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Yeast Phenotype Classifies Mammalian Protein Kinase C cDNA Mutants

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The phorbol ester receptor protein kinase C (PKC) gene family encodes essential mediators of eukaryotic cellular signals. Molecular dissection of their mechanisms of action has been limited in part by the lack of random mutagenesis approaches and by the complexity of signaling pathways in mammalian cells which involve multiple PKC isoforms. Here we present a rapid screen which permits the quantification of mammalian PKC activity phenotypically in the yeast *Saccharomyces cerevisiae*. Bovine PKCα cDNA is functionally expressed in *S. cerevisiae*. This results in a phorbol ester response: a fourfold increase in the cell doubling time and a substantial decrease in yeast colony size on agar plates. We have expressed pools of bovine PKCα cDNAs mutagenized by *Bal* 31 deletion of internal, amino-terminal, or carboxyl-terminal sequences and have identified three classes of mutants on the basis of their distinct yeast phenotypes. Representatives of each class were analyzed. An internal deletion of amino acids (aa) 172 to 225 displayed ligand-dependent but reduced catalytic activity, an amino-terminal truncation of aa 1 to 153 displayed elevated and ligand-independent activity, and a carboxyl-terminal 26-aa truncation (aa 647 to 672) lacked activity under any conditions. Additional mutations confirmed the distinct functional characteristics of these classes. Our data show that deletion of the V1 and C1 regions results in elevated basal catalytic activity which is still Ca^{2+} responsive. Internal deletions in the V2 and C2 regions do not abolish phorbol ester or Ca^{2+} regulation of PKC activity, suggesting that most of the C2 domain is not essential for phorbol ester stimulation and most of the regulatory domain is dispensable for Ca^{2+} regulation of PKC activity. These distinct activities of the PKC mutants correlate with a specific and proportional yeast phenotype and are quantified on agar plates by yeast colony size. This provides a phenotypic screen which is suitable to identify rare, randomly altered but active mammalian PKC mutants. It quantifies their catalytic and biological activities in response to PKC activators or inhibitors for a systematic mapping of PKC structure and function or PKC-drug interaction.

The calcium- and phospholipid-dependent protein kinase C (PKC) family is ubiquitously found in eukaryotes from yeasts to humans and plays important roles in the regulation of cell surface receptors, ion channels, secretion, neuronal plasticity and toxicity, and gene expression (17, 20). cDNA sequence analysis of the gene family defines four highly conserved regions, C1 to C4, and five variable regions, V1 to V5, between different subtypes (8). While the C1 region is critical for the regulation of PKC function and the C3 and C4 regions are critical for enzymatic activity, the roles of the C2 region and the variable regions V1 to V5 have not been defined (5, 16, 32, 37, 39). Many isoforms are activated by diacylglycerol, phospholipids, and Ca^{2+} in response to extracellular stimuli (34). At the same time, PKC plays a role in calcium mobilization, including calcium uptake in various cell types (26, 44, 45, 48). In addition, PKC is a major cellular receptor for tumor-promoting phorbol esters, which are resistent to degradation and are expected to cause protracted PKC activation which may ultimately result in tumor promotion (42). Consequently, PKC plays a growth-stimulatory role in many mammalian cells, while it has antiproliferative properties in others (12, 42).

The functional analysis of PKC action in mammalian experimental systems is complicated by multiple isoforms, cellular complexity, genetic inaccessibility, and lack of random mutagenesis approaches to generate catalytically active PKC forms (34). Potential members of the PKC family have been identified in other eukaryotes, including yeasts. In *Saccharomyces cerevisiae*, a related diacylglycerol-stimulated but phorbol ester-unresponsive protein activity with distinct catalytic properties has been described (23, 35); putative mammalian-like PKC isoforms have been described in a separate study (43). Independently, a related, essential yeast gene, *PKC1*, has been identified as having a role in osmotic stability and perhaps in bud morphogenesis (30, 31, 38).

To exploit the yeast *S. cerevisiae* as a minimally complex lower eukaryotic experimental model with excellent accessibility via molecular genetics and biochemistry, we have expressed bovine PKCα from cDNA to investigate the functional roles of its various structural domains. Expression products are fully functional proteins which display specific calcium-, phospholipid-, and phorbol ester-dependent catalytic activity in vitro and result in physiological consequences such as stimulation of Ca^{2+} uptake and cellular Ca^{2+} dependence (41). We observed that phorbol ester activation of bovine PKCα resulted in a fourfold increase in the yeast doubling time, and we determined whether this increase was correlated to PKC catalytic activity and could be used as a phenotype to screen for PKC mutants with altered enzymatic activity. When we expressed pools of randomly mutagenized bovine PKCα cDNAs in yeast cells, we observed that the yeast colony size was inversely proportional to the mutant catalytic and physiological activities. This phenotype defines distinct classes of mutations based on PKC function and provides a rapid screen to isolate PKC

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mutants with altered enzymatic activities and ligand responses.

MATERIALS AND METHODS

Duplicate measurements were routinely made in our experiments. All tests were independently performed several times with comparable results within a 10% error margin; representative data are shown.

Yeast strains and culture conditions. PKC expression plasmids and YEp51 or YEp52 as a control were introduced into *S. cerevisiae* 334 (*MATα pep4-3 prb1-1122 ura3-52 leu2-3,112 reg1-501 gal1*) (18) by lithium acetate transformation (22). Cells were routinely grown at 30°C on a culture roller or shaker in liquid culture or on 1.5% agar plates. The synthetic medium contained 2% glucose and was leucine free to select for stable propagation of the expression plasmids. Expressing yeast colonies were identified phenotypically by replica plating on medium containing or lacking phorbol-12-myristate-13-acetate (PMA). Phenol extracts of crude cell lysates were prepared to retransform *Escherichia coli* DH5α (15). PKC mutants were characterized by DNA sequencing, and defined plasmids were reintroduced into *S. cerevisiae* 334. These strains were used in all experiments shown. Cultures of 50 ml were routinely inoculated from freshly saturated cultures and grown for 16 h to an optical density at 600 nm (OD_{600}) of 0.4. Subsequently, transcription of PKC cDNAs was routinely induced with 2% galactose for 3 h in most experiments except where indicated otherwise. This results in up to a 700-fold induction of transcription in strain 334 within 3 h (18). PKC activation was routinely measured in response to a single dose of 1 μM PMA or in controls in the presence of a 1 μM concentration of the inactive isomer 4α-PMA (both from LC Services, Woburn, Mass.); both were added at the start of each experiment.

PKC mutagenesis and cDNA construction. The complete protein-coding region of bovine PKCα (39) was joined at the *Nco*I site at the translation initiation codon with a synthetic A-rich *Hin*dIII-*Nco*I adapter (5′-AGCTTAAAAAA-3′ and 3′-ATTTTTTGTAC-5′) to optimize the sequence upstream of the ATG codon for improved translation efficiency (7). The cDNA was truncated at the 3′ end by exonuclease *Bal* 31 digestion and joined with a synthetic blunt-end *Xba*I adapter (5′-TAACTAACTAAT-3′ and 3′-ATTGATTGATT AGATC-5′) which provides translation stop codons in all three reading frames. A complete protein-coding cDNA including 10 bp of the 3′ untranslated sequence was used for full-length PKCα expression. To create the mutation CD26, a truncated cDNA lacking coding sequences for 26 carboxyl-terminal amino acids (amino acids [aa] 647 to 672) but containing a newly created asparagine codon was prepared. Both cDNAs were inserted into the *Hin*dIII and *Xba*I sites under control of galactose-inducible *GAL10* transcriptional elements of the high-copy-number yeast episomal expression plasmid YEp52 containing the *LEU2* gene for selection (4). The mutation D172-225 lacking aa 172 to 225 was created by cleavage of the PKCα plasmid at a unique *Bam*HI site, *Bal* 31 digestion, and religation. To create the mutation ND153 lacking amino-terminal aa 1 to 153, the PKCα cDNA was truncated from the 5′ end by *Bal* 31 digestion, and a synthetic *Sal*I blunt-end adapter (5′-TCGACAAAAAAAAA AATGGCT-3′ and 3′-GTTTTTTTTTTTACCGA-5′) was joined to restore the methionine initiation codon and A-rich 5′ untranslated sequences for improved translation efficiency. The cDNA was inserted into the *Sal*I and *Xba*I sites of YEp51, which differs from YEp52 only by various cloning sites (4). Using the same strategies, we created additional deletion mutants lacking 84 (ND84), 118 (ND118), 140 (ND140), or 158 (ND158) amino-terminal amino acids, lacking 96 (CD96), 110 (CD110), 139 (CD139), or 149 (CD149) carboxyl-terminal amino acids, or lacking internal amino acids as specified by their position numbers (D186-189, D156-234, and D149-240) (Table 1). Methionine initiation codons had been restored in ND140 and ND153, methionine and alanine codons had been restored in ND84, ND118, and ND158, and leucine, threonine, and asparagine codons had been restored at the carboxyl terminus of CD149. After ligation, all plasmids were amplified in *E. coli* DH5α and were identified and confirmed by restriction analysis and DNA sequencing.

Phorbol ester binding. About 6×10^8 expressing cells were washed twice in 10 mM potassium phosphate buffer (pH 7.0)–20 mM EDTA and once in 1 M sorbitol and were resuspended in 10 ml of 10 mM potassium phosphate buffer (pH 7.0)–1.1 M sorbitol–0.5 mM $CaCl_2$. Cells were incubated with 1 U of Glusulase (β-glucuronidase/arylsulfatase from *Helix pomatia*; Boehringer Mannheim) for about 1 h under shaking at 30°C to remove the cell wall until test cell suspensions cleared in H_2O as a result of cell lysis (1). Spheroplasts (1.5×10^8) were incubated in 15-ml tubes in 1 ml of 2% glucose–2% galactose–1.1 M sorbitol–1 mg of bovine serum albumin per ml–75 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4) at 30°C for 90 min with 14 nM [^{3}H]phorbol-12,13-dibutyrate ([^{3}H] PDBu) at 25 Ci/mmol (Amersham), and nonspecific binding was determined in the presence of 20 μM unlabeled PDBu (LC Services). Spheroplasts were washed three times in ice-cold phosphate-buffered saline (PBS)–1 M sorbitol–50 mM HEPES (pH 7.4), and the cell-associated radioactivity was determined by liquid scintillation spectroscopy. Highest PKC levels resulted in specific binding of 5% of the [^{3}H] PDBu in the experiment.

Cell lysis. About 6×10^8 expressing cells were washed in PBS and resuspended in 1 ml of lysis buffer containing 1% Triton X-100, 50 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, pepstatin (10 μg/ml), leupeptin (40 μg/ml), aprotinin (10 μg/ml), and phenylmethylsulfonyl fluoride (200 μg/ml). Cell suspensions mixed with the same volume of acid-washed glass beads (450- to 500-μm diameter) were lysed mechanically by six 30-s vortexing steps interrupted by cooling on ice for at least 30 s in 50-ml screw-cap polypropylene tubes. The lysate was cleared by $4{,}000 \times g$ centrifugation for 15 min at 4°C and stored at −70°C for up to several weeks.

PKC catalytic activity. PKC catalytic activity was measured as described previously (19) by phosphorylation of 3 μg of the specific substrate peptide RFARKGSLRQKNV (GIBCO BRL) in the presence of 5 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) in 125 μl of fivefold-diluted cell extract for 30 min at 25°C with combinations of 160 μg of phosphatidylserine (Avanti Polar Lipids, Inc.) per ml, 1 μM PMA, and 5 mM $CaCl_2$. Phosphatidylserine in chloroform, with or without PMA, was evaporated under N_2 to dryness, resuspended in 20 mM Tris (pH 7.5), mixed, and sonicated at 4°C for 45 s before use. To terminate the reaction, the sample was placed on phosphocellulose paper (Whatman P81), which was repeatedly washed in 75 mM phosphoric acid. Bound radioactivity was determined by liquid scintillation spectroscopy.

$^{45}Ca^{2+}$ uptake. Expressing yeast cells after 16 h in 2% galactose medium were washed with PBS when they reached an OD_{600} of 0.5. Then 10^7 cells were suspended in 100 μl of 50 mM morpholine ethanesulfonic acid (MES; pH 6.5)–5 mM

$MgSO_4$ and incubated with 1 μM PMA for 1 h at 30°C under shaking on 96-well plates. Subsequently, uptake of 2 μCi of $^{45}Ca^{2+}$ at 300 μM $CaCl_2$ was measured for 2 h. Cells were rapidly transferred to Multiscreen filtration microtiter plates (Millipore) and washed four times with 200 μl of 10 mM MES–10 mM Tris–35 mM $CaCl_2$ (pH 6.0). Filters were dried, and cell-associated radioactivity was determined by liquid scintillation spectroscopy.

RESULTS

PKC mutagenesis and expression. To create bovine PKCα mutations with random alterations in major protein-coding domains, we prepared cDNA deletions internally within the C1, V2, and C2 coding regions and from the amino-terminal and carboxyl-terminal coding regions by exonuclease *Bal* 31 digestion, as shown for three examples in Fig. 1A. cDNAs were introduced into the high-copy-number episomal yeast expression plasmids YEp51 and YEp52 under control of galactose-inducible transcriptional elements (4). Pools of mutated plasmids were amplified in *E. coli* and purified for yeast transformation. Yeast colonies were screened on agar plates in the presence of 2% galactose to induce maximal cDNA transcription (18) and of 1 μM PMA to stimulate PKC catalytic activity (6). Under these conditions, expression of normal bovine PKCα resulted in a fourfold increase in the cell doubling time (Fig. 1B) and substantially reduced yeast colony size in comparison with wild-type yeast cells, which did not respond to PMA (Fig. 1C). Most of the yeast cells transformed with random PKC cDNA deletions were expected to express inactive PKC fragments and consequently displayed a wild-type yeast phenotype. However, about 1% of colonies transformed with internal or amino-terminal PKC deletions were reduced in size to various degrees in response to PMA by comparison with replica-plated colonies on PMA-free control plates. These colonies were chosen as candidates for the expression of functional PKC forms with different enzymatic activities and ligand responses. In contrast, transformation with carboxyl-terminal PKC truncations normally resulted in the wild-type yeast phenotype.

Primary structure of PKC mutants. Representative yeast colonies of each of the three types of PKC deletions were isolated, grown to saturation in liquid culture, diluted, and spread on agar plates; one example of each type is shown under various conditions in Fig. 1C. To determine the exact primary structure of the deletion mutants, plasmids from crude extracts of expressing yeast cells were introduced into *E. coli* (15), and amplified plasmids were purified and sequenced. All experiments in this study were performed with *S. cerevisiae* 334 which had been retransformed with these defined plasmids. The deduced primary structures for the examples shown are compared in Fig. 1A. The internal deletion is lacking aa 172 to 225 comprising the amino-terminal half of the PKC C2 domain, which is not essential for phorbol ester stimulation of PKC activity. The amino-terminal truncation is lacking 153 aa including the V1 domain and most of the C1 domain, in particular all 12 cysteine residues involved in phorbol ester binding (5, 37) and the pseudosubstrate sequence which is expected to inhibit the catalytic activity in the absence of activator (16). This structure provides an explanation for the phorbol ester-independent, elevated basal activity of this mutant (24, 33). The carboxyl-terminal truncation is lacking 26 aa (647 to 672) including the V5 and the end of the catalytic C4 domain (39), which results in inactivation of the kinase and explains the lack of PKC activity and the resulting normal yeast phenotype.

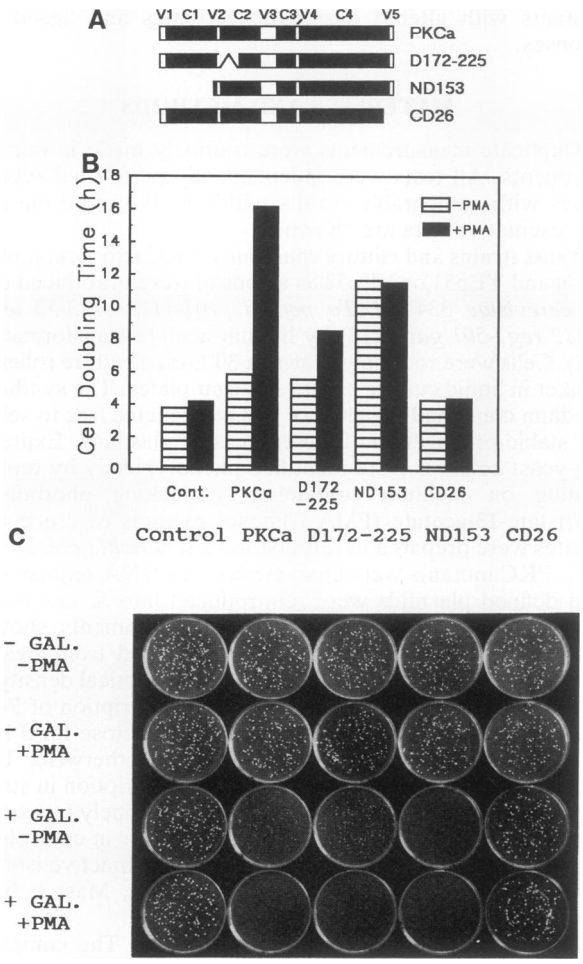

FIG. 1. (A) Primary structures of PKCα mutants. Deduced amino acid sequences of normal PKCα and deletion mutants D172-225, ND153, and CD26 are aligned for maximum homology. Regions of constant (C1 to C4) and variable (V1 to V5) amino acid sequences between PKC subtypes (7) are indicated by solid and open boxes, respectively. Deleted sequences have been omitted for emphasis or are indicated by the caret for D172-225. (B) Cell doubling times of yeast cultures expressing PKCα mutants. Freshly saturated cultures transformed with a control plasmid (Cont.) or expressing normal PKCα or deletion mutant D172-225, ND153, or CD26 were diluted to low cell densities. Cell growth was followed on a roller at 30°C to measurable cell densities of the logarithmic growth phase at an OD_{600} of 0.2 to 0.6. The medium contained 2% galactose to induce cDNA transcription and 1 μM PMA (+PMA) or the inactive isomer 4α-PMA (−PMA). Average doubling times were calculated. (C) Phenotypic screen of PKCα mutant activity by yeast colony size. Freshly saturated yeast cultures transformed with a control plasmid or expressing normal PKCα or deletion mutant D172-225, ND153, or CD26 were diluted, and comparable cell numbers were spread on agar plates. Colonies were grown at 30°C in the absence (−GAL.) or presence (+GAL.) of 2% galactose to induce cDNA transcription and in the presence of 1 μM PMA (+PMA) or the inactive isomer 4α-PMA (−PMA). Plates (9-cm diameter) were photographed after 3 days.

Yeast phenotype in response to PKC mutants. On galactose-free plates, as expected in the absence of cDNA transcription, all transformants displayed the wild-type pheno-

type, which was unaffected by PMA. Galactose stimulation of cDNA expression resulted for some PKC forms in specific, decreased yeast colony sizes which were either dependent on or independent of phorbol ester activation, as shown in Fig. 1C. Normal PKCα expression caused reduced colony sizes which were substantially further decreased to microscopically small sizes by phorbol ester activation. Internal PKC deletions such as D172-225 resulted in only slightly reduced colony sizes which were further decreased by phorbol ester stimulation to sizes slightly smaller than those of PKCα-expressing colonies in the absence of phorbol ester. Amino-terminal truncations such as ND153 led to microscopically small colonies independent of phorbol ester activation. Carboxyl-terminal truncations such as CD26 as well as control plasmids resulted in the wild-type phenotype under any condition (Fig. 1C). Microscopically small colonies expressing ND153 grew to visible sizes with rates independent of PMA. Colonies expressing PKCα grew even more slowly to visible sizes during continued incubation in the presence of PMA. Colony numbers were comparable in all experiments, indicating that yeast viability is not affected by any of the PKC forms (not shown).

PKC modulation of yeast doubling time. Cell doubling times were measured by OD_{600} reading of liquid cultures in the presence of 2% galactose (Fig. 1B) to test whether they correlate with the specific phenotypes (colony size) caused by normal and mutant PKCα. Control plasmids or carboxyl-terminal PKC truncations such as CD26 resulted in an average cell doubling time of 4 h in synthetic minimal medium, which is normal for *S. cerevisiae* 334 (18), and remained unaffected by phorbol ester. In the absence of phorbol ester, normal PKCα and the internal deletion D172-225 caused a 50% increase in the cell doubling time to 6 h, while amino-terminal truncations such as ND153 resulted in a phorbol ester-independent threefold increase to 12 h. Phorbol ester stimulation of the internal deletion mutant and normal PKCα resulted in two- and fourfold increases in the cell doubling time to 9 and 16 h, respectively. A normal 4-h doubling time was measured for all constructs in the absence of galactose (not shown). Our data indicate a clear correlation between the specific yeast colony sizes on agar plates and the cell doubling time, both of which are differentially modulated by normal PKCα and mutant PKC classes.

Protein gel analysis of PKC mutants. To test whether protein products of the predicted sizes are properly synthesized, detergent extracts of expressing cells were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels, and proteins were analyzed with PKCα-specific antibodies in immunoblots (46). PKCα was found to comigrate at 80 kDa with PKC isolated from rat brain (not shown), and the PKC deletions D172-225 and CD26 were detected as major protein bands of 70 and 75 kDa, respectively, consistent with their predicted sizes and with proper protein expression (Fig. 2A). ND153 resulted in two major bands of 60 and 65 kDa. The lower 60-kDa band was consistently more prominent and most closely corresponds to the predicted size of the protein based on the deletion of 153 aa. This heterogeneity, which has not been investigated, may be due to different levels of PKC phosphorylation as have been shown in mammalian cells (3). Since whole cell extracts had directly been loaded on SDS-gels without further purification, protein signals were low. Bands combined from two independent experiments have been photographed for optimal representation of relative migrations and should not be directly compared for quantification of protein products. Similar band intensities were reproducibly observed on nitrocellulose filters for

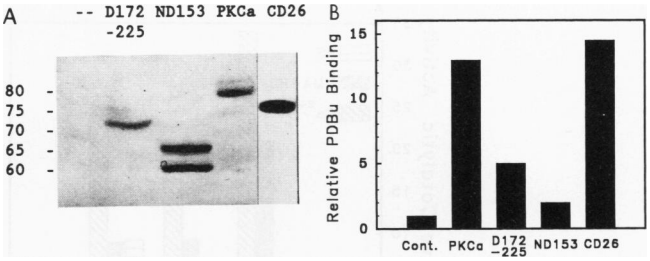

FIG. 2. (A) Immunoblot of PKCα mutants expressed in *S. cerevisiae*. Detergent extracts of yeast cells transformed with a control plasmid (−−) or expressing normal PKCα or deletion mutant D172-225, ND153, or CD26 were separated on SDS–8% polyacrylamide gels. Proteins were transferred to nitrocellulose and analyzed with specific antibodies to mammalian PKCα in immunoblots (27, 36). Size markers are indicated in kilodaltons. (B) Phorbol ester binding to cells expressing PKCα mutants. Levels of [^{3}H]PDBu binding to yeast spheroplasts transformed with a control plasmid (Cont.) or expressing normal PKCα or deletion mutant D172-225, ND153, or CD26 were compared.

normal PKCα and the 60-kDa (lower) band of ND153, which suggests comparable expression levels consistent with comparable PKC activities described below. Similar expression levels of normal PKCα and CD26 are also suggested by phorbol ester binding data in Fig. 2B, while band intensities of D172-225 were typically slightly reduced in immunoblots.

In vivo phorbol ester binding activity of PKC mutants. To directly test the function of the regulatory PKC domain, phorbol ester binding sites were determined after enzymatic removal of the yeast cell wall by exposure of yeast spheroplasts to [^{3}H]PDBu. Comparison of normal PKCα and PKC mutants (Fig. 2B) showed similar [^{3}H]PDBu binding levels for normal PKCα and CD26. Both were 13-fold elevated over background levels of control cells. Similar findings have been reported for PKC overexpression in transfected mammalian fibroblasts (25). Removal of the C-terminal 26 aa does not appear to affect PKC phorbol ester binding activity. In contrast, the amino-terminal 153-aa truncation resulted in background [^{3}H]PDBu binding levels consistent with the expected loss of the phorbol ester binding domain by this mutation (5, 24, 33, 37). The internal deletion of aa 172 to 225 led to phorbol ester binding levels reduced by 60% compared with normal PKCα, a potential consequence of the removal of parts of the C2 domain (5, 24).

In vitro catalytic activity of PKC mutants. To test whether the distinct phenotype of expressing yeast cells correlates with the catalytic activity of the PKC mutants, phosphorylation of the PKC-specific substrate [Ser-25]PKC_{19-31}, a derivative of the pseudosubstrate sequence (16), was measured in detergent cell extracts in response to combinations of phosphatidylserine, PMA, and calcium (13, 14). As shown in Fig. 3A, no activity was detected under any conditions for CD26, consistent with the inactivation of the kinase by the carboxyl-terminal 26-aa truncation. In the presence of phosphatidylserine alone, no catalytic activity was detected for normal PKCα, while D172-225 and ND153 displayed 4- and 10-fold-elevated levels, respectively. This finding suggests partial and significant elevation of their basal catalytic activities, respectively, as a consequence of the structural changes. Phorbol ester treatment of normal PKCα resulted in more than a 20-fold stimulation of catalytic activity, compared with a 2-fold stimulation of D172-225. This result indicates reduced responsiveness of D172-225 consistent

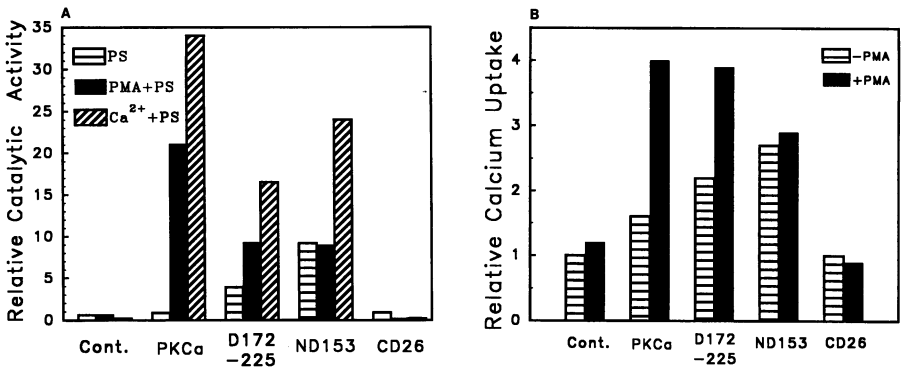

FIG. 3. (A) Comparison of PKC mutant catalytic activities in vitro. Phosphorylation of the pseudosubstrate derivative [Ser-25]PKC_{19-31} was measured in detergent extracts of cells transformed with a control plasmid (Cont.) or expressing normal PKCα or deletion mutant D172-225, ND153, or CD26. Catalytic activity was stimulated by combinations of phosphatidylserine (PS), the phorbol ester PMA, and calcium ions (Ca^{2+}). (B) Modulation of extracellular Ca^{2+} uptake by PKCα mutants. Yeast cells transformed with a control plasmid (Cont.) or expressing normal PKCα or deletion mutant D172-225, ND153, or CD26 were incubated with $^{45}CaCl_2$ to compare Ca^{2+} uptake in the presence of 1 μM PMA (+PMA) or the inactive isomer 4α-PMA (−PMA).

with its reduced phorbol ester binding (Fig. 2B). The catalytic activity of ND153 was PMA unresponsive, consistent with the loss of the phorbol ester binding domain in this mutant. Maximal catalytic activity of all three active PKC forms was stimulated by calcium, one of the basic characteristics of PKC (13, 14, 32, 41). Catalytic activities of the mutants (elevated basal activity of ND153; full and reduced phorbol ester-stimulated activities of normal PKCα and D172-225, respectively) and lack of activity for CD26 clearly correlate with the sizes of expressing yeast colonies.

Ca^{2+} uptake mediated by PKC mutants. Ca^{2+} influx has been reported in response to PKC activation in a variety of eukaryotic cells such as *Nitella syncarpa* plasmalemma (48), *Aplysia* bag cell neurons (45), rat ventricular myocytes (26), and rat pituitary cells (44). To test whether the measured catalytic activities and the yeast phenotype correlate with other biological responses mediated by PKC, $^{45}Ca^{2+}$ uptake into expressing cells was determined after PMA stimulation (2, 9). As shown in Fig. 3B, CD26 displayed the same basal response as did control cells, which was unaffected by phorbol ester and is consistent with the lack of PKC enzymatic activity caused by the carboxyl-terminal truncation. Normal PKCα displayed a 1.4-fold, D172-225 displayed a 2-fold, and ND153 displayed an almost 3-fold increase in Ca^{2+} uptake; overall, these values are comparable with basal catalytic and yeast phenotypic responses for these PKC forms. Stimulation of Ca^{2+} uptake by ND153 was unaffected by phorbol ester, consistent with its constitutive catalytic activity and the resulting yeast phenotype. After PMA stimulation of normal PKCα or D172-225, Ca^{2+} uptake increased to levels up to fourfold over the control cell background, suggesting almost comparable maximal responses for both PKC forms. These responses are consistent with the catalytic activities and phenotypic responses of the mutants: elevated basal activity of ND153, full and reduced phorbol ester-stimulated activities of normal PKCα and D172-225, respectively, and lack of activity for CD26.

DISCUSSION

The phenotypic screen for PKC activity. Our findings indicate that normal and mutant forms of bovine PKCα are functionally expressed in yeast cells and display distinct phorbol ester or constitutive catalytic and biological responses (or a lack thereof), all of which are specific for the different classes of mutations. These distinct levels of PKC activity cause a proportional increase in the cell doubling time which results in a proportional decrease in yeast colony size on agar plates. Even small differences in PKC activity are translated into visible differences in colony size (Fig. 1C), which permits the phenotypic classification of PKC mutants with altered basal or ligand-activated characteristics out of a large background of inactive PKC forms. Basal and phorbol ester-stimulated states of normal PKCα and mutant D172-225 define four levels of catalytic activity (Fig. 3) which result in proportional differences in yeast colony size (Fig. 1C) and demonstrate the resolution and sensitivity of the screen.

Correlation of PKC mutant activities. Overall, the distinct levels of PKC activity observed for the different mutants correlate well between catalytic and biological responses. Some existing variations are not unexpected when experiments performed in different time frames and under widely varying in vivo and in vitro conditions are compared. The high phorbol ester-stimulated Ca^{2+} uptake observed for the internal deletion D172-225 could be explained by different time dependencies of protein expression or altered downregulation compared with normal PKCα, which has not been addressed. The reduced phorbol ester binding of D172-225 compared with that of normal PKCα (Fig. 2B) may be a consequence of lowered phorbol ester affinity, which has been reported for one (5) but not for another (24) PKCα mutant, both of which carry deletions in the C2 domain. We have identified and classified additional mutations with the phenotypic screen which fall into the same three distinct functional classes (Table 1). Enzymatic activities were measured in vitro in response to PMA and Ca^{2+}, and modulation of the cell doubling time was compared in vivo in response to PMA. Catalytic and biological responses correlated well, as indicated by the symbols in Table 1.

PKC amino-terminal truncation mutants. Amino-terminal truncations of 84 to 158 aa are lacking the V1 domain and between the first half and most of the C1 region. All display a significantly elevated basal level of PKC activity. This is not unexpected after removal of the pseudosubstrate sequence (16), which has been studied by proteolysis (47) and cDNA mutagenesis (24, 33). All mutants are unresponsive to phorbol esters, consistent with the predicted role of the

TABLE 1. Effect of PMA and Ca^{2+} on mutant PKC activity[a]

PKC class	Activity		
	No activator	PMA	Ca^{2+}
PKCα	−	++	++
ND84	+	+	++
ND118	+	+	++
ND140	+	+	++
ND153	+	+	++
ND158	+	+	++
D186-189	(−)	+	+
D172-225	(−)	+	+
D156-234	(−)	+	+
D149-240	(−)	+	+
CD26	−	−	−
CD96	−	−	−
CD110	−	−	−
CD139	−	−	−
CD149	−	−	−

[a] PKC deletion mutants representing three distinct functional classes were tested for PMA and Ca^{2+} stimulation of in vitro catalytic activity and PMA-mediated increase of the cell doubling time as shown in detail in Fig. 3 and 1B, respectively, for the underlined mutants. Both in vitro and in vivo responses to PMA correlated well, and all activities measured are represented (in increasing levels) as −, (−), +, and ++.

cysteine repeats in the C1 region in phorbol ester binding (5, 37). The enzymatic activities of these mutants are still responsive to Ca^{2+}, which suggests that sequences in the C2 region (32) or in the catalytic domain are responsible for this function, which has not been accurately mapped (39).

PKC internal deletion mutants. Internal PKC deletions lacking between 4 and 92 aa including the V2 domain, 60% of the C2 region, and little of the C1 region (D186-189 to D149-240) have been identified (Table 1). All forms are still regulated by phorbol ester and Ca^{2+}, while their maximum enzymatic activities are reduced compared with that of normal PKCα. Our findings suggest that deletion of more than 90 aa from the PKC regulatory domain is compatible with the basic functional characteristics of PKCα. The somewhat elevated basal level may suggest a potential role of amino-terminal sequences in the C2 domain in the regulation of the kinase, a question which has not been addressed in reports of other PKC C2 domain deletions (5, 24). These sequences appear not to be essential for PKC activation and in particular not for Ca^{2+} binding (32, 39).

PKC carboxyl-terminal truncation mutants. All carboxyl-terminal truncations identified, ranging from CD26 to CD149, resulted in inactive proteins (Table 1) (41). These data indicate that PKCα enzymatic activity is lost after minimal truncation of the C4 region. Catalytic activity is not restored by increasing deletions, suggesting that essential catalytic sequences may have already been affected by a small truncation. In CD26, catalytic activity is already lost, although none of the putative autophosphorylation sites has been removed, as judged from autophosphorylation studies of PKCβII (11).

Homologous PKC action in yeast and mammalian cells. Mammalian PKC plays a growth stimulatory role in many mammalian cells (42) but has antiproliferative properties in other cell types (12), including yeast cells (this study). While putative mammalian PKC-like protein activity has been reported in yeast cells (43), we have not found any evidence for PMA-stimulated yeast PKC activity in any of our assays. This is consistent with the characteristics of the originally reported yeast PKC protein activity, which does not significantly respond to phorbol esters and displays altered substrate specificity compared with mammalian PKC (23, 35). The clear correlation between phorbol ester activation of bovine PKCα and the specific responses measured in various assays in this study compared with those of control cells indicate that the phenotypic screen quantifies mammalian PKC activity in the presently undefined yeast cellular background. Our results show that bovine PKCα expression causes a dramatic phorbol ester response in yeast cells, while only subtle physiological consequences have been reported in wild-type yeast cells (40). Consequently, phorbol esters are able reach and activate their target receptors in yeast cells. A gene, *PKC1*, with a role in osmotic stability has been identified in yeast cells; *PKC1* shares 53 and 51% amino acid identity with rat PKCβ1 and PKCγ, respectively (30, 31). *PKC1* and extragenic suppressors of the *BCK1* or *SKC*[d] locus may participate in the same pathway with a putative function in the cell cycle, perhaps in bud morphogenesis (10, 29, 38). *PKC1* may play a role in the regulation of a protein kinase cascade which includes the *BCK1* gene product. *BCK1* activates a pair of protein kinases encoded by the *MKK1* and *MKK2* genes, which are homologs of mammalian mitogen-activated protein kinase-kinase (21). This is believed to result in the activation of a yeast homolog of mammalian mitogen-activated protein kinase, which is encoded by the *MPK1* gene (28).

A rapid approach for PKC structure-function analysis and drug interaction. Our approach allows the evaluation of hundreds of individual colonies on a single culture plate, which permits the routine screening of thousands of individual mutations in one experiment. The experimental background created by normal variation in yeast colony size can be reduced by replica plating and successive screening steps, including liquid culture of promising mutant candidates, which allows the most sensitive quantification of PKC activity. The screen is particularly suitable for random mutagenesis strategies which introduce major structural changes and result in large numbers of inactive PKC forms. Rare mutations with various levels of PKC activity can be identified and quantified in this way. This strategy provides significant advantages over site-directed mutagenesis approaches, since large numbers of structural changes can be rapidly tested and changes are not limited to the structural predictions of the investigator. We expect that this screen will significantly facilitate and accelerate the dissection of the structural domains and their role in PKC function. It should also provide a rapid assay for large-scale screening of drugs such as tumor promoter agonists and antagonists on the basis of their interactions with PKC.

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