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 (α, βI, βII, γ, δ, ε, η, ζ, and θ) 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 PKCμ, selected peptides with basic amino acids at positions –6, –4, and –2. PKCα, βI, βII, γ, and η selected peptides with basic amino acid at positions +2, +3, and +4, but PKCδ, ε, ζ, and θ preferred peptides with hydrophobic amino acid at these positions. At position –5, the selectivity was quite different among the various isozymes; PKCα, γ, and ζ selected peptides with Arg at this position while other PKC isozymes selected hydrophobic amino acids such as Phe, Leu, or Val. Interestingly, PKCμ showed extreme selectivity for peptides with Leu at this position. The predicted optimal sequences from position –3 to +2 for PKCα, βI, βII, γ, δ, and η 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 PKCα, βI, βII, γ, δ, ζ, and θ were prepared and used for the determination of Km and Vmax for these isozymes. As judged by Vmax/Km 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) 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 isoforms of PKC have been identified in mammalian tissues and subdivided into conventional PKC (cPKC) members comprising α, βI, βII, and γ isoforms (activated by calcium, acidic phospholipid, and diacylglycerol (DAG)), novel PKCs (nPKC) comprising δ, ε, η, and θ (activated by DAG and acidic phospholipid but insensitive to calcium), and atypical PKCs (aPKC) (1–6). Another subgroup of PKCs may be defined by PKCμ, 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 phosphorylation sites and the use of synthetic peptides based on these sites, a consensus phosphorylation site motif for PKC was determined to be RXXS/TXXR, 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 IIIS, 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-1α was shown to be phosphorylated with much greater efficiency by nPKC than by cPKCs, nPKCε or η, or aPKCζ (9). Heterogeneous ribonucleoprotein A1 is efficiently phosphorylated by PKCζ but not by cPKCs or PKCε (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...
substrates for human PKCa, βI, βII, γ, δ, η, ι, and µ and found that each PKC isoform has a unique optimal substrate sequence. Furthermore, we prepared synthetic peptides based on the predicted optimal sequences for PKCa, βI, βII, γ, δ, η, ι, and µ and showed that these peptides are high affinity and selective substrates for the respective PKC isoforms.

EXPERIMENTAL PROCEDURES

Materials—DAG was purchased from Boehringer Mannheim. PS was purchased from Avanti Polar Lipids. Isozyme-selective antipeptide antibodies for PKCs, βI, βII, γ, δ, η, ι, and µ were purchased from Santa Cruz Biotechnology. PKC βII substrate peptide (PRKRGQSGVRRV) was purchased from Upstate Biotechnology Inc. Anti c-myc antibody was purchased from Oncogene Sciences. PS1 phosphocellulose paper was purchased from Whatman. [γ-32P]ATP (3000Ci/mmol) was obtained from DuPont NEN. Liquisint was purchased from National Diagnostics. A ferric imidodiacetic acid (IDA) bead 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-tri-methylamino-phenyl-phosphonium hexafluorophosphate (BOP/HOBt) N-hydroxybenzotriazole coupling protocols using Peptide BioSynthesizer (Minipore Model) as described previously (12).

Identification of Optimal Substrate Sequence for Nine PKC Isozymes—In order to determine optimal substrate sequences for each of nine human PKC isoforms (α, βI, βII, γ, δ, η, µ, and ι), we used a degenerate peptide library, comprising peptides of sequence: MAXXXXXXXXAAXKKK (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 MAXXXXXXXXARKKK, was also used to investigate the 9 PKC isoforms, 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 isoform 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-imidodiacetic 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 PKCa and µ. These two enzymes clearly selected for peptides with hydrophilic residues, with Gln and Lys strongly selected for peptides with Gly at p-1, while PKCγ had an extremely strong selectivity for peptides with Leu at position p-5. More than 40% of the phosphopeptide products of PKCγ had Leu at this position. At the p-2 position, both PKCs selected peptides with hydrophilic residues, with Glu and Lys preferred. PKCα selected for peptides with Gly at p-1, while PKCγ selected against peptides with Gly at p-1. Both PKCs selected peptides with hydrophobic amino acids at p+1, though PKCα selected Phe while PKCγ selected Val. At p+2, p+3, and p+4 positions, PKCa strongly selected for peptides with the basic amino acids Arg or Lys. In contrast, PKCγ preferred peptides with hydrophobic amino acids in both positions.

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 μl) contained 100 μM ATP with [γ-32P]ATP (5 μCi), 1 mM DTT, 5 mM MgCl2, 25 mM Tris-HCl (pH 7.5), 20 μg/ml PS, 10 μM DAG, 200 μM CaCl2 (for PKCs, βI, βII, and γ), 0.5 mM EGTA (for PKCβII) and anti-c-myc antibody 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 PS1 phosphocellulose paper and washed 4 times in 500 ml of 1% phosphoric acid. Incorporation of 32P 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 Km and Vmax, reaction rates were linear with respect to time for all conditions of peptide, and less than 10% of the peptide substrate was phosphorylated.
Human PKCα and -μ were expressed in Sf9 cells using baculovirus. A degenerated substrate library with the sequence Met-Ala-X-X-X-Arg-X-X-Ser-X-X-X-Ala-Lys-Lys (where X indicates any amino acid)

**FIG. 1.** Comparison of the substrate specificities of PKCα and PKCμ. Human PKCα and -μ were expressed in Sf9 cells using baculovirus. A degenerated substrate library with the sequence Met-Ala-X-X-X-Arg-X-X-Ser-X-X-X-Ala-Lys-Lys (where X indicates any amino acid
Specific Substrate Motifs of PKC Isozymes

TABLE I

Substrate specificities of protein kinase C isozymes

| PKC isozymes | -7 | -6 | -5 | -4 | -3 | -2 | -1 | 0 | +1 | +2 | +3 | +4 | +5 |
|--------------|----|----|----|----|----|----|----|----|----|----|----|----|----|
| PKCo        | R(2.1) | R/F(1.5) | R(2.1) | R(2.1) | R(2.1) | R(2.1) | Q(2.0) | G(2.0) | S | F(2.6) | R/(K(3.4) | R/(K(3.0) | K(2.3) | A(1.8) |
|             | L/F(1.7) | F(1.6) | R(1.8) | V(1.5) | G(2.0) | A/M(1.7) | M/L(1.8) | F(1.8) | R(1.9) | K(1.7) |
| PKCβI       | R/(K(1.5) | K(1.8) | L/R(2.1) | K/(R(2.3) | R(2.7) | Q(2.7) | G(2.0) | S | F(3.1) | K(2.5) | K(2.0) | F(1.8) | A(2.3) |
|             | K(1.6) | F(2.0) | F(1.5) | A(1.9) | M(1.9) | R(1.9) | F/(R(1.7) | V(1.6) | F(1.8) |
| PKCβII      | Y(1.8) | K(1.6) | L(1.9) | K(1.9) | R(2.2) | Q(2.0) | G(2.1) | S | F(2.0) | K(2.6) | K(2.6) | A(1.7) | M(1.5) |
|             | F(1.8) | K(1.6) | Q(1.5) | E(1.5) | M(2.1) | R(2.0) | Y(1.7) | F(1.5) | F/(K(1.6) | F(1.8) |
| PKCγ        | R(1.9) | R(1.9) | R(2.7) | R(3.5) | R(3.5) | R(2.4) | G(2.7) | S | F(2.1) | K(2.8) | K(2.4) | R(1.3) | A(1.6) |
|             | K(1.6) | F(1.5) | K(1.6) | Q(1.9) | M(1.5) | M(1.5) | S(2.1) | F(1.8) | V(1.5) | G(1.9) | G(2.2) | F(1.8) |
| PKCd        | A(1.6) | A/(R(1.7) | R(2.1) | K/(A(1.7) | R(2.1) | G(2.6) | S | F(2.4) | F/(V(1.5) | K/(M(1.6) | K(1.6) | F(1.8) |
|             | K(1.6) | E(1.9) | A(1.7) | Q(1.5) | K(1.5) | K(1.5) | M/V(1.8) | F(1.8) | G(1.9) | G(2.2) | F(1.8) |
| PKCe        | Y(1.9) | Y/(K(1.5) | X | R(1.7) | R(3.1) | R(2.7) | R(2.1) | R(2.1) | R(2.1) | R(2.1) | R(2.1) | R(2.1) | R(2.1) |
|             | E(1.6) | A/Q(1.5) | R(1.7) | K(1.6) | K(1.6) | K(1.6) | I/ M(1.7) | Y/(E(1.6) | F/(E(1.5) | F/(E(1.5) | |
| PKCη        | A(2.1) | R(2.0) | L/R(1.9) | R(1.9) | R(2.2) | R(2.2) | R(2.3) | F(2.4) | F(2.0) | R(2.1) | X | R(1.6) |
|             | R(1.8) | A(1.6) | R(1.6) | Q(1.9) | M(1.5) | M(1.5) | I/ M(1.7) | F(1.8) | Y/(E(1.6) | |
| PKCζ        | R(1.8) | R/(K(1.6) | F(2.5) | F(2.5) | K/(R(1.8) | K/(Y(1.8) | G(1.7) | S | F(2.6) | F/(V(1.8) | F(1.9) | F(1.7) | Y/(K(1.5) |
|             | L/R(1.6) | F(1.6) | R(1.8) | Y(1.6) | K/(M/Y(1.5) | M(2.0) | M(2.0) | M(2.0) | M(2.0) | F(1.8) | Y(1.6) | A(1.5) |
| PKCo        | A(1.9) | A/(K(1.7) | L(6.2) | V(2.2) | R | Q(2.4) | M(2.2) | S | V(2.0) | A/M(1.7) | F(1.8) | F(2.2) | F(1.9) |
|             | Y(1.5) | P(1.5) | V(3.3) | L(2.1) | K(2.1) | A(2.0) | L(1.6) | L(1.6) | L(1.6) | V(1.5) | V(1.5) | V(1.5) | A(1.6) | M(1.5) |

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 except 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.

The results obtained for other PKC isozymes are summarized in Table I. All PKC isozymes selected for peptides with hydrophobic amino acid at position +1, and all isozymes except PKCo selected for peptides with basic amino acid at the p–6, p–4 and p–2 positions. Interestingly, the predicted sequences for peptide substrates with Gln or Glu at the p–6, p–4 and p–2 positions, although in most cases Lys at this position was optimized, PKCo was selected for peptides with hydrophobic amino acids such as Phe, Leu, or Val. PKCβII and -γ 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 PKCζ. The predicted optimal sequences from p–3 to p+2 for PKCo, -βI, -βII, -γ, -δ, and -η 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 PKCo 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 PKCo, -β, and -γ). Most of these proteins have the motif R/K-X-R/K-R/K-X/S-Φ-R/K-R/K, where Φ 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 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 MAXXXXSXXXAKK, we found that PKCo, -βI, and -δ strongly selected for peptides with...
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 have been shown to be isozyme-specific substrates. eEF-1α is reported to be a specific substrate for PKCδ (9). The sequence of 426–436 from murine eEF-1α containing Thr-431 was compared with the predicted optimal substrate of PKCδ (Table III). PKCδ strongly selects for substrates with Arg at p−2, basic at p−2, hydrophobic at p−1, and Gly at p+4. The site in eEF-1α meets these criteria. The failure of cPKCs to phosphorylate this site could be explained.

### Table II

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

| PKC isozymes | Position |
|-------------|----------|
| PKCa optimal. | R R R K G |
| PKCa pseudo.  | S F R R K A |
| PKCβI optimal. | F K L R K G |
| PKCβI pseudo.  | S F K K F A |
| PKCβII optimal. | Y K L R K G |
| PKCβII pseudo. | S F K K A |
| PKCy optimal. | R R R R K G |
| PKCy pseudo.  | S F K K R K A |
| PKCc optimal. | A R R K K G |
| PKCc pseudo.  | A L R Q K V |
| PKCe optimal. | Y Y X R K M |
| PKCe pseudo.  | S F F Y G G |
| PKCδI optimal. | A R R K R Q |
| PKCδI pseudo. | A V R R R R |
| PKCy optimal. | R R R K Q G |
| PKCy pseudo.  | S F F Y F F |
| Ribosomal protein S6′ | R R R L S |
| PTP1B | R V V G S |
| Troponin I′ | K F R R P |
| Insulin receptor tyrosine kinase′ | N G R I L T |
| P-glycoprotein′ | R S T R S V |
| Kit/SCFR′ | A D K R R S V |
| Annexin II′ | P S A Y G S V K P Y T |

### 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

| Peptide/protein | position |
|-----------------|----------|
| MARCKS protein | R R K G S F |
| MARCKS protein | F K K R F S |
| MARCKS protein | K L S G F |
| MARCKS protein | L R M F S F |
| MARCKS protein | K L K K S F |
| MARCKS protein | K L S G F |
| MARCKS protein | L R M F S F |
| MARCKS protein | K L K K S F |
| MARCKS protein | K L S G F |
| MARCKS protein | L R M F S F |
| MARCKS protein | K L K K S F |
| Troponin I′ | R R R K G S F |
| Insulin receptor tyrosine kinase′ | N G R I L T |
| P-glycoprotein′ | R S T R S V |
| P-glycoprotein′ | L I R K R S T |
| Kit/SCFR′ | A D K R R S V |
| Annexin II′ | P S A Y G S V K P Y T |

a From Ref. 8.

b From Ref. 26.

c From Ref. 27.

d From Ref. 28.
e From Ref. 29.
f From Ref. 30.
g From Ref. 31.
h From Ref. 9.
i From Ref. 15.
MARCKS is not a good substrate for PKC because it has Leu at the position at which PKC shows greatest selectivity (Table III).

The optimal substrates for PKC family members selectively phosphorylate different subsets of the peptides. The optimal peptide for PKCε is in good agreement with the predicted optimal sequence of PKCε derived from the predicted optimal substrate sequence of PKCε. The glycogen synthase-derived peptide sequence is in good agreement with the theoretical peptides by PKCε.

The results we obtained for the specificities of the cPKCε and PKCβ3 are in good agreement with previous studies. The α-peptide is a Leu at the position broadens the number of kinases that could phosphorylate the substrate. The high specificity of the µ-peptide for PKCε can probably be explained by the lack of basic residues at p+2, p+3, and p+4 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 isoforms 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 isoforms 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 PKCα, PKCβ1, PKCδ, and PKCε were synthesized and shown to be excellent substrates for the respective enzymes.

Although each PKC isoform 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 (α, βI, βII, and γ) 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 (δ, ε, and η) and the nPKCζ also selected for substrates with basic residues at −6, −4, −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. PKCε 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 PKCε is 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
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 p
1 position for phosphorylation by cPKCs, consistent with known selectivity of these enzymes (21). The peptide lysine at p
1 has shown the importance of having basic residues at positions p
1, p
2, and p
3 for phosphorylation by the cPKCs, α and γ (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 isoforms are similar but not identical to the pseudosubstrate regions of the respective enzymes (Table II). Peptide substrates based on the pseudosubstrate regions of PKCa, βI, γ, and ε 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 PKCβI. 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 ε-peptide, these peptides are preferential substrates of the kinases for which they were designed (Table IV).

A few isoyme-specific PKC substrates have been previously reported. A synthetic peptide based on region 422–443 of eEF-1a (RFAVRDRMT*IVAVGVKVKAVDKK) was reported to be phosphorylated at Thr-431 by PKCδ but not by other PKC isoforms (9). Conversion of Met-428 (the p
2 position) to Lys made this peptide a good substrate for all PKC isoforms. These results are consistent with our finding that the cPKCs prefer substrates with basic residues at p
2 and p
3, whereas PKCδ 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 PKCμ. The peptide library results show that the two most critical residues for substrates of PKCμ are Leu at position p
5 and an aliphatic residue (preferentially Val) at position p
4 (Table I). PKCμ 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 PKCμ 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 PKCμ substrate based on the peptide library results (Table I). Syntide 2 (PLARTLS*VAGLPKGGK), a synthetic peptide derived from glycogen synthase, is also an efficient substrate of human PKCμ (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 PKCμ 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 βI-peptide is optimal for PKCβI but is a useful general substrate for all PKC isoforms (including PKCμ). The α-peptide (like the ε-pseudosubstrate peptide) is a general substrate for all PKCs except PKCδ. The μ-peptide is extremely specific for PKCμ, and the δ-peptide is relatively specific for PKCδ. The ε-peptide is phosphorylated by PKCα, PKCβI, and PKCμ 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-
Specific Substrate Motifs of PKC Isozymes

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

| Subdomain | Pocket | Substrate position | Residue no. |
|-----------|--------|--------------------|-------------|
| V         | VI     | VIII               | 129 133 170 203 |
|           |        |                    | 127 133 328 330 |
| PKCA      | F      | R                  | E           | E | ARG |
| PKCβI     | M      | Q                  | D           | D | ARG |
| PKCβII    | M      | Q                  | D           | D | ARG |
| PKCγ      | M      | Q                  | D           | D | ARG |
| PKCε      | M      | Q                  | D           | D | ARG |
| PKCθ      | M      | Q                  | D           | D | ARG |
| PKCε      | M      | Q                  | D           | D | ARG |
| PKCθ      | M      | Q                  | D           | D | ARG |
| PKCε      | M      | Q                  | D           | D | ARG |
| PKCθ      | M      | Q                  | D           | D | ARG |
| PKCε      | M      | S                  | E           | A | VAL |
| PKCθ      | M      | S                  | E           | A | VAL |
| PKCε      | M      | S                  | E           | A | VAL |
| PKCθ      | M      | S                  | E           | A | VAL |

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 PKCε 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 PKCε, which has fewer acidic residues in these pockets. For example, all the PKCs except PKCε and PKCθ have an Asp at position 203 of the p−5 pocket. The Ala rather than Asp at this position may explain why PKCθ is unique in its strong selection for peptides with Leu at p−5. Likewise, all the PKCs except PKCμ have an Asp at position 127 in the p−4 pocket and all except PKCμ select for peptides with a basic residue at p−4. PKCμ has a Met in the p−4 pocket and selects for substrates with Val at p−4. The p−2 pockets of PKCε and PKCθ 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 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.
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\(\delta\) 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 isoforms that can explain selectivity for in vivo and in vitro substrates. In addition, the selectivity of individual PKC isoforms 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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