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Phorbol Ester Activation of Functional Rat Protein Kinase C β-1 Causes Phenotype in Yeast

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Abstract The phorbol ester receptor protein kinase C (PKC) gene family encodes essential mediators of various eukaryotic cellular signals. The molecular dissection of its mechanisms of action has been limited in part by the genetic inaccessibility and complexity of signaling in mammalian cells. Here we present a novel approach to study rat PKC β-1 action in yeast, a simple lower eukaryotic genetic model. Expression of its cDNA in *Saccharomyces cerevisiae* introduces novel phorbol ester binding sites which stimulate a specific calcium- and phospholipid-dependent catalytic activity in vitro consistent with a fully functional protein which phosphorylates cellular yeast proteins in vivo. Phorbol ester activation of PKC β-1 in vivo results in biological responses which include stimulation of extracellular calcium uptake, changes in cell morphology, and an increase in the cell doubling time. These PKC functions are not affected by truncation of 12 amino terminal amino acids; however, they are completely abolished by truncation of 15 or more carboxyl terminal amino acids which likely result in inactivation of the kinase. The increase in the yeast doubling time caused by PKC β-1 activation provides a phenotype which can be exploited as a screen for the activity of random PKC cDNA mutations. Our findings indicate that rat PKC β-1 is functional in yeast and leads to biological responses which suggest compatible aspects of higher and lower eukaryotic signaling pathways and the feasibility of dissecting parts of the action of common signaling mediators in a simple genetic model.

Key words: Bal31 deletion, calcium uptake, cDNA expression, signal transduction, tumor promoter

The calcium- and phospholipid-dependent protein kinase C (PKC) family is ubiquitously found in eukaryotes from yeast to man and plays important roles in the regulation of cell surface receptors, ion channels, secretion, neuronal plasticity and toxicity, and gene expression [Huang, 1990; Houslay, 1991]. cDNA sequence analysis of the gene family defines four highly conserved regions C1–C4 and five variable regions V1–V5 between different subtypes [Coussens et al., 1986]. While the C1 and C3–C4 regions are critical for the regulation of PKC function and for enzymatic activity, respectively, the roles of the C2 and variable regions V1–V5 have not been defined [Parker et al., 1986; House and Kemp, 1987; Ono et al., 1989; Burns and Bell, 1991]. Many isoforms are activated by diacylglycerol, phospholipids, and Ca^{2+} in response to extracellular stimuli [Nishizuka, 1988]. At the same time PKC plays a role in calcium mobilization including calcium uptake in various cell types [Strong et al., 1987; Lacerda et al., 1988; Stojilkovic et al., 1988; Zherelova, 1989]. 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 [Rotenberg and Weinstein, 1991]. Consequently PKC plays a growth stimulatory role in many mammalian cells [Persons et al., 1988; Housey et al., 1988], while it has antiproliferative properties in others [Gescher, 1985]. The functional analysis of PKC action in mammalian experimental systems is complicated by multiple isoforms, cellular complexity, and genetic inaccessibility [Nishizuka, 1988; Parker et al., 1989].

Potential members of the PKC family have been identified in other eukaryotes including yeast. In *Saccharomyces cerevisiae* a related diacylglycerol-stimulated but phorbol ester-unresponsive protein activity with distinct catalytic

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properties has been described [Ogita et al., 1990; Iwai et al., 1992] as well as putative mammalian-like PKC isoforms in a separate study [Simon et al., 1991, 1992]. Independently, a related, essential gene PKC1 has been identified in yeast with a cell cycle-specific role in osmotic stability and perhaps in bud morphogenesis which is however not complemented by mammalian PKC isoforms when disrupted [Levin et al., 1990; Fields and Thorner, 1991; Levin and Bartlett-Heubusch, 1992]. Consequently this gene does not encode a functional homolog of mammalian PKC isoforms.

Since functional homologs of mammalian PKC isoforms have not been established at the gene level in yeast to this date while a variety of mammalian PKC cDNAs are available, we tested whether rat PKC β-1 could be functionally expressed in yeast and whether this would result in physiological consequences. We found that expression of its cDNA in *S. cerevisiae* introduces novel phorbol ester binding sites which stimulate a specific calcium- and phospholipid-dependent catalytic activity in vitro consistent with a fully functional protein which also phosphorylates cellular yeast proteins in vivo. Phorbol ester activation of PKC β-1 in vivo results in biological responses which include stimulation of extracellular Ca^{2+} uptake, changes in cell morphology, and an increase in the cell doubling time. These PKC functions are not affected by truncation of 12 amino terminal amino acids; however, they are completely abolished by truncation of 15 or more carboxyl terminal amino acids which likely results in inactivation of the kinase.

METHODS

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

PKC Mutagenesis and cDNA Construction

The complete protein-coding region of rat protein kinase C β-1 [Housey et al., 1987] was isolated as a 2441 base pair *Bss*HII (endfilled)-*Hind*III fragment and was inserted into the *Sal*I (endfilled) and *Hind*III sites under control of galactose-inducible *GAL10* transcriptional elements of the high copy number yeast episomal expression plasmid YEp51 containing the *LEU2* gene for selection [Broach et al., 1983]. The truncation mutation CD15 lacking coding sequences for 15 carboxyl terminal aa was created by exonuclease *Bal*31 digestion from the 3′-end. Sequences were joined with the synthetic blunt end—*Xba*I adaptor 5′-TAACTAACTAAT-3′ and 3′-ATTGATTGATTAGATC-5′ which created new codons for Leu, Thr, and Asn followed by a translation stop codon. The truncated cDNA was re-inserted into plasmid YEp51 at the *Xba*I site [Broach et al., 1983]. The truncation mutation ND12 lacking coding sequences for 12 amino terminal aa was created by exonuclease *Bal*31 digestion from the 5′-end. Poly A-rich 5′-untranslated sequences, the translation initiation codon for Met, and a codon for the second aa Ala were reintroduced with the synthetic *Sal*I—blunt end adaptor 5′-TCGACAAAAAAAAAAATGGC-TCAAAAAAAAAAATGGCT-3′ and 3′-GTTTTT-TTTTTTACCGAGTTTTTTTTTTTACCGA-5′. The truncated cDNA was reinserted into plasmid YEp51 at the *Sal*I site [Broach et al., 1983]. After transformation plasmids were amplified in *Escherichia coli* DH5 alpha and were identified and confirmed by restriction analysis and DNA sequencing.

Yeast Strains and Culture Conditions

PKC expression plasmids and YEp51 as a control were introduced into *S. cerevisiae* strain 334 [*MAT alpha; pep4–3; prb1–1122; ura3–52; leu2–3,112; reg1–501; gal1*] [Hovland et al., 1989] by lithium acetate transformation [Ito et al., 1983]. Cells were routinely grown at 30°C on a culture roller or shaker in leucine-free synthetic medium containing 2% glucose in liquid culture or on 1.5% agar plates to select for stable propagation of the expression plasmids. For many experiments 50 ml cultures were inoculated from freshly saturated cultures and grown for 18 h to an optical density of 0.5 $O.D._{600}$. Transcription of PKC cDNAs was routinely induced in the presence of 2% glucose with 2% galactose during this time which results in up to 1,500-fold induction of transcription in strain 334 since it carries mutations that prevent glucose repression [Hovland et al., 1989]. PKC activation was routinely measured in response to a single dose of 1 μM PMA (phorbol-12-myristate-13-acetate) or in controls (−PMA) in response to 1 μM of the inactive isomer 4 alpha-PMA (both LC Services, Woburn, MA) which were added at the start of each experiment.

Phorbol Ester Binding

About 6×10^8 expressing cells were washed once in H_2O and subsequently in 10 mM K-phosphate buffer, pH 7.0, 20 mM EDTA, 10 mM DTT, and finally in 1 M sorbitol. Cells were resuspended in 10 ml of 10 mM K-phosphate buffer, pH 7.0, 1.1 M sorbitol, and 0.5 mM $CaCl_2$, and were incubated with 1 U glusulase (β-glucuronidase/arylsulfatase from *Helix pomatia,* Boehringer Mannheim, Indianapolis, IN) for about 1 h under shaking at 30°C to remove the cell wall until test cell suspensions cleared in H_2O due to cell lysis [Beggs, 1978]. This preparation provided best results when compared to other enzymes such as zymolyase from *Arthrobacter luteus.* 1.5×10^8 spheroplasts were incubated in 15 ml tubes in 1 ml 2% glucose, 2% galactose, 1.1 M sorbitol, 1 mg/ml BSA, and 75 mM Hepes, pH 7.4 at 30°C for 90 min with 13 nM [^{3}H]PDBu at 20 Ci/mmol (Amersham, Arlington Heights, IL). Nonspecific binding was determined in the presence of 20 μM of unlabeled PDBu (LC Services) and was subtracted for each sample. For Scatchard analysis [Scatchard, 1949] unlabeled PDBu was added varying from 5 nM to 1 μM. Spheroplasts were washed three times in ice-cold PBS, 1 M sorbitol, and 50 mM Hepes, pH 7.4, and 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 were resuspended in 0.5 ml lysis buffer containing 1% Triton X-100, 50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 10 μg/ml pepstatin, 40 μg/ml leupeptin, 10 μg/ml aprotinin, and 200 μg/ml PMSF. Cell suspensions mixed with the same volume of glass beads (450–500 μm) 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*g* centrifugation for 15 min at 4°C and stored at −70°C for up to several weeks.

In Vitro Catalytic Activity

PKC catalytic activity was measured as described by Hu et al [1990] by phosphorylation of 3.8 μg of the specific substrate peptide RFARKGSLRQKNV (Gibco BRL) in the presence of 1.25 μCi [γ-^{32}P]ATP (3,000 Ci/mmol) in 63 μl of 5-fold diluted cell extract for 30 min at 25°C with combinations of 160 μg/ml phosphatidylserine (Avanti Polar Lipids, Inc.), 1 μM PMA, and 5 mM $CaCl_2$. In the experiment shown in Figure 3b no calcium had been added, PMA was 1 μM, and the phosphatidylserine concentration varied up to a maximum level of 960 μg/ml. Phosphatidylserine in chloroform, with or without added 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) and was repeatedly washed in 75 mM phosphoric acid. Bound radioactivity was determined by liquid scintillation spectroscopy.

In Vivo Protein Phosphorylation

Two hundred fifty milliliter cultures of expressing cells at 0.15 $O.D._{600}$ were incubated in synthetic low (1 mM) phosphate medium [Sherman, 1991] for 14 h and subsequently with 2% galactose and 1 μM PMA for 3 h. Cells were washed and resuspended in 1 ml phosphate-free medium at high cell density of 100 $O.D._{600}$ with 90 μCi $^{32}PO_4$ (Amersham), 2% galactose, and 1 μM PMA for 1 h before they were collected by centrifugation [Warner, 1991]. A nuclei-enriched cell fraction was prepared as described by Aris and Blobel [1991]; however, only one Ficoll step gradient purification step (at the reported volume) was performed. The other volumes had been reduced to 50% of the reported levels; 0.5 g of wet cells were resuspended, the cell wall was removed by zymolyase treatment and cells were lysed with a homogenizer in 20% Ficoll 400, 1 mM $MgCl_2$, and 20 mM K-phosphate, pH 6.5. Cell nuclei were enriched on a 30/40/50% step gradient (1.5/1.5/1.5 ml) by centrifugation for 1 h at 10^5g, collected from the 40% layer, washed, and stored at −70°C for several days. Phosphoproteins were separated on SDS 15% polyacrylamide gels, electrophoretically transferred to nitrocellulose, and detected by autoradiography.

$^{45}Ca^{2+}$ Uptake

10^7 expressing cells were washed with PBS and were suspended in 100 μl of 50 mM MES, pH 6.5, and 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 $^{45}Ca^{2+}$ in the presence of 300 μM $CaCl_2$ was measured for 1.5 h. Cells were rapidly trans-

ferred to Multiscreen filtration microtiter plates (Millipore), washed four times with 200 μl of 10 mM MES, 10 mM Tris, 100 mM NaCl, 25 mM KCl, and 35 mM $CaCl_2$, pH 6.0 and filters were dried and cell-associated radioactivity was determined by liquid scintillation spectroscopy.

RESULTS

To create yeast expression constructs we inserted the complete protein coding cDNA of rat PKC β-1 [Housey et al., 1987] under the control of galactose-inducible *GAL10* transcriptional promoter elements into the high copy number episomal plasmid YEp51 [Broach et al., 1983]. In addition two controls, truncation mutants lacking 12 amino terminal or 15 carboxyl terminal amino acids (aa) were prepared by *Bal*31 exonuclease treatment and were named ND12 or CD15, respectively. Plasmids were stably propagated in transformed *S. cerevisiae* strain 334 [Hovland et al., 1989] by selection in leucine-free 2% glucose medium and transcription was routinely induced with 2% galactose for 18 h in most experiments. Control YEp51-transformed cells were used to determine the experimental background.

To test whether normal and truncated PKC protein products of the predicted primary structures are properly expressed, detergent extracts of transformed cells were separated on SDS polyacrylamide gels and proteins were analyzed in immuno blots with PKC-specific antibodies [Huang and Huang, 1986] as shown in Figure 1. Expression of normal PKC β-1 resulted in a single antigenic protein band of 80k MW which comigrated with PKC isolated from rat brain (not shown). Expression of PKC truncation mutants CD15 and ND12 resulted in a single protein band of 78k or 75k MW, respectively. As expected substantial changes in migration are not observed at this level of resolution due to the small truncations by 15 and 12 aa, respectively. However the ND12 protein band consistently migrated faster than the CD15 protein, suggesting that truncation of the PKC amino terminus and carboxyl terminus result in distinct changes in protein migration on SDS polyacrylamide gels which do not strictly correlate with the extent of the truncation in a linear fashion. The predicted primary structures of both truncation mutants had been confirmed by cDNA sequencing. Expression levels and/or stabilities of normal and truncated PKC forms appear to be overall comparable based on band intensities observed on

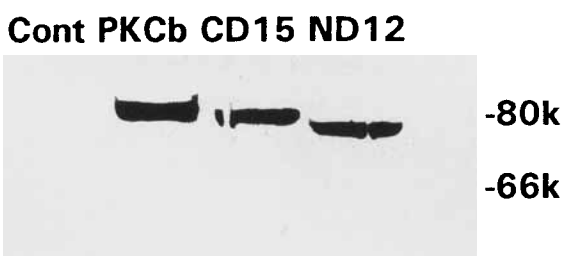


Fig. 1. Immunoblot of normal and truncated rat PKC β-1 expressed in yeast. Detergent extracts of cells transformed with control plasmids (Cont.) or of cells expressing normal PKC β-1 (PKCb) or truncation mutants ND12 lacking 12 amino terminal aa or CD15 lacking 15 carboxyl terminal aa were separated on SDS 8% polyacrylamide gels. Proteins were transferred to nitrocellulose and analyzed in immunoblots [Towbin et al., 1979] by alkaline phosphatase staining with specific antibodies to mammalian PKC [Huang and Huang, 1986]. Size markers are indicated in daltons.

the original immunoblot of cell extracts in Figure 1 and based on similar levels of phorbol ester binding to expressing yeast cells in vivo (Fig. 2a).

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]phorbol-12,13-dibutyrate ([^{3}H]PDBu). As shown in Figure 2a expression of normal PKC β-1 resulted in [^{3}H]PDBu binding levels which were more than 10-fold elevated over background levels of control transformed yeast cells as observed upon PKC expression in transfected mammalian fibroblasts [Knopf et al., 1986]. Truncation of the amino terminus by 12 aa or of the carboxyl terminus by 15 aa as represented by the mutants ND12 and CD15, respectively, resulted in similar PDBu binding activity as shown in Figure 2a. These data suggest that the truncations do not interfere with the function of the PKC regulatory domain and that expression levels are comparable between the different PKC forms. Scatchard analysis in Figure 2b suggests $.5 \times 10^4$ PDBu-binding sites per yeast cell (10 μm diameter) with a low affinity of 80 nM K_d for normal PKC β-1. Affinity constants have been reported to vary in mammalian cell types depending on the experimental conditions from subnanomolar K_ds to low affinities of 60 nM K_d in PB-3c murine mast cells [Mazurek et al., 1987]. PKC expression levels in yeast appear to be somewhat lower than in the richest mammalian PKC sources (such as rat brain, not shown) but overall in the same order of magnitude as in many mammalian cells.

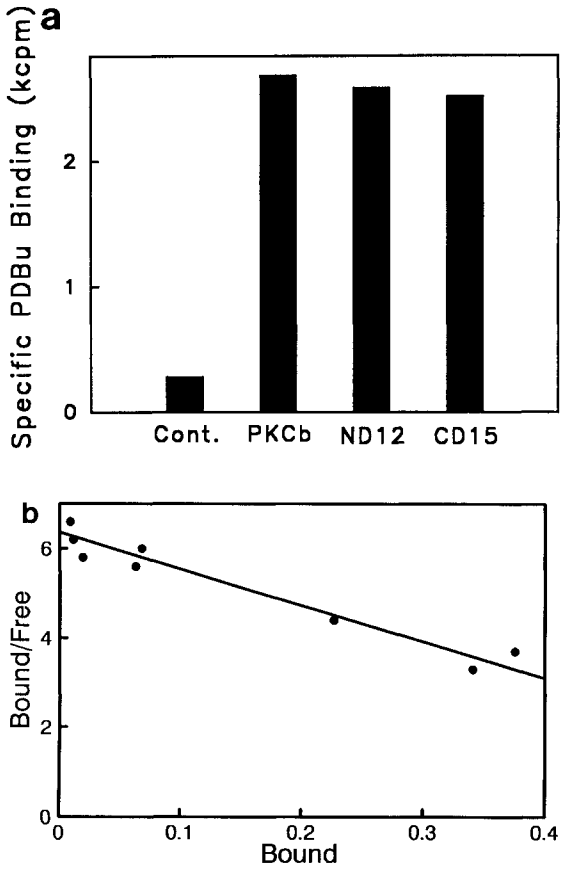


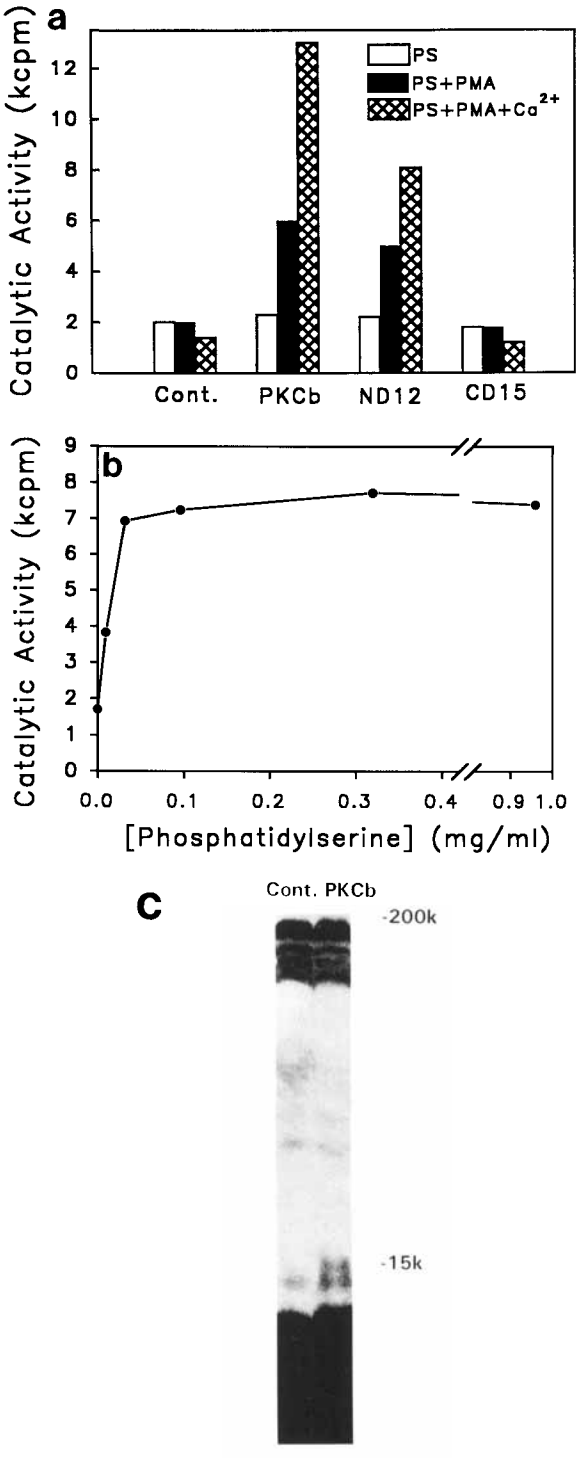
Fig. 2. **a:** [^{3}H]PDBu binding to yeast spheroplasts expressing normal and truncated PKC β-1. [^{3}H]PDBu binding to yeast spheroplasts transformed with control plasmids (Cont.) or expressing normal PKC β-1 (PKCb) or truncation mutants ND12 or CD15 was compared. Background binding determined in the presence of a 2,000-fold excess of unlabeled PDBu has been subtracted. **b:** Scatchard analysis of [^{3}H]PDBu binding to yeast spheroplasts expressing PKC β-1. Bound: PDBu (pmol); Free: [PDBu] (μM).

To compare the enzymatic activity of normal and truncated PKC forms, phosphorylation of the PKC-specific substrate [ser^{25}]PKC$_{19-31}$, a derivative of the pseudosubstrate sequence [House and Kemp, 1987], was measured in detergent cell extracts in response to combinations of phosphatidylserine, the phorbol ester PMA (phorbol-12-myristate-13-acetate), and Ca^{2+} [Hannun et al., 1985; Hannun and Bell, 1990]. As shown in Figure 3a in the presence of phosphatidylserine alone only a background level of phosphorylation was detected in all samples including control cells which likely represents the activity of other endogenous yeast protein kinases. A 7-fold or 4-fold stimulation of catalytic activity was measured after the addition of calcium and a 3-fold or 2.5-fold increase was observed after PMA stimulation for normal PKC β-1 and ND12, respectively, while no changes were observed for CD15 or in control cells under any conditions. PKC β-1 catalytic activity was phospholipid-dependent as shown in Figure 3b and was stimulated by diacylglycerol (not shown) similar to PMA. Our findings with ND12 indicate that while maximum catalytic activity is somewhat reduced by truncation of 12 amino terminal aa, this mutation is still compatible with a calcium- and phospholipid-dependent and phorbol ester-responsive enzymatic activity and shares the basic catalytic characteristics of PKC β-1 [Hannun et al., 1985; Hannun and Bell, 1990]. Truncation of 15 carboxyl terminal aa in CD15 however results in undetectable levels of enzymatic activity suggesting that sequences essential for PKC catalytic activity have been affected.

To measure PKC β-1 catalytic activity in vivo, expressing cells were metabolically labeled with ^{32}P and nuclei-enriched cell fractions from Ficoll step gradients [Aris and Blobel, 1991] were analyzed on SDS polyacrylamide gels as shown in Figure 3c. While the pattern of most yeast phosphoproteins did not change, a protein band of 15k MW was detected after PMA stimulation of PKC β-1 which was not visible in control cells. A comparable phosphoprotein was detected albeit weakly in whole cell extracts (not shown) suggesting that the phosphorylated 15k protein may be a yeast nuclear protein. While this experiment does not demonstrate PKC β action in the yeast nucleus a role of PKC β has been implicated in mammalian cell nuclei [Rogue et al., 1990]. Our data indicate PKC β-1 catalytic activity in vivo and the presence of yeast protein substrates which may be responsible for the observed biological responses.

One biological response, stimulation of Ca^{2+} uptake has been reported upon PKC activation in a variety of eukaryotic cells such as *Nitella syncarpa* plasmalemma [Zherelova, 1989], *Aplysia* bag cell neurons [Strong et al., 1987], rat ventricular myocytes [Lacerda et al., 1988], and rat pituitary cells [Stojilkovic et al., 1988]. To test whether the enzymatic activities observed for normal and truncated PKC β-1 forms correlate with biological responses mediated by PKC, $^{45}Ca^{2+}$ uptake into expressing cells was determined after PMA stimulation [Borbolla and Pena, 1980; Eilam et al., 1990]. As shown in Figure 4a, CD15 displayed the same basal response as 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 PKC β-1 and ND12 both displayed an almost two-fold increased level of Ca^{2+} uptake shown in Figure 4a which corresponds to the in vitro enzymatic properties of these PKC forms and suggests an overall correlation between PKC catalytic and biological activities.



PKC plays a growth stimulatory role in many mammalian cells [Persons et al., 1988; Housey et al., 1988] but has antiproliferative properties in other cell types [Gescher, 1985]. When we investigated the effect of PKC β-1 on the doubling time of expressing yeast cells we observed an almost three-fold increase upon phorbol ester activation as shown in Figure 4b which was reversible and did not affect cell viability (not shown). A similar response was measured for ND12 while CD15 resulted in cell doubling times indistinguishable from control transformed cells. Our data show a correlation between two biological responses, $^{45}Ca^{2+}$ uptake and modulation of the yeast cell doubling time for all PKC forms and suggest that while deletion of 12 amino terminal aa still allows virtually normal biological PKC β-1 function removal of 15 carboxyl terminal aa results in complete loss of enzymatic and biological activity.

To explore potential changes in yeast cell morphology in response to PKC activation individual expressing yeast cells were investigated by phase-contrast microscopy. In the absence of galactose (not shown) or PMA all PKC forms resulted in a wild-type phenotype as shown in Figure 4c. Phorbol ester activation of normal PKC β-1 and ND12 caused a phenotype represented by string-like cellular structures. This phenotype has not been well established in yeast and does not offer an obvious explanation for the underlying molecular mechanism which remains to be elucidated by future studies. However this response suggests phorbol ester-stimulated activity of normal PKC β-1 and ND12 and

Fig. 3. **a:** Catalytic activity of normal and truncated PKC β-1 in vitro. Phosphorylation of the pseudosubstrate derivative $[ser^{25}]PKC_{19-31}$ was measured in detergent extracts of yeast cells transformed with control plasmids (Cont.) or expressing normal PKC β-1 (PKCb) or truncation mutants ND12 or CD15. Enzymatic activity is shown for combinations of phosphatidylserine (PS) alone or combinations of PS and PMA or PS, PMA, and calcium ions (Ca^{2+}). **b:** Phosphatidylserine dependence of PKC β-1 enzymatic activity in vitro. Phosphorylation of the pseudosubstrate derivative $[ser^{25}]PKC_{19-31}$ was measured in detergent cell extracts with varying phosphatidylserine concentrations up to 960 μg/ml. **c:** Phosphorylation of yeast proteins in vivo by PKC β-1. After metabolic labeling with ^{32}P nuclei-enriched fractions of PMA-stimulated control (Cont.) and PKC β-1 expressing (PKCb) yeast cells were separated on SDS 15% polyacrylamide gels and phosphoproteins were analyzed by autoradiography. Size markers are indicated in daltons.

loss of function for CD15 consistent with the other enzymatic and biological functional assays described above.

DISCUSSION

Our findings indicate that normal and amino or carboxyl terminal truncation mutants of rat PKC β-1 are faithfully synthesized in yeast and result in cellular phorbol ester binding sites. Expression products display substrate-specific calcium-, phospholipid-, and phorbol ester-responsive enzymatic activity in cell extracts, phosphorylate yeast protein in vivo, and result in proportional biological consequences. Three responses which were measured in vivo: $^{45}Ca^{2+}$ uptake, the modulation of the cell doubling time, and the altered cell morphology all correlate well with the enzymatic activities measured in vitro. Compared to normal PKC β-1, truncation of 12 amino terminal aa (ND12) resulted in somewhat reduced enzymatic but normal biological activity. Enzymatic properties were calcium-, phospholipid-, and phorbol ester-responsive and display the basic characteristics of PKC β-1 while truncation of 15 carboxyl terminal aa (CD15) results in loss of enzymatic and biological activity. Further carboxyl terminal truncation consistently results in inactive proteins and does not restore PKC enzymatic activity which has been tested for PKC β-1 mutants lacking 18, 32, 42, 56, and 67 aa (not shown).

Our findings indicate that the amino terminal 12 aa of PKC β-1 are not essential for PKC function while removal of 15 carboxyl terminal aa results in loss of PKC enzymatic activity. This defines the boundary of the region essential for PKC β-1 enzymatic activity within 15 aa from the carboxyl terminus based on functional criteria. This is not necessarily obvious from aa sequence comparisons of the known PKC isoforms and consequently different interpretations have led to different predictions for the end of the PKC catalytic C4 region. Coussens et al. [1986] proposed a boundary only 2 aa up-

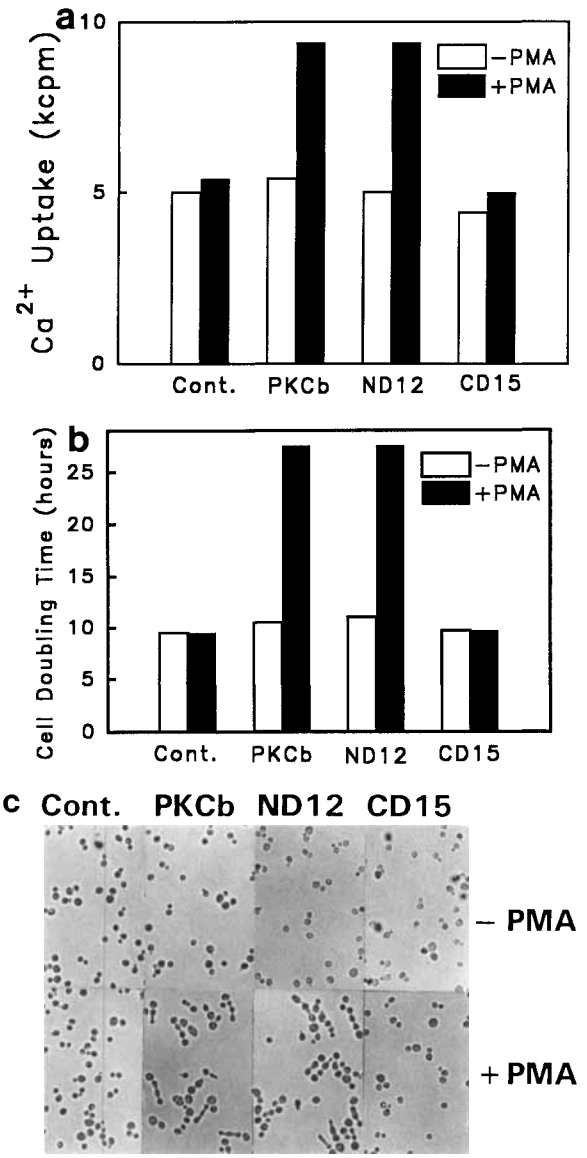


Fig. 4. **a:** Stimulation of extracellular Ca^{2+} uptake by normal and truncated PKC β-1 in vivo Yeast cells transformed with control plasmids (Cont) or expressing normal PKC β-1 (PKCb) or truncation mutants ND12 or CD15 were incubated for 1 5 h with $^{45}CaCl_2$ to compare Ca^{2+} uptake in the presence of PMA (+PMA) or the inactive isomer 4α-PMA (−PMA) **b:** Modulation of cell doubling time by normal and truncated PKC β-1 in vivo Yeast cells transformed with control plasmids (Cont) or expressing normal PKC β-1 (PKCb) or truncation mutants ND12 or CD15 were cultured to saturation in 2% glucose and subsequently diluted to low cell densities Cell growth was monitored on a culture roller at 30°C to O D $_{600}$ measurable cell densities (0 2–0 6) of the logarithmic growth phase The medium was glucose-free and contained 2% galactose to induce cDNA transcription as well as 3% glycerol which results in a 10 h doubling time for strain 334 and allows maximum modulation by PKC The result of PKC activation was studied in the presence of a single dose of 1 μM PMA (+PMA) compared to the inactive isomer 4α-PMA (−PMA) Average cell doubling times were calculated **c:** Induction of yeast morphology changes by PMA activation of normal and truncated PKC β-1 Yeast cells transformed with control plasmids (Cont) or expressing normal PKC β-1 (PKCb) or truncation mutants ND12 or CD15 were cultured in 3% glycerol/2% galactose medium for 13 h in the presence of 1 μM PMA (+PMA) or the inactive isomer 4α-PMA (-PMA) Cells were fixed in 3 7% formaldehyde, and were photographed in the phase-contrast microscope The length of the panels corresponds to 0 5 mm

stream of the PKC alpha carboxyl terminus while other studies projected it more than 50 aa upstream [Parker et al., 1989; Osada et al., 1990]. Some reports have defined it 4 aa from the PKC β-1 carboxyl terminus [Kikkawa et al., 1987; Ono et al., 1988], consistent with our data which are based for the first time on functional criteria.

Overall PKC activities correlate well between enzymatic and biological responses of the truncation mutants. While biological responses to ND12 are comparable to normal PKC β-1 its catalytic activity appears to be lowered by one quarter compared to normal levels. Enzymatic in vitro studies may not always properly reflect in vivo PKC function or full biological responses may be reached with somewhat reduced enzymatic activity. The PKC β-1 amino terminus may enhance the enzymatic activity and perhaps benefit the conformation or stability of the protein. Serine and threonine autophosphorylation sites have been determined in the V1, V3, and C4 regions of PKC β-2 [Flint et al., 1990] and homologous sites are found in the PKC β-1 sequence. It should be noted that while all putative autophosphorylation sites are still present in both mutants, enzymatic activity is lost in the mutant CD15 but little affected in ND12.

The biological responses found in yeast after PKC β-1 activation have been observed in various other eukaryotic organisms and likely indicate related signaling pathways in higher and lower eukaryotes. Our findings suggest that phorbol ester-activated rat PKC β-1 stimulates a yeast Ca^{2+} uptake mechanism which may share some characteristics of a proposed cell surface cation channel [Borbolla and Pena, 1980; Eilam et al., 1990]. Such a mechanism is energy independent, exploits an electrochemical gradient, and correlates with intracellular acidification [Eilam et al., 1990]. Ca^{2+} is subsequently transported into the yeast vacuole (the major cellular calcium store) in an energy-dependent process via the Ca^{2+}/H^{+} antiporter [Ohsumi and Anraku, 1983] which is fueled by a proton gradient generated by the vacuolar H^{+}-ATPase [Eilam et al., 1990]. Ca^{2+} plays an essential role in the regulation of the yeast cell cycle comparable to its obligatory role in mediating G1 events in mammalian cells [Iida et al., 1990]. Consequently the PKC β-1-stimulated Ca^{2+} uptake observed in this study in yeast may be causally related to PKC modulation of the average cell doubling time since PKC action up- or down modulates cell growth in mammalian cells [Rotenberg and Weinstein, 1991; Gescher et al., 1985].

The endogenous PKC background in yeast is still controversial. We have not observed any evidence for phorbol ester-responsive yeast PKC activity in any of our assays. This is consistent with a described PKC-related yeast protein activity which does not significantly respond to phorbol esters and displays distinct catalytic properties including substrate specificity [Ogita et al., 1990; Iwai et al., 1992]. In combination with the specific responses observed in our assays these findings suggest that mammalian PKC isoforms can be selectively activated in yeast by phorbol ester and studied in the presently undefined yeast cellular background. The related, essential yeast gene *PKC1* plays a role in cell cycle-specific osmotic stability; however, it is not complemented by mammalian PKC isoforms including β, when disrupted [Levin et al., 1990; Fields and Thorner, 1991; Levin and Bartlett-Heubusch, 1992] and consequently does not represent a functional yeast homolog of mammalian PKC β. The function of *PKC1* characterized in yeast suggests a role in a common pathway with a related gene *BCK1*, possibly in bud morphogenesis [Levin and Bartlett-Heubusch, 1992; Lee and Levin, 1992]. The normal catalytic function of rat PKC β-1 and its biological responses in yeast suggest that it may, in response to phorbol esters, participate in a pathway shared with putative functional yeast homologs to mammalian PKC isoforms which have not yet been established at the gene level. The report of mammalian-like PKC forms in yeast [Simon et al., 1991, 1992] suggests that such homologs may exist and rat PKC β-1 may activate the putative signaling pathway in which they may participate. The future dissection of these interactions should be facilitated by the experimental advantages of the yeast model. The knowledge gained should help to elucidate the yeast signaling pathway involved as well as the molecular mechanisms of mammalian PKC action.

Since the phenotype observed in yeast correlates with PKC β-1 enzymatic activity it can be utilized as a simple screen for PKC function based on the colony size of expressing cells on agar plates (not shown). This screen is particularly suitable for random mutagenesis strategies to test the phenotype (including phorbol ester regulation) of large numbers of mutations which will not be limited to the biased structural

predictions of the investigator. We expect that the yeast model will significantly facilitate and accelerate the dissection of the molecular roles of the homology regions in the PKC primary structure.

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