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A phorbol ester-responsive PKC-ζ generated by fusion with the regulatory domain of PKC-δ

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Abstract

A hybrid molecule generated by fusing the regulatory domain of PKC-δ with the catalytic domain of PKC-ζ is, like PKC-δ but unlike PKC-ζ, a phorbol ester-dependent enzyme. However, the substrate specificity of this hybrid resembles that of PKC-ζ. Expression of mammalian PKC-δ, but not PKC-ζ, in the fission yeast *Schizosaccharomyces pombe* causes growth retardation and phorbol esters amplify the PKC-δ phenotype without affecting that of PKC-ζ (Goode et al., submitted). The chimaeric molecule also inhibited growth and this effect was phorbol-ester dependent. Both the hybrid and PKC-δ holoenzyme, in contrast to PKC-ζ, down-regulate upon prolonged exposure to phorbol esters in vivo. Thus, this hybrid retains the regulatory properties conferred by PKC-δ but the catalytic properties of PKC-ζ. This regulatable chimaeric molecule will be useful in assessing the function of PKC-ζ.

Key words: Isotype regulation; Phosphorylation; Protein kinase C; Protein kinase C-ζ; Substrate specificity; *Schizosaccharomyces pombe*

1. Introduction

Mammalian protein kinase C (PKC) consists of a family of at least ten polypeptides encoded by nine genes [1,2]. Within the family, three distinct groups have been identified based on both structural and functional criteria [2]. PKC-α, -β_1, -β_2, and -γ, which were the first to be cloned, have been termed classical PKCs (cPKC); PKC-δ, -ε, -η and -θ are novel PKCs (nPKC) and PKC-ζ and -λ are atypical (aPKC). In general, the PKC molecule is divided into four constant regions (C_1–C_4) separated by five variable regions (V_1–V_5). Functionally the enzyme consists of the amino terminal regulatory domain, which contains the C_1 and C_2 regions, and the carboxy terminal catalytic domain, containing the C_3 and C_4 regions. The hinge region V_3 connects these functional domains. The C_1 region contains a sequence with homology to a consensus PKC substrate site but lacking a phosphate accepting serine or threonine (see [1]). This sequence, known as the pseudosubstrate site (PSS), has been proposed to function in maintaining the enzyme in an inactive state through a non productive association with the catalytic domain (discussed in [5,6]). The C_1 region of the cPKCs and nPKCs also contains two cysteine-rich sequences that have been shown to be the site of interaction with the phospholipid and diglyceride/phorbol ester activators [3,4]. However, the aPKC sequences contain only one cysteine-rich region in C_1 and it has been established that PKC-ζ differs from the other PKCs in dependency on lipid activators, especially phorbol esters, both in vitro [7–9] and in vivo [10,11].

The distinct lipid dependence of PKC-ζ and our consequent lack of understanding of its regulation has limited our appreciation of its functional significance in vivo. An approach to this issue has developed from our previous work on chimaeric PKC mutants which retain their regulatory properties [12]. Here we demonstrate that a chimaeric PKC-δ/ζ molecule retains δ-dependent phorbol ester regulation in vitro whilst the substrate specificity of this mutant, as specified by the PKC-ζ kinase domain, is conserved. In vivo this chimera, in contrast to PKC-ζ, is responsive to phorbol esters and induces a growth phenotype upon expression in *Schizosaccharomyces pombe*.

2. Experimental

2.1. Cell strain and culture

The yeast strain Ade6.704, Leu1-32, Ura4-4, h^- was routinely cultured at 32°C in rich YE medium or in synthetic Edinburgh minimal medium supplemented with 2% (w/v) glucose, 100 μg/ml adenine and leucine, and free of uracil (minimal selective medium) to select for the stable propagation of the expression plasmids [13]. Transformation was by electroporation using the manufacturer's instructions (Bio-Rad Pulse

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Abbreviations: PKC, protein kinase C; PSS, pseudosubstrate site; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PS, phosphatidylserine.

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Controller) and the transformants were spread onto minimal selective plates containing 1 M sorbitol and 1 μM thiamine. Individual colonies were plated onto minimal selective plates with no additives or containing 100 ng/ml 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or 1 μM thiamine to assay for a growth phenotype. Cultures were routinely started by picking a colony into minimal selective medium containing thiamine and culturing overnight until mid-log phase. Cells were then washed to remove thiamine, rediluted into fresh minimal selective medium with the appropriate additives (thiamine or TPA) and cultured for a further 24–48 h as described in the text. Growth status was assessed by measuring the OD_{595} (0.4 units = 10^7 cells/ml).

2.2. Expression plasmids

The pREP4 expression plasmid is a derivative of pREP1 [14] except that the *Nde*I site in the multiple cloning site has been replaced by a *Bal*I site and the selectable marker is the URA4 gene. Expression in this plasmid is driven by the nmt1 promoter which expresses at 8-fold the level of the ADH promoter and is repressed 80-fold by thiamine [14,15]. All subcloning methods used were as described [16]. The cDNA for PKC-δ was excised from the pREP3X-PKC-δ plasmid (Goode et al., submitted) as an *Sph*I–*Bam*HI fragment and ligated into pREP4 digested with the same enzymes. The PKC-ζ cDNA was excised as an *Xba*I fragment from pBluescript-PKC-ζ [11] and, after filling the recessed ends with the Klenow fragment of DNA polymerase I, was subcloned into a *Bal*I–*Sma*I digested vector. The PKC-ζA119E substitution was made using PCR (D.K. Ways and P.J.P., unpublished) essentially as described for other mutations [17,18] and was subcloned from pBluescript into pREP4 using an identical strategy as for PKC-ζ. This substitution, in the PSS of PKC-ζ, is equivalent to the A25E and A161E substitutions of PKC-α [17] and PKC-η [19], respectively. Both of these mutant molecules are constitutively active being co-factor independent for activity [17,19]. To construct the chimaeric molecule, the 2.2-kb *Sph*I–*Sca*I fragment (containing the promoter of the vector and the aminoterminal sequence of PKC-δ up to and including the V3 region) was isolated from pREP3X-PKC-δ and subcloned into pREP4-PKC-ζ which had been digested with ClaI, blunt ended with Klenow and then digested with SphI (which contained the V_3, C_3, V_4, C_4 and V_5 domains of PKC-ζ but lacked V_1, C_1, V_2 and C_2).

2.3. Protein extracts and PKC assays

Denatured protein extracts were made from equivalent numbers of cells by standard techniques [13] and SDS-PAGE and western blotting were performed as previously [20]. Polyclonal rabbit isotype-specific PKC-δ and -ζ antibodies directed against the carboxy termini (see [20] and references therein), and a polyclonal antiserum against the V3 region of PKC-δ (A.R. Olivier, S. Kiley and P.J.P., unpublished) were followed by donkey anti rabbit horse radish peroxidase-conjugated antibodies and the ECL detection system (Amersham International).

Native proteins for PKC activity analysis were extracted from mid log phase cell strains cultured in the absence of thiamine for 30 h essentially as described [13] except that the extraction buffer contained 20 mM Tris pH 8.0, 5 mM EDTA, 5 mM EGTA, 0.3% (v/v) β-mercaptoethanol, 0.5% (v/v) Triton X-100, 10 mM benzamidine, 50 μg/ml PMSF, 50 μg/ml ovalbumin and 100 μg/ml leupeptin. After clearing by centrifugation, the extracts were loaded onto a 1 ml HiTrap Heparin column (Pharmacia) using an FPLC. After washing with 5 column volumes of buffer (20 mM Tris pH 8.0, 2 mM EDTA, 5 mM EGTA, 0.3% (v/v) β-mercaptoethanol, 0.1% (v/v) Triton X-100 and 10 mM benzamidine), proteins were eluted with a linear 0–1.5 M NaCl gradient (15 × 0.5 ml 1 min fractions). 5 μl of column fractions were assayed using 250 μg/ml of the serine containing δPSS peptide as substrate in the presence or absence of phosphatidylserine (PS, 1.25 mg/ml) and TPA (250 ng/ml) as described previously [21]. Protein concentrations were measured by Bradford assay (Bio-Rad) and used to correct for variation in extraction and recovery.

Peak activity fractions of the PKC-δ and -δ/ζ profiles were compared with respective vector control fractions for kinase activity (in the presence of PS/TPA) against a titration of the α, δ and ζPSS peptides, centred around K_m values expected from previous work [21]. From preliminary experiments, it was known that PKC-ζ eluted between 1.1–1.4 M NaCl. Fraction 12 of the PKC-ζ and -ζA119E profiles were also compared with vector controls for kinase activity (in the presence or absence of PS and TPA) using 250 μg/ml (178 μM) δPSS, 500 μg/ml (255 μM) αPSS or 375 μg/ml (259 μM) ζPSS peptides as substrates (see [21] and references therein).

3. Results and discussion

Given the lack of response of PKC-ζ to the classic PKC activator, TPA [7,10,11], and our inability to activate this isotype by other means in a whole cell context, we were interested to develop a regulatable form of PKC-ζ. The protein kinase specificity of PKCs, and therefore function, is determined in part by the catalytic domain of the enzyme. Therefore a chimaeric molecule was created, replacing the regulatory domain of PKC-ζ with that of PKC-δ, in the hope that an enzyme with the output of PKC-ζ but regulated in a PKC-δ fashion would result. *S. pombe* was chosen as an expression vehicle because the low background of TPA-dependent kinase activity (Goode et al., submitted) assists in interpretation when compared with mammalian cells which generally express several PKC isotypes (e.g. [20,22]).

Mammalian PKC-δ, -ζ, the PKC-δ/ζ chimera and a constitutive PKC-ζ mutant, PKC-ζA119E, were subcloned into the pREP4 vector which uses the powerful but thiamine repressible nmt1 promoter to drive expression [14]. These plasmids were transformed into *S. pombe* cells by electroporation, and individual transformants were picked into 1 ml of minimal selective liquid medium in the absence of thiamine. Denatured extracts, collected after 30 h of culture, were analysed for expression of the relevant mammalian PKC by western blotting using isotype-specific PKC antisera (data not shown).

3.1. Regulation and substrate range of expressed proteins

Representative colonies expressing PKC-δ, -ζ, -δ/ζ or -ζA119E and vector control were expanded in liquid culture (in the absence of thiamine). Triton X-100 soluble proteins extracted from 100 ml of these cultures were loaded onto a 1 ml HiTrap Heparin column and eluted with a 0–1.5 M NaCl gradient. Fractions were assayed for activity dependent on the PKC co-factors PS and TPA using the δPSS peptide as substrate. Fractions were also analysed by SDS-PAGE and Western blotting using the carboxy terminal PKC-δ or -ζ specific antisera. Column profiles with relevant western blots are shown in Fig. 1. PKC-δ elutes at 0.7–0.9 M NaCl which accounts for the main peak of co-factor dependent activity, with the catalytic domain fragment eluting at 0.7 M and generating the co-factor independent activity. The main peak of the chimaeric protein elutes at 0.9–1.1 M NaCl correlating with the activity peak. Catalytic domain fragments elute at 0.8–0.9 M but the associated activity is relatively low compared with that of the full length enzyme. PKC-ζ and -ζA119E elute with the peak protein at 1.2–1.3 M NaCl but no kinase activity is associated with these proteins when using the δPSS substrate. Cata-

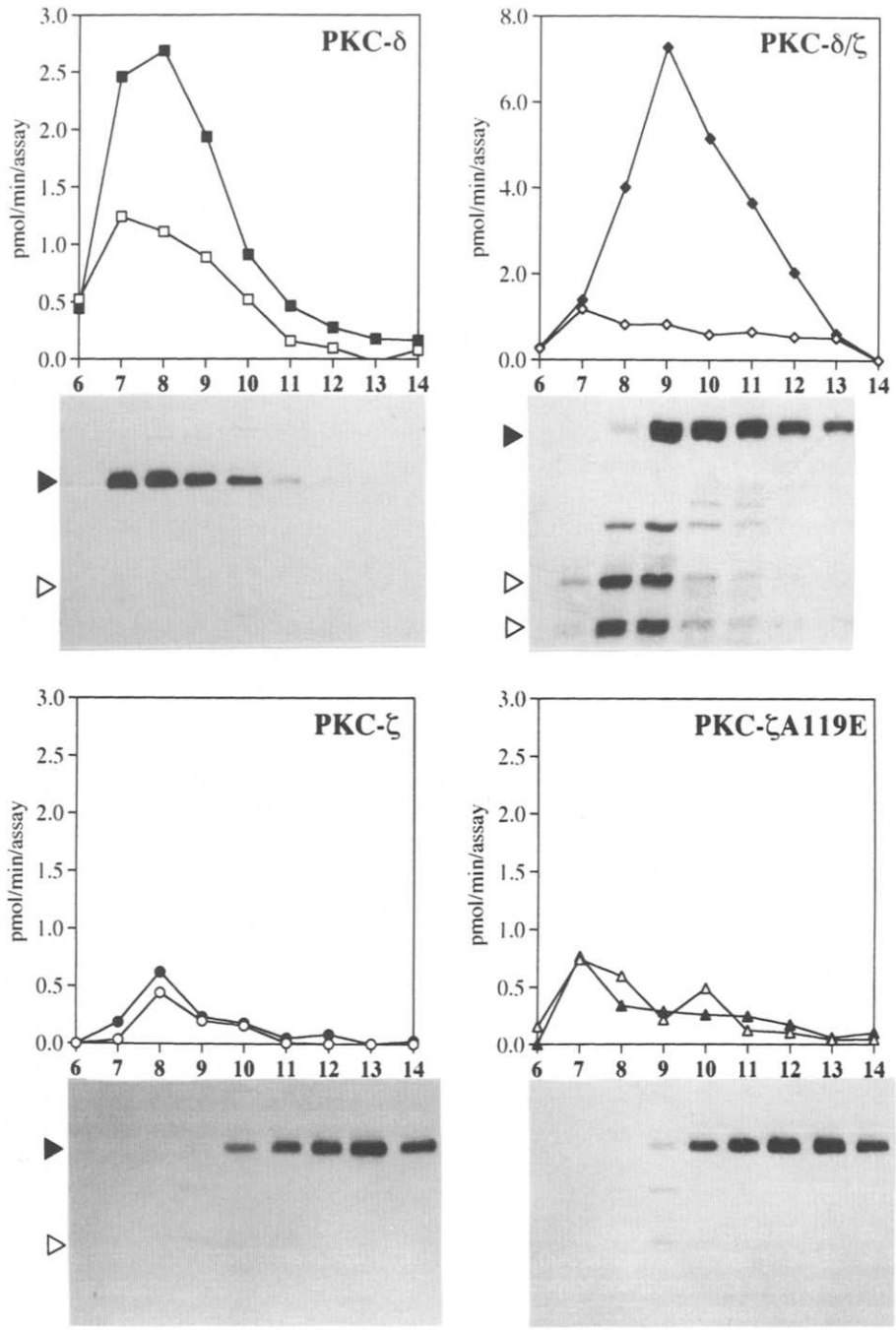

Fig. 1. Activity profiles of column fractions using the δPSS substrate. 5 μl of column fractions of extracts from vector control, PKC-δ, -ζ, -δ/ζ and -ζA119E cells were assayed for co-factor dependent activity (open symbols, − PS/TPA; filled symbols, + PS/TPA) using the δPSS as substrate. After subtraction of background counts and correction for protein concentration, results are plotted as net activity (vector control activity has been subtracted). Each fraction represents a 0.1 M increase in NaCl concentration (fraction 0 = 0 M). Figures are means of duplicate samples. Aliquots of the same fractions were denatured and analysed by SDS-PAGE and western blotting. The positions of the full length proteins and catalytic domain fragments are indicated by filled and open arrowheads, respectively.

lytic domain fragments for both of these enzymes elute at 0.7–0.9 M NaCl and may account for the small peaks of activity in these fractions. Thus these four enzymes can be separated by a single liquid chromatography step. The PKC-δ regulatory domain renders the PKC-ζ catalytic domain co-factor dependent for activity.

Since the kinase domain contributes to the determination of substrate specificity, and the kinase domain of PKC-δ and the hybrid are different, the ability of the PKC-δ/ζ chimera and of PKC-δ to phosphorylate a panel of pseudosubstrate site peptides was assessed. Peak fractions of PKC-δ (fraction 8) and of PKC-δ/ζ (fraction 10) were compared with the same fractions from vector control chromatograms. The amount of PKC in the rel-

evant fractions was equilibrated by densitometer scanning of western blots probed with a polyclonal antiserum directed against the V3 region of PKC-δ, which is present in both of these molecules (data not shown). The results (net values after correction for protein concentration and subtraction of the appropriate vector control values) are shown in Fig. 2. PKC-δ exhibits a higher apparent affinity for all three peptides than does the chimera. PKC-δ shows a preference for both the ζPSS and δPSS peptides over the αPSS peptide (apparent K_m values for these peptides were 1.3, 2.9 and 28 μM, respectively). The apparent K_m values for these peptides of PKC-δ/ζ although weaker follow a similar pattern but with clear preference for the ζPSS (K_m values for the ζ, δ and αPSS peptides were 4.3, 18 and 80 μM, respectively). The PKC-δ/ζ hybrid exhibits higher V_{max} values for the αPSS and ζPSS peptides than for δPSS (values of 0.65, 0.38 and 0.17 pmol/min/μg protein) which is the opposite to the activity pattern of PKC-δ (values of 0.23, 0.27 and 0.34 pmol/min/μg for the same peptides). The K_{cat} values (V_{max}/K_m) show that there is a clear divergence in specificity with respect to the δPSS, with relative K_{cat} values of 1:0.6:0.05 for PKC-δ on the ζ, δ and α peptides, respectively, while PKC-δ/ζ shows ratios of 1:0.11:0.11 in the same analysis.

The δ/ζ hybrid demonstrates higher V_{max} values for the α and ζPSS peptides than for the δPSS peptide. This pattern may reflect the substrate preference of the PKC-ζ kinase domain and could partially explain the lack of detection of PKC-ζ and -ζA119E kinase activity when using the δPSS as substrate (Fig. 1). To establish the substrate preference of PKC-ζ, we determined the ability of the PKC-ζ holoenzyme and the constitutive mutant to phosphorylate the same panel of PSS peptides.. Peak fraction 12 of PKC-ζ and -ζA119E eluants, which contain similar amounts of these PKC-ζ enzymes (Fig. 1), were compared with the same fraction from the vector controls. Neither of these enzymes phosphorylated the δPSS (Fig. 3 and above). However, the constitutive PKC-ζ mutant, and to a lesser extent the holoenzyme, phosphorylated the ζPSS peptide; the αPSS peptide is also a substrate if somewhat poorer (Fig. 3). The PKC-ζ holoenzyme appears to be a poor kinase. However, these assays have been optimised for the activation of PKC-δ and not PKC-ζ. It is therefore not surprising that the ζ holoenzyme is not active in these assays. Indeed, the activity associated with the PKC-ζ mutant is constitutive, showing no difference in the presence or absence of activators (data not shown). In the absence of activation of the holoenzyme, the constitutive mutant can be considered as representing the intrinsic peptide preference of PKC-ζ. Thus PKC-ζ exhibits distinct peptide substrate preferences. PKC-ζA119E is expressed at approximately 24 ± 16% of the level of the δ/ζ chimera (as determined by densitometer tracing of western blots from 4 experiments). If this lower level of expression of PKC-ζA119E is considered, the observed pattern of peptide phosphorylation and the lack of perceived δPSS phosphorylation are consistent quantitatively with the activity of the δ/ζ chimera.

We have shown that the hybrid molecule is active in vitro and has a substrate preference resembling that of PKC-ζ, but appears to be regulated like PKC-δ (it can be purified as a phorbol ester dependent kinase). To confirm the TPA-dependence of PKC-δ/ζ, a titration with TPA was carried out and comparison made with PKC-δ. Both show TPA-dependence over the range 0.2–25 ng/ml with PKC-δ appearing more sensitive (data not shown).

3.2. *Growth effects in* S. pombe

These studies were initiated to obtain a regulatable form of PKC-ζ which could be used in whole cell systems. Since the molecules are expressed in *S. pombe* on a tightly repressible promoter, the consequence of expression can be assessed. We have shown previously that mammalian PKC-δ produces a growth inhibition in *S. pombe* which is enhanced by phorbol esters whereas

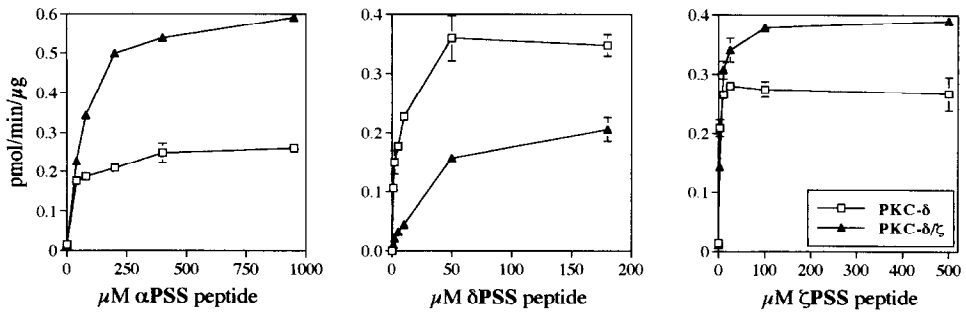

Fig. 2. PKC-δ/ζ and PKC-δ have distinct substrate specificities. Fraction 8 of PKC-δ (□) and fraction 10 of PKC-δ/ζ (▲) were assayed in the presence of PS/TPA using titrations of the serine containing αPSS, δPSS and ζPSS peptides as substrates. After correction for protein concentration, the appropriate vector control values were subtracted. The amount of PKC in the respective fractions was compared by western blotting followed by densitometer scanning and the values were corrected for relative PKC concentration. Values for apparent K_m and V_{max}, calculated on the basis of inverse plots of substrate concentration and activity, are included in the text.

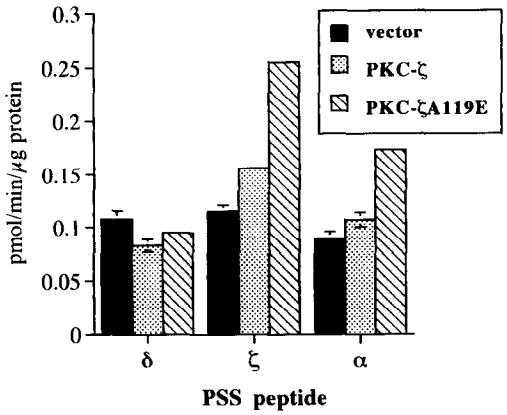

Fig. 3. PKC-ζA119E and PKC-ζ specificity towards PSS peptides. Fraction 12 from vector control, PKC-ζ and PKC-ζA119E cells (as indicated) were assayed using the δPSS, ζPSS and αPSS peptides as substrates, as described in section 2. Data shown, from assays performed in the presence of PS/TPA, was not significantly different from co-factor independent values. Results are means and ranges of duplicate samples.

PKC-ζ has no detectable effect (Goode et al., submitted). Representative colonies of vector controls or transformants expressing PKC-δ, -ζ, -δ/ζ or -ζA119E were spread onto minimal selective solid media with or without 1 μM thiamine. PKC-δ expression produces a significant reduction in colony size and the chimaeric molecule repeatably produced a slight growth inhibition (Fig. 4, compare plates A and B). Expression of PKC-ζ and -ζA119E produced no growth effect (Fig. 4, plates A and B and data not shown).

PKC-δ is activated by phorbol esters both in vitro and in vivo [20,21,23] and we have shown that the chimaeric molecule is activated by TPA in vitro. To ascertain if the chimera was also activated by TPA in vivo, the effect of TPA treatment on the transformed *S. pombe* strains was determined. Cells were streaked onto minimal selective plates which contained 100 ng/ml TPA (Fig. 4, plate C and data not shown). The colony size of vector controls

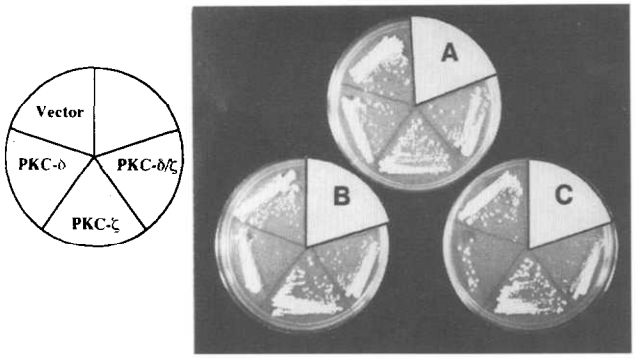

Fig. 4. Growth effects of PKC-δ, -ζ and -δ/ζ and response to TPA. Colonies transformed with pREP4, pREP4-PKC-δ, -ζ or -δ/ζ were plated onto minimal selective medium with 1 μM thiamine (A), no additive (B) or 100 ng/ml TPA (C) and incubated at 32°C for 3 days.

and PKC-ζ and -ζA119E was not affected by TPA whereas the yeast strain expressing the chimaeric molecule was further growth inhibited and PKC-δ transformants ceased growth (some large colonies seen with PKC-δ plated on TPA reflect loss of functional PKC-δ since the vast majority of colonies are seen as a feint lawn). Therefore, the chimaeric molecule produces a phenotype in *S. pombe* and is responsive to TPA. A form of PKC-ζ (PKC- ζA119E) which is constitutively active, and has the same substrate specificity as the δ/ζ chimera, produces no growth effects in this biological assay. This apparent inconsistency can be explained by the lower levels of expression of PKC-ζA119E compared with PKC-δ/ζ.

A characteristic of sustained TPA treatment is the down-regulation of most PKC isotypes and this phenomenon has been linked to the ability to activate the relevant PKC. For example, PKC-ζ does not down-regulate and is not activated by TPA [10,11] whereas PKC-δ both down-regulates and is activated [20,23]. To determine if the hybrid molecule behaves like PKC-δ in vivo, its response to TPA in *S. pombe* was analysed. Extracts taken from cells grown in liquid culture for 30 h in the presence or absence of TPA (without thiamine) were examined by western blotting. TPA causes the down-regulation of PKC-δ and the levels of PKC-δ/ζ protein are reduced by approximately 80% whereas PKC-ζ does not down-regulate (Fig. 5). The PKC-δ regulatory domain therefore confers TPA sensitivity to the chimaeric molecule in this biological context. An implication of this finding is that the kinase specificity of PKC-ζ, as defined by that of the chimera, is appropriate for triggering the process of down-regulation. This phenomenon has an active char-

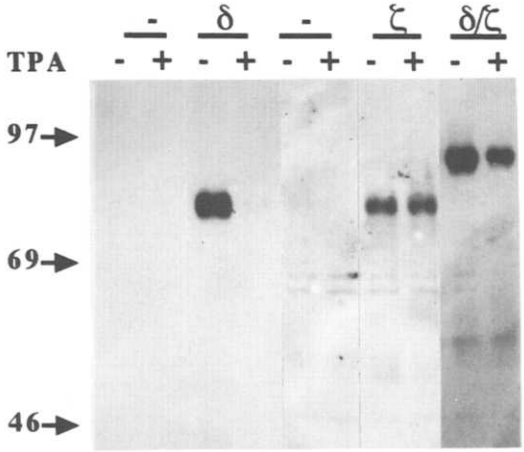

Fig. 5. TPA causes the down-regulation of PKC-δ and -δ/ζ but not PKC-ζ. Extracts from vector controls (−) and cells expressing PKC-δ, PKC-ζ or PKC-δ/ζ cultured in the presence or absence of TPA (+/−) for 30 h (in absence of thiamine) were analysed by SDS-PAGE and Western blotting using isotype-specific anti-PKC-δ (lanes 1–4) or PKC-ζ (lanes 5–10) antibodies. Arrows indicate molecular mass (kDa). PKC-δ and -ζ migrate at 76–80 kDa whereas the chimaeric molecule has an increased molecular mass of approximately 87 kDa due to duplication of the V_3 region during construction.

acteristic (NTG and PJP, manuscript in preparation) that is fulfilled by PKC-δ/ζ.

Thus PKC-δ expression in *S. pombe* produces a growth inhibition which can be amplified by phorbol ester treatment whereas PKC-ζ expression has no effect on growth. A hybrid molecule which contains the regulatory domain of PKC-δ and the catalytic domain of PKC-ζ behaves like PKC-δ in this assay although the growth effects are less marked. The PKC-δ regulatory domain renders the chimaeric molecule TPA sensitive for both phenotypic effects and down-regulation.

In conclusion, a chimaeric molecule has been constructed which retains the characteristics of its constituent halves. A PKC has been generated which has the substrate specificity of PKC-ζ but can be regulated both in vitro and in vivo by phorbol esters, in contrast to the PKC-ζ holoenzyme. Since there are no proven activators for PKC-ζ which can be used in a whole cell context, this molecule should greatly assist in the elucidation of the function of PKC-ζ.

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