptides with Leu at p-5. Likewise, all the PKCs except PKC $\mu$  have an Asp at position 127 in the p-4 pocket and all except PKC $\mu$  select for peptides with a basic residue at p-4. PKC $\mu$  has a Met in the p-4 pocket and selects for substrates with Val at p-4. The p-2 pockets of PKC $\zeta$  and PKC $\mu$  are less acidic than those of the rest of the PKCs, and these two enzymes select for peptides with Gln rather than Lys at p-2. Several PKC isoforms selected

substrates with either hydrophobic amino acids (Leu or Phe) or Arg at the p-5 position. The selection for hydrophobic amino acids may be explained by a hydrophobic residue in this pocket (e.g. a Met conserved in the PKC family members, Table V). The selection for Arg could be rationalized if the aliphatic part of the Arg side chain interacts with the hydrophobic Met in the pocket, while the guanidium group interacts with hydrophilic residues (Asp, Gln).

The differences in selectivity of the various PKCs at the p+2 and p+3 pockets can also be rationalized. All the PKCs except PKC $\mu$  have acidic residues in these pockets (Table V). This can explain why the cPKCs strongly select substrates with basic residues at p+2 and p+3 and why PKC $\mu$  fails to select for substrates with basic residues at these positions. PKCs  $\delta$ ,  $-\epsilon$ , and  $-\zeta$  weakly select for substrates with basic residues at p+2 and p+3 but prefer substrates with hydrophobic residues at these positions. This might be explained by subtle changes in the packing of residues in these regions such that the surfaces of the Phe residues in these pockets are more available for contact with substrate side chains (positions 54 and 198).

In summary, the oriented peptide library approach has provided information about substrate specificity of PKC isozymes that can explain selectivity for *in vivo* and *in vitro* substrates. In addition, the selectivity of individual PKC isozymes can be rationalized on the basis of analogies to the PKA/PKI crystal structure. Ultimately, these models will be testable by mutational studies and by co-crystals of PKC/peptide complexes.

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**Determination of the Specific Substrate Sequence Motifs of Protein Kinase C  
Isozymes**

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