Recombinant C1b domain of PKCδ triggers meiotic maturation upon microinjection in *Xenopus laevis* oocytes

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Abstract The C1 domains are 50 amino acid sequences present in protein kinase C (PKC) isozymes that are responsible for binding of phorbol esters and the lipid second messenger diacylglycerol (DAG). We found that bacterially expressed C1b domain of PKCδ induces germinal vesicle breakdown (GVBD) when microinjected into *Xenopus laevis* oocytes. Injection of the C1b domain of PKCδ significantly enhanced insulin- but not progesterone-induced maturation. Interestingly, the PKCδ C1b domain markedly synergized with normal Ras protein to induce oocyte maturation when both proteins were co-injected in oocytes. Our results demonstrate that the purified C1b domain of PKCδ is sufficient to promote meiotic maturation of *X. laevis* oocytes probably through activation of components of the insulin/Ras signaling pathway.

Key words: Protein kinase C; C1 domain; Oocyte; *Xenopus laevis*

1. Introduction

The C1 domains are 50/51 amino acid motifs that were initially identified in the regulatory domain of protein kinase C (PKC) isozymes, a family of kinases that play a critical role in signaling events controlling cell proliferation, death and differentiation [1–5]. C1 domains possess the motif $HX_{12}CX_2CX_nCX_2CX_4HX_2CX_7C$, where H is His, C is Cys, X is any other amino acid, and *n* is 13–14. This domain is duplicated in tandem (C1a and C1b) in the calcium-dependent or 'classical' PKCs (cPKCα, βI, βII, and γ), in the calcium-independent or 'novel' PKCs (nPKCδ, ε, η, and θ) and in the PKC-related protein PKCμ (PKD). A single C1 domain is present in 'atypical' PKCs (aPKCζ and λ/ι) and in proteins unrelated to PKCs, including α- and β-chimaerins, RasGRP, *Caenorhabditis elegans* Unc-13 and mammalian Munc-13 isoforms [4,6].

Mutagenesis and structural studies have revealed that the C1 domains in the regulatory domain of cPKC and nPKCs are responsible for the binding of phorbol esters and the second messenger diacylglycerol (DAG) [1,7–9]. Using isolated C1 domains of PKCs expressed in bacteria it was established that the 50 amino acid region is the minimum domain for phorbol ester binding [1,7–10]. The ligand binding properties of isolated C1 domains are similar to those of the corresponding holoenzymes [10,11]. Mutations in Cys or His that are essential for folding of the domain abolished phorbol ester/DAG binding [1,9]. Binding of phorbol esters/DAG to intact PKCs or isolated C1 domains is dependent on phospholipids, and phosphatidylserine is the most efficient lipid for reconstitution of binding [7,10,12]. Although highly homologous, individual C1 domains have shown remarkable differences in ligand recognition, suggesting that significant structural variations might exist among the different C1 domains that confer unique properties in each case [13,14].

The C1 domains are implicated in the association of cPKCs and nPKCs to membranes after elevation of DAG levels or phorbol ester treatment, a process known as 'translocation'. Allosteric activation of PKC involves the association of the enzyme to membranes, and this association requires the C1 domain. Interestingly, studies have demonstrated non-equivalent roles for C1a and C1b domains in PKC translocation [15,16]. PKC isozymes distribute to different intracellular compartments, and there is strong evidence that association to specific proteins upon translocation is a key mechanism that determines localization for each isozyme [17,18]. Several proteins have been identified that associate to different regions in the regulatory domain of PKCs, including association to the C1 domain [4,17,19–23]. Importantly, several studies have demonstrated that the regulatory domain in PKC isozymes can indeed evoke cellular responses, and in many cases even mimic the action of the full-length protein [24–28]. This is relevant because there is little evidence for substrate specificity for PKC isozymes, and suggests a role for the regulatory domain in conferring isozyme specificity through protein–protein association. The complexity of the interactions that occur through the C1 domain and its potential for conferring isozyme specificity is a subject of intense investigation.

In this paper we explore the hypothesis that C1 domains of PKCs have in vivo functions using *Xenopus laevis* oocytes as a model system. *X. laevis* oocytes are arrested at the G2/M transition of the first meiotic prophase. Insulin and progesterone induce oocytes to enter metaphase and undergo meiotic maturation involving germinal vesicle breakdown (GVBD) [29,30]. The possibility of inducing maturation by microinjection of various oncogene products makes the *Xenopus* oocyte system a very useful experimental model for the analysis of in vivo function(s) of various cellular gene products in the progression through the eukaryotic cell cycle. This model has

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Abbreviations: PKC, protein kinase C; PDBu, phorbol 12,13-dibutyrate; DAG, diacylglycerol; GST, glutathione *S*-transferase; GVBD, germinal vesicle breakdown; PI3K, PI3 kinase

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been widely used to evaluate the relationship of PKC with other signaling pathways [21,31,32]. The data presented here show that an isolated C1b domain of the novel PKCδ induces maturation of oocytes, and accelerates insulin-induced maturation.

2. Materials and methods

2.1. Materials

Glutathione-Sepharose 4B beads, benzamidine-Sepharose, reduced glutathione and isopropyl-1-thio-β-D-galactopyranoside (IPTG) were purchased from Pharmacia Biotech Inc. (Piscataway, NJ, USA). Ampicillin, insulin and progesterone were obtained from Sigma. Bovine plasminogen free thrombin and gonadotropin were from Calbiochem-Novabiochem (La Jolla, CA, USA). All other chemicals were of high quality.

2.2. Expression of the PKCδ C1b domain in Escherichia coli

The PKCδ C1b domain was expressed as a glutathione *S*-transferase (GST) fusion protein in *E. coli*. Generation of the expression vector and expression of the recombinant protein are described elsewhere [9,10]. Briefly, a mouse cDNA fragment encoding the PKCδ C1b domain [9] was generated by polymerase chain reaction and subcloned in-frame into the pGEX-TK vector (Pharmacia Biotech Inc.) to get the pGEXδ plasmid. In order to express the recombinant C1 domain as a GST fusion protein, JM101 cells were transformed with the pGEXδ plasmid and then grown in 1 l of LB medium containing 50 μg/ml ampicillin. Expression of the GST fusion protein was induced by the addition of 0.5 mM IPTG after the bacteria reached an A_{600} of 0.5. Five hours later cells were pelleted at $4000\times g$ and resuspended in 20 ml of phosphate-buffered saline containing 3 mM dithiothreitol. Cells were lysed by homogenization, passed two times through a French press and sonicated. After centrifugation of the lysate ($15\,000\times g$ at 4°C, 45 min), the supernatant was collected and incubated for 1 h at 4°C with 10 ml of a 50% glutathione-Sepharose 4B suspension. The glutathione-Sepharose 4B beads were collected by centrifugation ($500\times g$, 5 min), and the GST fusion protein was eluted with 1 ml of 10 mM reduced glutathione in 50 mM Tris–Cl, pH 8.0. The elution step was repeated an additional three times, and the collected fractions were pooled. In order to cleave the PKCδ C1b domain from the GST partner, the solution was then treated with thrombin (250 U, 4 h). This condition was sufficient to cleave all the recombinant C1 domain, as judged by gel electrophoresis. Thrombin was then removed by incubation with benzamidine-Sepharose beads according to the manufacturer's instructions (Pharmacia). The beads were then pelleted, and the supernatant purified by HPLC on C4 silica gel using a gradient of 0.05% trifluoroacetic acid/H_2O : 0.05% trifluoroacetic acid in 90/10 CH_3CN/H_2O, from 75:25 to 25:75 over 30 min. The C1 domain and the residual GST eluted at 14.8 min and 18 min, respectively. The purified PKCδ C1b domain was then refolded as described previously [10]. Briefly, the C1 domain was dissolved in 0.05% trifluoroacetic acid, pH 2.5, and 2.5–3 molar equivalents (based on weight of protein) of Zn^{2+} (as 50 mM $ZnCl_2$) were added, and the pH was slowly raised to 6.0 with NaOH. The solution was desalted and concentrated on Centricon-3 filters. A single peak was found in the HPLC profile after purification. A single band was also observed after Coomassie blue staining of SDS–polyacrylamide gels (see [10] for a figure with HPLC profile and Coomassie blue staining). The activity of the protein was confirmed by [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) binding, using the method of Sharkey and Blumberg [9–11,33]. This purified C1 domain has been also used for X-ray crystallography analysis and structure determination [8].

2.3. Mutants of the PKCδ C1b domain

Mutants in position 22 of the PKCδ C1b domain (which corresponds to position 252 in the full-length protein) were generated by site-directed mutagenesis using the U.S.E. Mutagenesis kit (Pharmacia Biotech Inc.). In these mutants, Trp-252 was replaced by either Gly (W252G-PKCδ C1b) or Phe (W252F-PKCδ C1b). The details of the generation of these mutants are described elsewhere [9]. Expression of the GST-PKCδ C1b mutants in *E. coli* and purification of the recombinant protein were done as described above for the wild-type PKCδ C1b domain.

2.4. Expression and purification of non-transforming Ras

Expression and purification to homogeneity of non-transforming H-Ras protein (Gly-12) was done as we have previously described [34,35].

2.5. Expression and purification of the SH2 domain of p85 (PI3 kinase (PI3K))

The SH2 domain of p85 (residues 333–428) of PI3K was cloned into the bacterial expression vector pQE9 (Qiagen), and expressed and purified to near homogeneity as previously described [36].

2.6. Oocyte preparation, microinjection and maturation

Adult female *X. laevis* (purchased from Nasco, Fort Atkinson, WI, USA) were stimulated to ovulate by microinjecting 50 U of serum gonadotropin 3 days before oocyte extraction. Ovarian fragments were surgically removed from frogs anesthetized by hypothermia. Full grown, stage VI oocytes were manually dissected into ND-96 medium (5 mM HEPES, 96 mM NaCl, 1 mM $MgCl_2$, 2 mM KCl, 1.8 mM $CaCl_2$, pH 7.8, 10 μg/ml penicillin and 10 μg/ml streptomycin sulfate). The oocytes were allowed to recover overnight in the same buffer before further treatment, and were constantly maintained at 20°C. For induction of meiotic maturation, groups of 10 to 12 oocytes were incubated in ND-96 medium without KCl in the presence of progesterone (15 μM) or insulin (7.5 μM). In another set of experiments, 30 to 60 nl of PKCδ C1b protein or mutated forms of the domain (1 μg/μl solutions made in 20 mM Tris–HCl, pH 7.5) were microinjected into oocytes. Controls were microinjected with buffer. For co-injection experiments, oocytes were first injected with 30 nl of purified normal H-Ras protein or the SH2 domain of p85 (PI3K) (1 μg/μl) and then allowed to recover by incubating for 30 min in ND-96 medium before injecting them again with 30 nl of purified PKCδ C1b protein.

Meiotic maturation was assayed by scoring the disappearance of the nucleus (GVBD) in oocytes fixed with 10% trichloroacetic acid [37]. The absence of the nucleus correlated with the appearance of a white spot in the animal pole. Periodic, routine assays indicated that the absence of the nucleus correlated with the appearance of a white spot on the animal pole and with activation of histone H1 kinase activity (MPF kinase) in clarified extracts of the oocytes [38,39].

2.7. Determination of protein concentration

Protein was determined by the method of Bradford [40], using bovine serum albumin as standard.

3. Results

In our previous studies we have shown that the PKCδ C1b domain (Fig. 1) is the minimum domain for phorbol ester binding in PKCδ [9,10]. We expressed the PKCδ C1b domain in *E. coli* as a GST fusion protein, and recovered the protein from bacterial lysates using glutathione-Sepharose 4B beads. The GST partner was removed by thrombin digestion and the recombinant protein purified by HPLC as described in Section 2. Phorbol ester binding analysis for the purified PKCδ C1b domain using the radioligand [^{3}H]PDBu revealed a dissociation constant (K_d) of 0.8 ± 0.1 nM ($n=5$), which is similar to that observed with the holoenzyme [10,11]. The PKCδ C1b domain is therefore properly folded after purification, as previously confirmed by X-ray crystallography [8].

231 280

PKCδ C1b domain HRFKVYNYMSPTFCDHCGSLLWGLVKQGLKCEDCGMNVHHKCREKVANLC

Fig. 1. Sequence of the PKCδ C1b domain. The amino acid subjected to mutagenesis (Trp-252) is underlined.

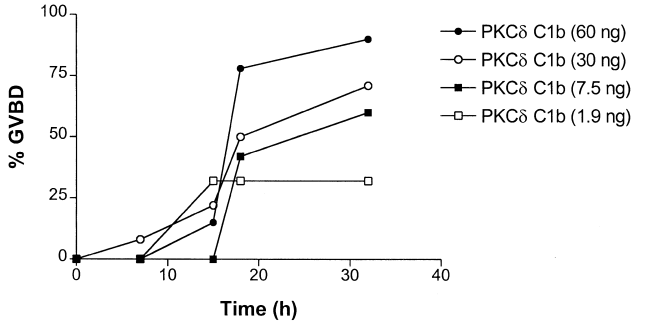

Fig. 2. Induction of maturation of *X. laevis* oocytes by microinjection of PKCδ C1b domain. Oocytes (10 to 12 for each experimental condition) were injected with different amounts of the PKCδ C1b domain in 30 nl of 20 mM Tris–HCl, pH 7.5 buffer, and GVBD was monitored for 32 h. Closed circles, 60 ng; open circles, 30 ng; closed squares, 7.5 ng; open squares, 1.9 ng. Injection of buffer alone did not induce any maturation. Results are expressed as a percentage of oocytes reaching GVBD. The induction of oocyte maturation by the PKCδ C1b domain was observed in at least five independent experiments.

We evaluated the effect of microinjection of the purified PKCδ C1b domain into full grown, stage VI *X. laevis* oocytes. Interestingly, the PKCδ C1b domain induced meiotic maturation of the oocytes in a dose-dependent manner as determined by GVBD (Fig. 2). We next assessed the effect of a mutant of the PKCδ C1b domain, in which Trp-252 was replaced by Gly (W252G-PKCδ C1b). This mutant has approximately 30-fold lower binding affinity for [^{3}H]PDBu compared to the wild-type PKCδ C1b domain [9]. We observed that W252G-PKCδ C1b was less efficient than wild-type PKCδ C1b at inducing oocyte maturation (Fig. 3). We have also used a mutated PKCδ C1b domain in which Trp in position 252 has been replaced by Phe (W252F-PKCδ C1b). This mutant has similar binding affinity for [^{3}H]PDBu as the non-mutated domain [9]. When microinjected into oocytes, W252F-PKCδ C1b induced maturation with similar potency as the wild-type PKCδ C1b domain (Fig. 3).

In order to begin elucidating the mechanism by which the PKCδ C1b domain induces oocyte maturation, we first evaluated whether microinjection of the PKCδ C1b domain affects maturation induced by either insulin or progesterone. Activation of insulin or progesterone receptor induces oocyte maturation through different signaling pathways. While progesterone induces maturation mainly through inhibition of the cAMP/PKA-dependent pathway, insulin activates maturation through a receptor tyrosine kinase Ras-dependent mechanism [36,41–43]. Interestingly, when the PKCδ C1b domain was injected into oocytes, we observed that it markedly accelerates the maturation induced by insulin (Fig. 4A). As expected, similar results were observed when the mutant W252F-PKCδ C1b was injected (data not shown). On the other hand, microinjection of the PKCδ C1b domain did not affect the kinetics of GVBD mediated by progesterone (Fig. 4B), suggesting that the effect is specific for the insulin pathway.

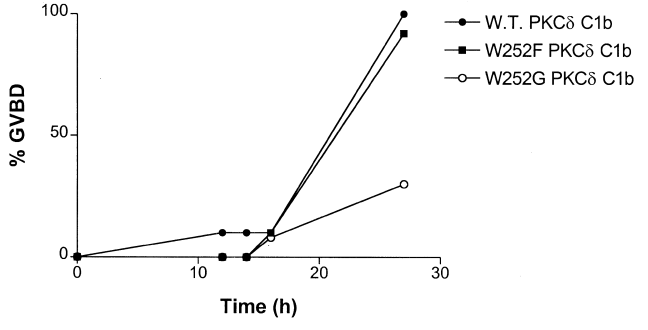

Fig. 3. Effect of PKCδ C1b domain mutants on oocyte maturation. Each C1 domain (30 ng) was injected in groups of 10–12 oocytes and GVBD was monitored. Results are expressed as a percentage of oocytes reaching GVBD. Closed circles, wild-type (W.T.) PKCδ C1b domain; open circles, W252G-PKCδ C1b domain; closed squares, W252F-PKCδ C1b domain. Similar results were observed in two additional experiments.

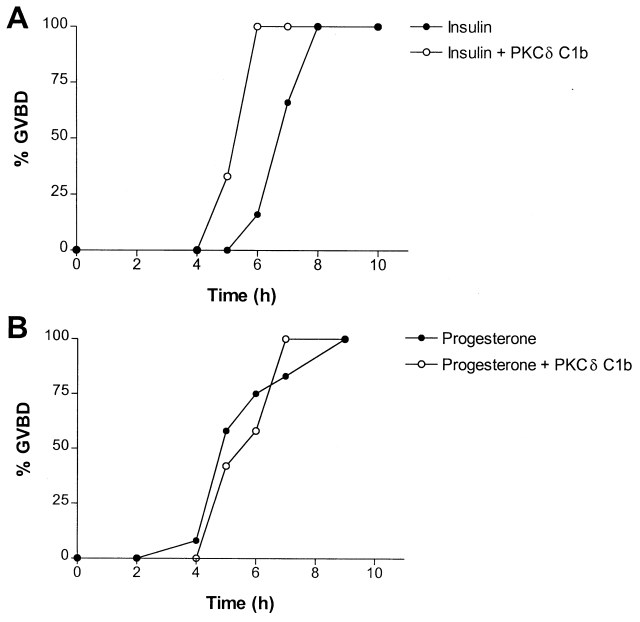

Fig. 4. Effect of microinjection of PKCδ C1b domain on insulin- (panel A) or progesterone- (panel B) induced maturation. Each group of 10–12 oocytes was microinjected with 30 ng of PKCδ C1b domain (open circles) or vehicle (closed circles). Insulin (7.5 μM) or progesterone (20 μM) were added 30 min later, and GVBD was monitored for the times indicated in the figure. Results are expressed as a percentage of oocytes reaching GVBD. Essentially identical results were obtained in three independent experiments. Experiments using 0.1 μM progesterone gave similar results.

In the next set of experiments we evaluated whether the PKCδ C1b domain affects the effect of Ras on maturation. In contrast to transforming Ras variants, normal Ras proteins (Gly-12) poorly induce maturation in oocytes [36]. Interestingly, our experiments reveal that microinjection of the PKCδ C1b domain markedly potentiates the effect of normal H-Ras on oocyte maturation (Fig. 5A). In order to further elucidate the mechanism of action of the PKCδ C1b domain, we assessed whether the effect on oocyte maturation could be affected by the SH2 domain of p85 (PI3K). We have previously shown that the SH2 domain of p85 (PI3K) blocks insulin-induced maturation of *Xenopus* oocytes [36]. When co-injected with the PKCδ C1b domain, the SH2 domain of p85 did not block meiotic maturation, although it completely blocked the effect of insulin under the same experimental condition (Fig. 5B). Taken together, these experiments indicate that the PKCδ C1b domain exerts its effect on oocytes through activation of the Ras-dependent insulin pathway.

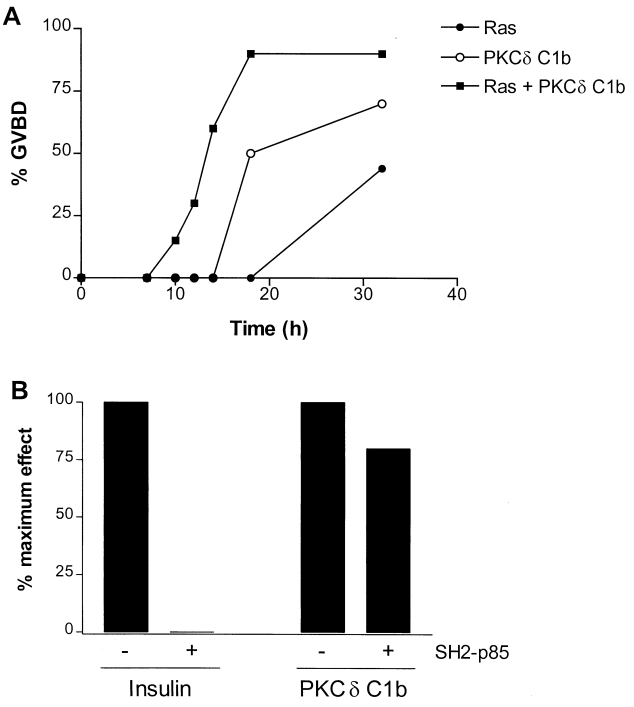

Fig. 5. Effect of Ras and the SH2 domain of p85 (PI3K) on oocyte maturation induced by the PKCδ C1b domain. Panel A: Oocytes (in groups of 10–12) were microinjected with 30 ng of PKCδ C1b domain (open circles), 30 ng Ras (Gly-12) (closed circles) or 30 ng PKCδ C1b together with 30 ng Ras (Gly-12) (closed squares), and GVBD was monitored for the times indicated in the figure. Results are expressed as a percentage of oocytes reaching GVBD. Two additional experiments gave essentially the same results. Panel B: Oocytes in groups of 10 were microinjected with the SH2 domain of p85 (PI3K) (+) or vehicle (−). After 30 min, insulin (7.5 μM) was added or 30 ng of PKCδ C1b was injected, and GVBD was monitored as previously described. Results were expressed as percentage of the maximum effect observed with insulin or with the PKCδ C1b domain. A second experiment gave similar results.

4. Discussion

Our experiments have clearly shown that the PKCδ C1b domain induces meiotic maturation in *X. laevis* oocytes. This is relevant because it suggests that regions within the regulatory domain of PKCs could trigger biological responses. Most of the PKC responses have been attributed to phosphorylation events mediated by the C-terminal kinase domain, whereas the main role of the regulatory domain in PKCs is to keep the catalytic domain of the enzyme in a 'close' inactive state. Upon binding of phorbol esters or DAG to the C1 domain, PKC becomes active. In this active state, PKC binds a series of proteins through the regulatory domain which are probably critical for determining the intracellular localization of the activated enzyme [4,17,18].

Despite the critical role of the kinase domain in catalysis, several reports have shown that PKC-mediated responses could be attributed to the PKC regulatory domain. For example, expression of the regulatory domain of PKCδ induces remarkable changes in cell growth and affects malignant transformation in mammary cancer cells [25,26]. The regulatory domains of PKCα and PKCε can regulate phospholipase D activity [28,44]. It was also reported that overexpression of the C1 domains of PKCε is sufficient to inhibit Golgi-specific sulfation reactions [45]. In another interesting study, Zeidman et al. [27] have shown that the C1 domains of PKCε induce neurite-like processes in neuroblastoma cells, an effect that is independent of the catalytic domain. Furthermore, the C1a and C1b domains in PKCε seem to have distinct roles in this process, suggesting unique interaction properties and functional roles for each C1 domain. Several reports have found that C1 domains of PKCs bind specifically to proteins [20,22,23]. Functional roles have also been demonstrated for C1 domains of phorbol ester unresponsive proteins. For example, the C1 domain of Raf plays an essential role in the interaction with Ras and in the regulation of the Ras cascade [46]. Likewise, the C1 domain of phorbol ester unresponsive atypical PKCs interacts with proteins that regulate their activation [19]. Taken together, these results revealed a very complex functional role for the C1 domains besides their well-known role in lipid interactions and phorbol ester/DAG binding.

We found that microinjection of the PKCδ C1b domain into oocytes potentiates insulin and Ras-mediated maturation, but not progesterone-induced maturation. In contrast, Pawelczyk et al. have reported that an isolated C1b domain of PKCγ inhibits oocyte maturation after insulin (but not progesterone) stimulation [21]. It is interesting that despite the high degree of homology between the C1b domains of PKCγ and PKCδ, both exert opposite responses in the same model. This is not surprising, however, considering the differential properties of individual C1 domains as phorbol ester receptors and in protein–protein interaction. Indeed, C1 domains could be phorbol ester/DAG unresponsive (e.g. those in atypical PKCs, c-Raf or Vav) or even bind phorbol ester analogs with unique patterns of recognition [2,4,13,14,16]. Striking differences in ligand binding properties have also been reported for C1a and C1b domains of PKC isozymes, including the C1 domains of PKCγ [16,47–49]. The differential roles of C1 domains have also been highlighted in cell models, as reported in experiments showing that disruption of either C1a or C1b domain of a single PKC isozyme distinctly affects translocation, thereby suggesting that each C1 domain has unique membrane interaction properties [15,16]. In some cases, such as in PKCε or the novel phorbol ester receptor β2-chimaerin, C1 domains are critical for determining subcellular compartmentalization after phorbol ester/DAG activation [45,50]. These conclusions are also supported by our experiments showing that a mutant PKCδ C1b domain with reduced affinity for phorbol esters (W252G-PKCδ) is less effective at inducing oocyte maturation. The low phorbol ester affinity of this mutant is due to a reduced ability to associate to membranes (M.G. Kazanietz and P.M. Blumberg, unpublished data), as also reported for mutants in hydrophobic amino acids in the PKCα C1 domain [51]. Therefore, we can speculate that association to membranes is probably required for the C1 domain to exert its effect in oocytes.

It is interesting that the PKCδ C1b domain acts selectively on the insulin pathway but not on the progesterone pathway in *Xenopus* oocytes, as also reported with the PKCγ C1b domain. Moreover, the PKCδ C1b domain markedly synergizes with Ras to promote meiotic maturation. These experiments not only rule out the possibility of a non-specific effect of the PKCδ C1b domain after microinjection, but also establish its specificity for the insulin-Ras pathway. Remarkably, the SH2 domain of p85 (PI3K) does not inhibit the effect of the PKCδ

C1b domain, despite its strong inhibition of the insulin response. The SH2 domain of p85 binds to insulin receptor substrates [36], which suggests that the effect of the PKCδ C1b domains occurs further downstream. Although the experiments presented in this paper do not fully elucidate the mechanisms by which the PKCδ C1b domain induces oocyte maturation, one can envision several hypothetical scenarios that may explain its effect. One possibility is that the C1 domain is involved in a protein–protein interaction that results in the activation of the Ras cascade, as described for the Raf–Ras interaction. Importantly, several reports have revealed a functional interaction between Ras and PKC pathways for oocyte maturation as well as cooperation of Ras and PKC for malignant transformation [42,52,53]. Second, it may also be possible that expression of the PKCδ C1b domain disrupts an intramolecular association in PKC leading to its activation, including the potential for disrupting PKC homodimer formation [54]. A third possibility is that the PKCδ C1b domain affects PKC signaling by regulating DAG levels in membranes. Although further studies will be ultimately necessary to determine the precise mechanism of action of the PKCδ C1b domain in oocyte maturation, this study underscores a potential role for motifs in the PKC regulatory domains as regulators of signaling events, and highlights the concept that C1 regions can be effector domains that confer selectivity in PKC action.

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