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Protein kinase C in *Saccharomyces cerevisiae*: Comparison with the mammalian enzyme

(diacylglycerol/phorbol ester/myelin basic protein)

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ABSTRACT **Protein kinase C (PKC) was detected in the yeast *Saccharomyces cerevisiae* with bovine myelin basic protein as the phosphate acceptor. The enzyme was purified at least 500-fold by a four-step column chromatographic procedure (phenyl-Sepharose CL-4B, Mono Q, Heparin-5PW, and hydroxyapatite). The molecular mass was approximately 90 kDa, as estimated by gel-filtration analysis. Yeast PKC was activated by the simultaneous addition of Ca^{2+}, diacylglycerol, and phosphatidylserine. Free arachidonic acid alone could activate the enzyme to some extent. However, yeast PKC did not respond significantly to tumor-promoting phorbol esters. GTP did not serve as phosphate donor. The yeast enzyme showed substrate specificity distinctly different from that of mammalian PKCs. H1 histone and protamine were poor substrates. With myelin basic protein as a model substrate, yeast PKC phosphorylated threonyl residues preferentially, whereas rat brain PKCs phosphorylated seryl residues preferentially. Further studies should elucidate the role of yeast PKC in cellular regulation and cell cycle control.**

Protein kinase C (PKC) plays a crucial role in the signal transduction pathways that control various physiological processes (1). Biochemical, molecular cloning, and immunocytochemical analysis has revealed the existence of multiple subspecies of PKC in various mammalian tissues (for review, see ref. 1) as well as in other organisms such as *Xenopus laevis* (2), *Dictyostelium discoideum* (3, 4), sea urchin eggs (5), and *Drosophila* (6, 7). Thorner *et al.* (8) have proposed the existence of an enzyme in *Saccharomyces cerevisiae* that possesses properties similar to those of mammalian PKC. This yeast enzyme would utilize protamine as substrate and require Ca^{2+}, diacylglycerol (DG), and phosphatidylserine (PS) for its catalytic activity. In addition, these authors have isolated a gene that encodes a nucleotide sequence that is about 50% identical to the sequence of the mammalian PKC (8). However, neither the enzymological properties nor the genetic and structural features of yeast PKC (yPKC) have been documented in detail. In the present studies we have confirmed the existence of PKC in the yeast *S. cerevisiae*. This enzyme, unlike mammalian PKCs, preferentially phosphorylates bovine myelin basic protein (MBP) rather than H1 histone and protamine and shows enzymological characteristics distinctly different from those of mammalian PKCs. yPKC will be described here and compared with the α subspecies of rat brain PKC (αPKC), which is the most common species in mammalian tissues and cell types.

MATERIALS AND METHODS

Yeast Strain and Culture. *S. cerevisiae* (strain IFO 10430; MATa *pho 9-1 leu 2-3, 112 his 4-519 canl*) was obtained from the Central Research Laboratories, Takeda Chemical Industries (Osaka) and cultured in the YPD medium [glucose (20 g/liter)/polypeptone (20 g/liter)/yeast extract (10 g/liter)] with continuous agitation at 30°C. This strain contained low protease activity. The cells, grown at 30°C for 16–20 hr until late-logarithmic phase, were employed for the present studies.

Chemicals. All chemicals were of analytical grade. Bovine brain MBP, protamine sulfate (from herring), kemptide, and soybean trypsin inhibitor were purchased from Sigma. [γ-^{32}P]ATP and [γ-^{32}P]GTP were obtained from New England Nuclear. Phospholipids, DGs, and free fatty acids were purchased from Serdary Research Laboratories (London, ON, Canada). Phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDBu), and 4α-phorbol 12,13-didecanoate (4αPDD) were the products of LC Services (Woburn, MA). Calf thymus H1 histone was prepared by the method of Oliver *et al.* (9). The peptide substrates employed in the present studies were synthesized using an Applied Biosystems peptide synthesizer model 430A and were as follows: Gln-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu [MBP-(4–14), Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala [S6-(232–239)], and Val-Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu {epidermal growth factor receptor-(650–658) [EGFR-(650–658)]}. These sequences are from MBP, ribosomal S6 protein, and EGFR, respectively, as indicated. Protease inhibitors such as leupeptin, antipain, pepstatin, and chymostatin were purchased from Peptide Institute (Osaka). (*p*-Amidinophenyl)methanesulfonyl fluoride hydrochloride (*p*-APMSF) was purchased from Wako Pure Chemical (Osaka). Other chemicals were obtained from commercial sources.

Enzyme and Assay. αPKC was purified from the rat brain cytosol as described (10). yPKC and αPKC were routinely assayed by measuring the incorporation of $^{32}P_i$ into bovine MBP from [γ-^{32}P]ATP under the conditions essentially as described (11). The standard reaction mixture (50 μl) contained 20 mM Tris Cl (pH 7.5), 10 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP, MBP (200 μg/ml), PS (80 μg/ml), diolein (DO; 8 μg/ml), and 10 μM $CaCl_2$. Basal activity was measured in the presence of 0.5 mM EGTA instead of PS, DO, and $CaCl_2$. After incubation for 10 min at 30°C, the reaction was termi-

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Abbreviations: PKC, protein kinase C; yPKC, yeast protein kinase C; αPKC, α subspecies of rat brain protein kinase C; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol-12,13-dibutyrate; 4αPDD, 4α-phorbol 12,13-didecanoate; MBP, myelin basic protein; DG, diacylglycerol; PS, phosphatidylserine; DO, diolein; DTT, dithiothreitol; *p*-APMSF, (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride; EGFR, epidermal growth factor receptor.

nated by spotting 40 μl of the reaction mixture onto P81 paper (Whatman). The paper was washed for five 5-min periods by immersion in about 10 ml of 75 mM H_3PO_4. Phosphorylation of H1 histone, protamine sulfate, and various synthetic peptides was similarly assayed. When casein was employed as substrate, 25% (wt/vol) trichloroacetic acid-insoluble materials were collected on a nitrocellulose filter. The radioactivity was quantitated using a scintillation spectrometer by Cerenkov counting.

Phospho Amino Acid Analysis. Phosphorylated amino acids were identified under the conditions described by Hunter and Sefton (12). Briefly, MBP and H1 histone were extensively phosphorylated by either yPKC or αPKC in the presence of PS (80 μg/ml), DO (8 μg/ml), and 10 μM $CaCl_2$ and subjected to SDS/PAGE analysis. The radioactive MBP or H1 histone was excised and hydrolyzed in 6 M HCl at 100°C for 3 hr. The hydrolyzates were subjected to thin layer electrophoresis (Polygram CEL-300) in the presence of authentic samples of phosphoserine, phosphothreonine, and phosphotyrosine, as described by Hunter and Sefton (12). The standards were stained with ninhydrin, and the ^{32}P-labeled amino acids were detected with autoradiography.

Molecular Mass Estimation. The molecular mass of yPKC was estimated by the gel-filtration procedure with a HiLoad 16/60 Superdex-200 column (1.6 × 60 cm), which was connected to an FPLC system (Pharmacia), equilibrated with 20 mM Tris Cl (pH 8.0) containing 0.5 mM EGTA, 0.5 mM EDTA, 2 mM dithiothreitol (DTT), and 200 mM NaCl. Samples (2 ml) were applied, and the proteins were eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected. Blue dextran was measured by absorbance at 625 nm for the void-volume determination. Aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7 kDa) were used as molecular mass standards.

RESULTS

Purification of yPKC. All manipulations were carried out at 0–4°C. Cells [100 g (wet weight)] were suspended in 200 ml of 50 mM Tris Cl (pH 8.0) containing 1 mM EGTA, 1 mM EDTA, 5 mM DTT, leupeptin (100 μg/ml), 50 μM *p*-APMSF, antipain (0.5 mg/ml), pepstatin (0.5 mg/ml), chymostatin (0.5 mg/ml), and soybean trypsin inhibitor (0.5 mg/ml). The cell suspension was homogenized for 3 min by using a Bead-Beater (Biospec Products, Bartlesville, OK) with 200 g of glass beads (0.5-mm diameter).

The resulting homogenate was centrifuged for 60 min at 100,000 × *g* and filtered through glass wool. The supernatant (180 ml, 2.1 g of protein), brought to 2 mM $MgCl_2$ and 2 mM $CaCl_2$, was applied on a phenyl-Sepharose CL-4B column (Pharmacia, 2.6 × 10 cm) previously equilibrated with 20 mM Tris Cl (pH 8.0) containing 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM EGTA, 1 mM EDTA, 2 mM DTT, leupeptin (50 μg/ml), 50 μM *p*-APMSF, antipain (10 μg/ml), pepstatin (10 μg/ml), chymostatin (10 μg/ml), and soybean trypsin inhibitor (10 μg/ml) at a flow rate of 0.3 ml/min. After washing the column with 50 ml of the equilibration buffer, the enzyme was eluted batch-wise with 125 ml of 20 mM Tris Cl (pH 8.0) containing 10 mM EGTA, 2 mM DTT, leupeptin (50 μg/ml), 50 μM *p*-APMSF, antipain (10 μg/ml), pepstatin (10 μg/ml), chymostatin (10 μg/ml), and soybean trypsin inhibitor (10 μg/ml) at a flow rate of 0.3 ml/min. The protein kinase in this eluate did not require PS and DG for enzymatic activity presumably due to some lipids that contaminated the preparation. The enzyme fraction (125 ml, 130 mg of protein) was directly applied on a Mono Q HR 16/10 column (Pharmacia, 1.6 × 10 cm) that was connected to an FPLC system previously equilibrated with 20 mM Tris Cl (pH 8.0) containing 0.5 mM EGTA, 0.5 mM EDTA, 2 mM DTT, 5% (wt/vol) glycerol, leupeptin (50 μg/ml), and 50 μM *p*-APMSF (buffer A). The column was washed with 100 ml of buffer A at a flow rate of 3 ml/min. yPKC was eluted by application of a 360-ml linear concentration gradient of NaCl (0–0.5 M) in buffer A at a flow rate of 3 ml/min. Fractions (6 ml) were collected. When each fraction was assayed with MBP as phosphate acceptor in the presence of PS, DO, and Ca^{2+}, one major and several minor peaks appeared (Fig. 1).

Fractions 24–32 of the major peak (54 ml, 14.5 mg of protein) were pooled, diluted with 7 vol of buffer A, and applied on a TSK Heparin-5PW column [Toyo Soda (Tokyo), 2.15 × 15 cm]. After washing with 200 ml of buffer A, PKC was eluted by application of a 240-ml linear concentration gradient of NaCl (0–0.8 M) in buffer A at a flow rate of 4 ml/min. Fractions (4 ml) were collected. A single peak of yPKC was eluted at about 0.2 M NaCl. This enzyme fraction contained some cAMP-dependent protein kinase, which could be removed by the following step.

The active fractions (16 ml, 880 μg of protein) were pooled and then loaded directly on a packed column of hydroxyapatite (KOKEN, 0.78 × 10 cm) equilibrated with 20 mM potassium phosphate (pH 7.5) containing 0.5 mM EGTA, 0.5 mM EDTA, 10% glycerol, 2 mM DTT, leupeptin (20 μg/ml), and 50 μM *p*-APMSF (buffer B). The column was washed with 15 ml of buffer B at a flow rate of 0.4 ml/min. yPKC was then eluted by application of a 84-ml linear concentration gradient of potassium phosphate (pH 7.5, 20–250 mM) in buffer B. Fractions (1 ml) were collected. Fig. 2*A* shows a typical elution profile of the enzyme, which appeared again as a major peak with some shoulders. Rat brain PKC can be resolved into three subspecies by hydroxyapatite column chromatography (10), which are given in Fig. 2*B* for comparison. It is presently unknown whether these several yPKC peaks represent multiple species of the enzyme or are simply proteolytic artifacts. Structural analysis should provide the answer to this question. For further studies, the major peak, fractions 36–48 (protein at 14 μg/ml), was employed. Some inhibitors and lipids that contaminated the eluate from phenyl-Sepharose CL-4B column interfered with the quantitative measurement of yPKC activity. If the recovery of the enzyme from the phenyl-Sepharose CL-4B column was 100%, then this enzyme was purified at least 500-fold. By using MBP as a common phosphate acceptor protein, the yPKC preparation showed one-third the specific activity of an apparently pure αPKC preparation, although the phosphorylation sites by these enzymes were different (see below).

Molecular Mass. The molecular mass of the main yPKC fraction was estimated to be about 90 kDa by gel filtration. This value is slightly larger than that of mammalian PKCs (77–80 kDa).

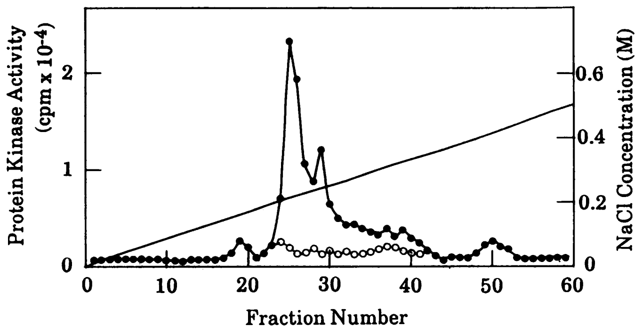

FIG. 1. yPKC elution profile from a Mono Q column. The active yPKC fraction from a phenyl-Sepharose column was subjected to Mono Q HR 16/10 column chromatography and yPKC activity was assayed. ●, In the presence of 10 μM $CaCl_2$, PS (80 μg/ml), and DO (8 μg/ml); ○, in the presence of 0.5 mM EGTA (instead of $CaCl_2$, PS, and DO).

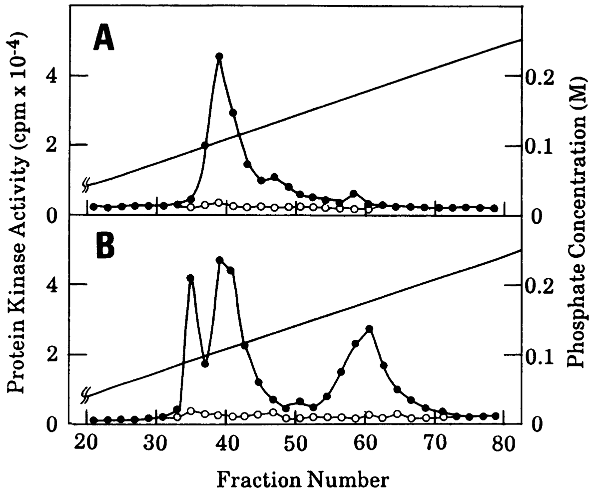

FIG. 2. Hydroxyapatite column chromatography of PKCs from *S. cerevisiae* and rat brain. yPKC from Heparin-5PW and rat brain PKCs from DE52 (10) were subjected to hydroxyapatite column chromatography and the PKC activity was assayed. (*A*) yPKCs from *S. cerevisiae*. (*B*) PKCs from the rat brain. ●, In the presence of 10 μM $CaCl_2$, PS (80 μg/ml), and DO (8 μg/ml); ○, in the presence of 0.5 mM EGTA (instead of $CaCl_2$, PS, and DO).

Requirement of Ca^{2+} and Lipids. With MBP as phosphate acceptor, yPKC *per se* was catalytically inactive and almost absolutely required PS, DO, and Ca^{2+} to exhibit full enzymatic activity, as shown in Fig. 3*A*. DO alone showed no stimulation but enhanced the PS-dependent activity over a wide range of Ca^{2+} concentrations. In the presence of EGTA instead of Ca^{2+}, yPKC showed a substantial activity, but Ca^{2+} in the micromolar range was needed for maximum activity. Fig. 3*B* shows the kinetic properties of αPKC for comparison. With MBP as phosphate acceptor, αPKC showed some activity with DO in the absence of PS at higher Ca^{2+} concentrations. H1 histone was a poor substrate for yPKC (see below), but the reaction kinetics was similar to that observed with MBP as shown in Fig. 3*C*. Fig. 3*D* shows the kinetic properties of αPKC with H1 histone. In the presence of higher concentrations of Ca^{2+}, PS could be replaced by other phospholipids such as phosphatidylinositol and phosphatidic acid for both yPKC and αPKC. Other DGs including 1,2-dioctanoylglycerol, 1,2-dimyristoylglycerol, and 1-oleoyl-2-acetylglycerol could activate yPKC as well as mammalian PKCs in the presence of PS and Ca^{2+}.

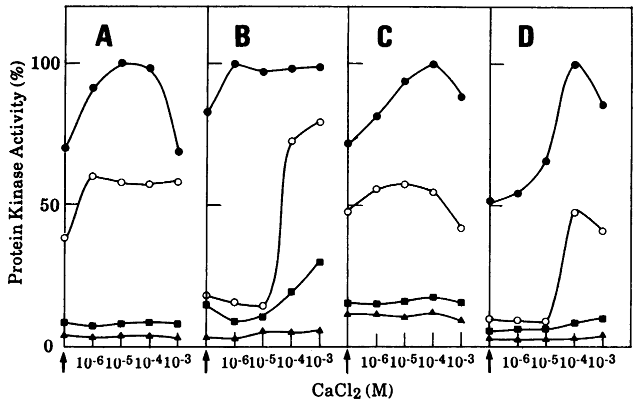

FIG. 3. Activation of PKC by PS and DO at various concentrations of Ca^{2+}. PKC was assayed with MBP or H1 histone. (*A*) yPKC activity with MBP. (*B*) αPKC activity with MBP. (*C*) yPKC activity with H1 histone. (*D*) αPKC activity with H1 histone. ●, In the presence of PS (40 μg/ml) and DO (8 μg/ml); ○, in the presence of PS (40 μg/ml) alone; ■, in the presence of DO (8 μg/ml) alone; ▲, in the absence of PS and DO. Where indicated with arrows, 1 mM EGTA was added instead of $CaCl_2$. Results were normalized to the maximum activity obtained in the presence of PS and DO at the optimum concentration of $CaCl_2$.

Lack of Activation by Phorbol Ester. Tumor-promoting phorbol esters such as PMA and PDBu can substitute for DG and activate mammalian PKCs in the presence of Ca^{2+} and PS (13). It is particularly worth noting that yPKC was not significantly activated by PMA or PDBu. Fig. 4 shows the response of yPKC and αPKC to active and inactive phorbol esters under comparable conditions. 4αPDD was inactive toward yPKC and αPKC.

Activation by Arachidonic Acid. Although the physiological significance has not been fully clarified, the mammalian PKCs are activated substantially *in vitro* by free unsaturated fatty acids, such as arachidonic acid and oleic acid, in the absence of added Ca^{2+} and PS (14, 15). The kinetics of this activation varies greatly with the subspecies of PKC and also with the phosphate acceptors employed (16). Analogously, yPKC was activated by arachidonic acid, with MBP or H1 histone as phosphate acceptor, although the optimum arachidonic acid concentrations for these reactions were not identical, as shown in Fig. 5. Under the same conditions, αPKC responded to the fatty acid with H1 histone but not with MBP as phosphate acceptor. Arachidonic acid methyl ester was always inactive.

Phosphate Donor and Acceptor. yPKC utilized ATP but not GTP as phosphate donor. The physiological substrate of yPKC in yeast remains unknown at present, but the enzyme could phosphorylate several proteins and synthetic oligopeptides containing seryl and threonyl residues. Among various phosphate acceptors examined, MBP was the best model substrate for yPKC. H1 histone, which is normally employed for the assay of mammalian PKCs, did not serve preferentially as a substrate. Protamine, which is a unique substrate for mammalian PKCs since its phosphorylation does not depend on PS, DG, or Ca^{2+} (17), was also a poor substrate for yPKC. This reaction of yPKC with protamine, if any, was dependent on the presence of PS, DO, and Ca^{2+}. The relative activities of various phosphate acceptors are shown in Table 1. Synthetic polypeptides such as MBP-(4–14), S6-(232–239), EGFR-(650–658), and kemptide, which are frequently used as model substrates for the assay of mammalian PKCs, were practically inactive for yPKC.

Phospho amino acid analysis of MBP and H1 histone that were phosphorylated by yPKC was performed. The results in Fig. 6 indicate that yPKC preferentially phosphorylated threonyl residues, whereas αPKC phosphorylated seryl res-

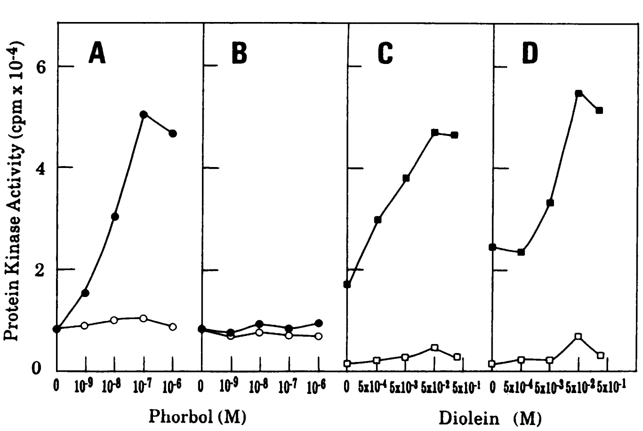

FIG. 4. Response to phorbol esters and DG. αPKC and yPKC were assayed with MBP as phosphate acceptor in the presence of PS (40 μg/ml) and 10 μM $CaCl_2$ at various concentrations of phorbol ester derivatives and DO. (*A* and *C*) αPKC activity. (*B* and *D*) yPKC activity. ●, PMA; ○, 4αPDD; ■, DO; □, DO in the absence of PS.

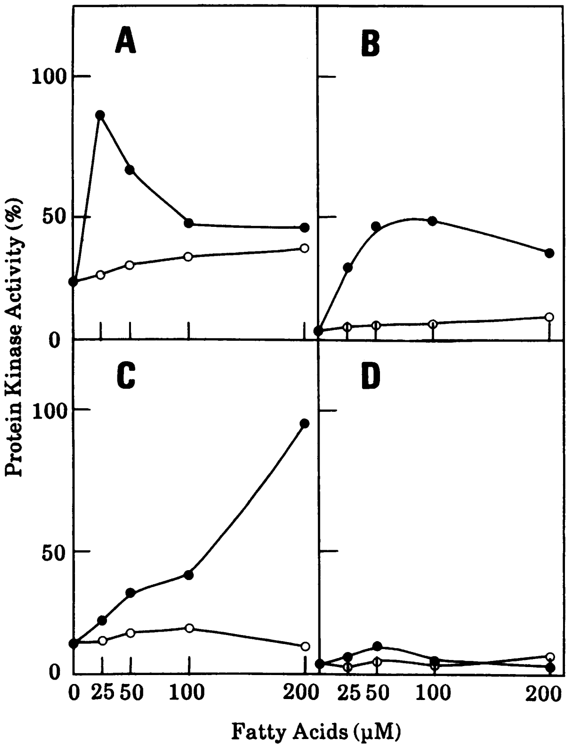

FIG. 5. Activation of PKC by free arachidonic acid and its methyl ester. αPKC and yPKC were assayed in the presence of 10 µM $CaCl_2$ and various concentrations of fatty acids. (*A*) yPKC activity with H1 histone. (*B*) yPKC activity with MBP. (*C*) αPKC activity with H1 histone. (*D*) αPKC with activity MBP. ●, Arachidonic acids; ○, methyl arachidonate. Results were normalized to the maximum activity that was obtained in the presence of PS (80 µg/ml), DO (8 µg/ml), and 10 µM $CaCl_2$.

idues in these proteins. The results are consistent with the fact that yPKC did not react with MBP-(4–14), which does not contain a threonyl residue.

DISCUSSION

PKC is distributed ubiquitously in many tissues and organs in mammals and other higher organisms and appears to play pivotal roles in the control of various cellular activities, including growth and differentiation (1). To clarify further the biochemical basis of action of yPKC in gene expression and cell cycle control, we searched for the existence of PKC in lower eukaryotic cells such as yeast, since genetic manipulation of yeast is much easier. However, all attempts to detect PKC in yeast were unsuccessful until recently. Thorner *et al.* (8) proposed the existence of a PKC in *S. cerevisiae* that phosphorylates protamine and has enzymological characteristics similar to those of mammalian PKC. In the present studies, we have confirmed the presence of PKC in this organism, but the yPKC described herein utilizes MBP rather than H1 histone and protamine and has enzymological properties clearly different from those of the PKC family thus far isolated from mammalian tissues. The results presented above apparently show multiple species of yPKC, although a proteolytic artifact cannot be ruled out until structural analysis is done. The striking differences between yPKC and the mammalian PKC are that these enzymes clearly differ from each other in their substrate specificity and in their responses to tumor-promoting phorbol esters. It is possible that the yPKC described above is a different enzyme than that reported by Thorner *et al.* (8). Thorner *et al.* (8) also isolated one gene from *S. cerevisiae* that showed 50% nucleotide sequence homology with the mammalian PKC sequence. However, the structural and genetic characterization of the yPKC remains to be done. Although the activation signal of yPKC is presently unknown, further enzymological and genetic studies may elucidate the physiological role of this protein kinase pathway in growth and cell cycle control.

Table 1. Substrate specificity of yeast and mammalian PKCs

Substrate	Activity, %	
	yPKC	αPKC
MBP	100	100
H1 histone	12	30
Protamine sulfate	8	193
Casein	2	5
MBP-(4-14) (QKRPSQRSKYL)	2	140
S6-(232–239) (RRLSSLRA)	3	106
EGFR-(650–658) (VRKRTLRRL)	6	43
Kemptide (LRRASLG)	4	6

PKC activity was assayed with MBP (200 µg/ml), H1 histone (200 µg/ml), protamine (400 µg/ml), casein (400 µg/ml), MBP-(4–14) (40 µM), S6-(232–239) (200 µM), EGFR-(650–658) (40 µM), or kemptide (20 µM) as phosphate acceptors. The amounts of enzyme used in each assay were 6.8 ng (αPKC) or 36 ng (yPKC). The specific activities of αPKC and yPKC used in this experiment were 320 nmol/min per mg and 70 nmol/min per mg, respectively, with MBP as phosphate acceptor. The PKC activities with various substrates were expressed as a percentage of that obtained with MBP.

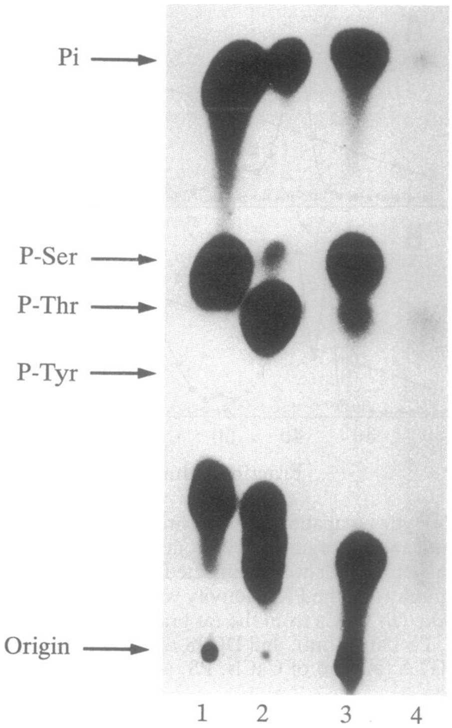

FIG. 6. Phospho amino acid analysis. The phosphorylated amino acids in MBP and H1 histone were analyzed. Lanes: 1, MBP phosphorylated by αPKC; 2, MBP phosphorylated by yPKC; 3, H1 histone phosphorylated by αPKC; 4, H1 histone phosphorylated by yPKC. Radioactive spots near the origin presumably represent phosphopeptides due to incomplete acid hydrolysis. P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

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