Determination of the calcium-binding sites of the C2 domain of protein kinase Cα that are critical for its translocation to the plasma membrane

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The C2 domain is a conserved protein module present in various signal-transducing proteins. To investigate the function of the C2 domain of protein kinase Cα (PKCα), we have generated a recombinant glutathione S-transferase-fused C2 domain from rat PKCα, PKC-C2. We found that PKC-C2 binds with high affinity (half-maximal binding at 0.6 μM) to lipid vesicles containing the negatively charged phospholipid phosphatidylserine. When expressed into COS and HeLa cells, most of the PKC-C2 was found at the plasma membrane, whereas when the cells were depleted of Ca^{2+} by incubation with EGTA and ionophore, the C2 domain was localized preferentially in the cytosol. Ca^{2+} titration was performed *in vivo* and the critical Ca^{2+} concentration ranged from 0.1 to 0.32 μM. We also identified, by site-directed mutagenesis, three aspartic residues critical for that Ca^{2+} interaction, namely Asp-187, Asp-246 and Asp-248. Mutation of these residues to asparagine, to abolish their negative charge, resulted in a domain expressed as the same extension as wild-type protein that could interact *in vitro* with neither Ca^{2+} nor phosphatidylserine. Overexpression of these mutants into COS and HeLa cells also showed that they cannot localize at the plasma membrane, as demonstrated by immunofluorescence staining and subcellular fractionation. These results suggest that the Ca^{2+}-binding site might be involved in promoting the interaction of the C2 domain of PKCα with the plasma membrane *in vivo*.

Key words: calcium signalling, phosphatidylserine, signal transduction.

INTRODUCTION

C2 domains are sequence motifs that were originally identified as the second of four conserved domains in the α, β and γ isoforms of mammalian Ca^{2+}-dependent protein kinase C (PKC) [1–5]. They contain approx. 130 residues and are present in a large variety of proteins besides PKC involved in intracellular signalling. This variety includes proteins involved in regulating vesicle transport (such as synaptotagmin, rabphilin-3A or UNC-13), proteins implicated in lipid modification, e.g. cytosolic phospholipase A2, Cγ, Cβ, Cδ and phosphoinositide 3-kinase, and several GTPase-activating proteins, among others (reviewed in [6]).

By analogy with other domains, it has been postulated that C2 domains are structural modules involved in mediating intermolecular interactions. It has been demonstrated that the C2 domain can bind to different ligands including Ca^{2+}, phospholipids, inositol phosphates and intracellular proteins [7–12]. However, the physiological role of C2 domains in proteins that contain them is largely unknown.

The structures of several C2 domains have been determined by X-ray crystallography and NMR [10,13–15]. C2 domains form two distinct topological folds named I and II. Each structure forms an eight-stranded anti-parallel β-sandwich consisting of a pair of four-stranded β-sheets. The key difference between the two topologies is in their β-strand connectivity: the first strand of topology I occupies the same structural position as the eighth β-strand of topology II, which shifts the order of homologous strands in the primary structure. Although these structural studies have provided excellent information, the mechanism by which Ca^{2+} binding increases the domain's affinity for anionic lipids is still unknown.

As mentioned above, PKC is a family of related protein kinases, which includes at least 11 mammalian isoforms and three yeast enzymes. Basically they contain four conserved domains, C1–C4 [1], and can be classified into three groups on the basis of their structure and their regulation by cofactors. The first group includes the classical isoforms (α, β and γ), which can be distinguished from the other groups because their function is regulated by Ca^{2+} and diacylglycerol. Its C2 domain contains a putative Ca^{2+}-binding site and is predicted to contain the type I topology. The second group corresponds to the novel mammalian (δ, ε, η and θ) and the yeast PKCs, which are structurally similar to the classical isoforms except that their function is not regulated by Ca^{2+} and the C2 domain bears the type II topology. The third group comprises the atypical PKC isoforms (ζ, λ and μ); they are regulated neither by diacylglycerol nor by Ca^{2+}, and lack the C2 domain [6,16].

So far, very little is known about the physiological role of the C2 domain in the different PKC isoforms. It has been shown that the C2 domain of PKC βII exhibits Ca^{2+}-induced binding to mixed vesicles containing phosphatidylcholine and phosphatidylserine with a half-maximal binding calculated for Ca^{2+} at 4.2 μM [14]. Recently, Edwards and Newton [17] have demonstrated that the mutation of two aspartic residues (probably involved in the Ca^{2+}-binding site) to arginines changes the Ca^{2+}-dependent phosphatidylserine binding to PKC βII, although the affinity of the enzyme for Ca^{2+} was not directly measured. Thus

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GST, glutathione S-transferase; HA, haemagglutinin; MEM, modified Eagle's medium; PKC, protein kinase C.

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this mutation did not change the affinity of the enzyme for membranes in the absence of Ca^{2+}. Furthermore the authors showed that mutation of the highly basic β-sheet behind the Ca^{2+}-binding site had no effect on the interaction of PKC with Ca^{2+} or membranes. In connection with the role of the C2 domain in novel isoforms, Dekker and Parker [11] found that the GTPase-activating protein GAP-43 interacts directly with PKCδ at the Vo/C2 region in the presence of Ca^{2+}. All these experiments support the idea that Ca^{2+} is regulating the function of the C2 domain in different PKC isoenzymes, although there is no information about what residues in this domain are implicated in this interaction.

In the present study we examined the function of the C2 domain of PKCα. We report that the isolated C2 domain binds to acidic phospholipids with high affinity in a Ca^{2+}-dependent manner. We also demonstrate that in the presence of cytosolic Ca^{2+} the domain is translocated to the plasma membrane of different cell lines (COS1 and HeLa cells). Site-directed mutagenesis in three aspartic residues located in a putative Ca^{2+}-binding site has demonstrated that neutralization of the charge in this domain abolished both Ca^{2+} and lipid binding. Experiments *in vivo* showed that these mutants could not localize at the plasma membrane. This suggests that these residues are directly involved in the interaction of the domain with Ca^{2+} and have an important role translocating the protein to the lipid surface.

EXPERIMENTAL

Construction of expression plasmids

The DNA fragment corresponding to the C2 domain of PKCα (residues 158–285) was amplified by using PCR with oligonucleotides 5PS and 3PS (CAAGAATTCAAGAGGGGGCGGATTTAC and CAAAAGCTTGTATTCACCCTCCTCTTG respectively). PKCα cDNA was a gift from Dr. Y. Nishizuka and Dr. Y. Ono (Kobe University, Kobe, Japan). The resulting 381 bp PCR fragment was subcloned into the *Eco*RI and *Hin*dIII sites of the bacterial expression vector pGEX-KG, in which the inserts were fused to glutathione S-transferase (GST) [18]. Mutations in the C2 domain, replacing aspartic residues at positions 187 and 246–248 with asparagines, were generated by PCR mutagenesis [19]. The resulting DNA fragments encoding the two mutants were subcloned into the same expression vector. All constructs were confirmed by DNA sequencing. For studies *in vivo*, all constructs, both wild-type and mutant domains, were subcloned into pCGN vector [a gift from Dr. M. Tanaka (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY)]. This vector contains the cytomegalovirus promoter and multicloning sites that allow the expression of genes fused 3′ to the haemagglutinin (HA) epitope [20].

Expression and purification of the GST–PKC-C2

The pGEX-KG plasmid containing wild-type or mutant PKC-C2 domains was transformed into HB101 *Escherichia coli* cells. The bacterial cultures (at a D_{600} of 0.6) were induced with 0.2 mM isopropyl β-D-thiogalactoside (Boehringer Mannheim, Mannheim, Germany) for 5 h at 30 °C. The cells were lysed by sonication in PBS containing protease inhibitors (10 mM benzamidine, 1 mM PMSF and 10 μg/ml trypsin inhibitor). The soluble fraction of the lysate was incubated with glutathione–Sepharose beads (Pharmacia Biotech) for 30 min at 4 °C. The beads were washed with PBS and the proteins were eluted with 15 mM glutathione in 50 mM Tris, pH 8.0. The eluates were desalted and concentrated with a Centricon-30 centrifugal filter unit. Protein concentration was determined with the method described by Lowry et al. [21].

Preparation of phospholipids

Lipid vesicles were generated by mixing chloroform solutions of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (phosphatidylcholine) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (phosphatidylserine) (Avanti Polar Lipids) at the desired composition and dried from the organic solvent under a stream of nitrogen and then dried further under vacuum for 60 min. 1,2-Dipalmitoyl-L-3-phosphatidyl-*N*-methyl[^{3}H]choline (Dupont; specific radioactivity 56 Ci/mmol) was included in the lipid mixture as a tracer, at approx. 3000–6000 c.p.m./μg of phospholipid. Dried phospholipids were resuspended in buffer containing 50 mM Hepes, pH 7.2, 0.1 M NaCl and 0.5 mM EGTA by vigorous vortex-mixing and subjected to direct probe sonication (three cycles of 30 s each). The suspension was centrifuged for 20 min at 14000 ***g*** to remove aggregated material. Other lipids used were 1,2-dioleoyl-*sn*-glycerol (diacylglycerol), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine(phosphatidylethanolamine) and 1,2-dioleoyl-*sn*-glycero-3-phosphate (phosphatidic acid).

Phospholipid binding measurements

A standard assay [7] contained 20 μg of PKC-C2 bound to glutathione–Sepharose beads. Beads were prewashed with the respective test solutions and resuspended in 0.1 ml of buffer containing 50 mM Hepes, pH 7.2, 0.1 M NaCl, 0.5 mM EGTA and 20 μg of the corresponding lipids. The mixture was incubated at room temperature for 15 min with vigorous shaking, then centrifuged briefly in a tabletop centrifuge. The beads were washed three times with 1 ml of the incubation buffer without liposomes. Liposome binding was then quantified by liquid-scintillation counting of the beads. Free Ca^{2+} concentration was estimated from total Ca^{2+} and EGTA concentrations by using computer software developed by Fabiato [22]. Contaminating Ca^{2+} in the buffer solution was determined by using a dual-wavelength spectrophotometer (Bio-Logic Co., Claix, France) as described by Dani et al. [23]. The selected wavelength pair was 660.4 and 680.1 nm, with the metallochromic indicator Arsenazo III. A standard Ca^{2+} solution was used for calibration. The contaminating Ca^{2+} concentration was determined as 5 μM; it was taken into account in calculating the final free Ca^{2+} concentration.

Ca^{2+} binding assay

$^{45}Ca^{2+}$ was measured with an overlay assay, as described by Mahadevan et al. [24]. Basically, the C2 domain (5 μg) was bound to nitrocellulose paper in a Bio-Dot SF microfiltration apparatus (Bio-Rad). The nitrocellulose filter was washed twice with overlay buffer [10 mM imidazole (pH 8.0)/100 mM NaCl] containing 10 mM EDTA to remove any protein-bound bivalent cations. The filter was then washed four times with overlay buffer to remove the EDTA. The $^{45}Ca^{2+}$ overlay was performed by incubating the filters for 30 min at room temperature in overlay buffer containing 0.40 μCi/ml $^{45}Ca^{2+}$ (Amersham) in the presence of unlabelled $CaCl_2$ (10 and 100 μM) and lipid vesicles containing 25% (mol/mol) phosphatidylserine. BSA (5 μg) was bound to the nitrocellulose membrane and used as a background control to subtract the non-specific $^{45}Ca^{2+}$ binding to the lipid vesicles. The filter was rinsed rapidly with 50% (v/v) ethanol in water, then air-dried and exposed to X-ray film for 24 h. For quantitative analysis, the filters were analysed by densitometry.

Cell culture and transfection

COS1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal calf serum (FCS). HeLa cells were maintained in Modified Eagle's medium (MEM) supplemented with 10% (v/v) FCS. Transfection was performed with calcium phosphate, as described by Wigler et al. [25]. In brief, DNA was diluted in Tris/EDTA buffer containing 125 mM $CaCl_2$. The mixture was then diluted in Hepes/Na_2HPO_4 buffer and left at room temperature for 30 min before being added to the culture medium. At 12 h after transfection, media were aspirated and washed three times with PBS. Afterwards, COS1 cells cultures were fed with DMEM supplemented with 5% (v/v) FCS, and HeLa cell cultures were fed with MEM supplemented with 10% (v/v) FCS; 2 μg of each cDNA was used for transfection.

Indirect immunofluorescence

Cells were fixed with 3.7% (v/v) formaldehyde in PBS for 1 h at room temperature, or overnight at 4 °C, and then permeabilized with 0.1% (v/v) Triton X-100 in PBS for 3 min at room temperature. The coverslips were incubated for 1 h at 37 °C with the 12CA5 primary antibody (anti-HA) from Babco, diluted in 1% (w/v) BSA in PBS, followed by a 1 h incubation with the secondary antibody [affinity-purified fluorescein-conjugated goat anti-(mouse IgG)]. The stained cells were observed and photographed with a Leitz fluorescence microscope.

Cell lysis and Western blot analysis

Cells were resuspended in 50 mM Tris, pH 7.4, containing 10 mM NaCl, 1 mM dithiothreitol, 1 mM Na_3VO_4, 50 mM NaF, 1 mM PMSF, 10 μg/ml soybean trypsin inhibitor and 10 mM benzamidine. They were lysed by sonication. The cytosolic fraction was the supernatant after 1 h of centrifugation at 100000 ***g*** at 4 °C. The Triton X-100-soluble particulate fraction was prepared by extraction of the pellet with the same buffer containing 1% (v/v) Triton X-100 and a subsequent centrifugation for 1 h at 100000 ***g***. The remaining pellet was the Triton X-100-insoluble fraction. The protein samples were subjected to SDS/PAGE [15% (w/v) gel] by the method of Laemmli [26] and transferred to nitrocellulose membranes. Immunoblot analysis of the epitope-tagged transiently expressed proteins was performed with anti-HA antibody (12CA5) by using enhanced chemiluminescence reagents.

RESULTS

PKC-C2 binds phospholipids in a phosphatidylserine/Ca^{2+}-dependent manner

To study the function of the C2 domain of PKCα (PKC-C2), we generated a recombinant fusion protein in which a fragment encompassing residues 158–285 of PKCα was N-terminally fused to GST. By using glutathione–Sepharose beads, PKC-C2 was purified to homogeneity (Figure 1A, lane 3). Our first aim was the characterization *in vitro* of the biochemical properties of this domain. We studied the dependence of phospholipid binding on the Ca^{2+} concentration by using a panel of Ca^{2+}/EGTA buffers (Figure 1B) and phospholipid vesicles containing phosphatidylcholine and phosphatidylserine (3:1, mol/mol). An optimal fit was obtained for a co-operative model with the Hill equation. In three independent experiments with different preparations of buffers and PKC-C2, half-maximal binding was observed at 0.6 μM Ca^{2+} with a Hill coefficient of 4.5. Thus Ca^{2+} binding to

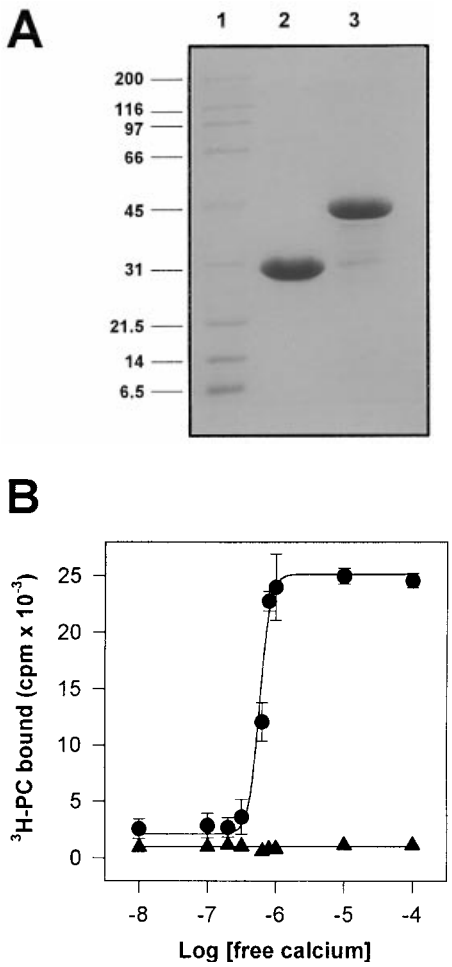

Figure 1 Purification of PKC-C2 and lipid binding assay

(**A**) Purification of PKC-C2. SDS/PAGE [15% (w/v) gel] of molecular mass markers containing: lane 1, myosin, β-galactosidase, phosphorylase *b*, BSA, ovalbumin, carbonic anhydrase, trypsin inhibitor, lysozyme and aprotinin (200, 116, 97, 66, 45, 31, 21.5, 14 and 6.5 kDa respectively; molecular masses are indicated at the left); lane 2, GST control; lane 3, PKC-C2 purified by glutathione transferase affinity chromatography. (**B**) Ca^{2+} concentration dependence of phospholipid binding to the C2 domain of PKCα. Binding of ^{3}H-labelled PS/PC liposomes to GST (▲) and to PKC-C2 (●) was studied as a function of the Ca^{2+} concentration, with the free Ca^{2+} concentration stabilized by Ca^{2+}/EGTA buffers as described in the Experimental section. Error bars indicate the S.E.M. for triplicate determinations.

Table 1 Phosphatidylserine-dependent binding of PKC-C2

The binding of PKC-C2 to small unilamellar vesicles containing phosphatidylcholine and an increasing mole percentage of phosphatidylserine was measured in the presence of 100 μM free Ca^{2+}. Binding was monitored with ^{3}H-labelled phosphatidylcholine. Each experiment was performed in triplicate and the results given are the averages for three independent experiments.

	[^{3}H]Phosphatidylcholine bound (c.p.m.)	
[Phosphatidylserine] (%)	PKC-C2	GST
0	544 ± 59	216 ± 48
10	3504 ± 138	814 ± 113
20	10704 ± 661	1262 ± 540
30	9246 ± 396	1500 ± 280
40	9140 ± 751	1234 ± 613

Table 2 Phospholipid-dependent binding of PKC-C2

The binding capacity of the PKC-C2 domain was measured as described previously, using 70% phospatidylcholine (PC) and 30% different lipids (mol/mol): phospatidylserine (PS), phosphatidic acid (PA), phosphatidylethanolamine (PE) and diacylglycerol (DAG). Binding was monitored by using ^{3}H-labelled PC. Every experiment was performed in triplicate and the results are the averages for three independent experiments.

	[^{3}H]Phosphatidylcholine bound (c.p.m.)	
Phospholipid	PKC-C2	GST
PC	1500 ± 244	952 ± 122
PS	9224 ± 685	1164 ± 69
PA	11 200 ± 700	1648 ± 312
PE	1630 ± 130	868 ± 130
DAG	1217 ± 230	960 ± 120

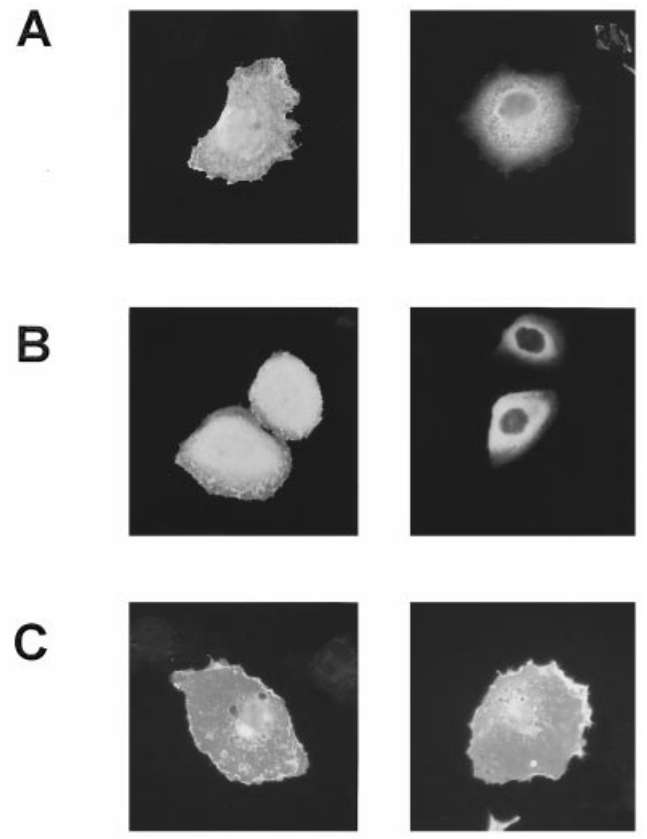

Figure 2 Subcellular localization of PKC-C2 and HRas

Cells were transiently transfected with PKC-C2 into COS1 (**A**) and HeLa cells (**B**). Afterwards they were incubated in DMEM supplemented with 5% (v/v) FCS and MEM supplemented with 10% (v/v) FCS respectively. After 24 h, cells were fixed directly [(**A**) and (**B**), left panels] or they were incubated for 1 h in a Ca^{2+}-free Dulbecco's PBS medium supplemented by 20 mM Hepes, pH 7.3; 2 min before fixing, 5 mM EGTA and 3 μM A23187 were added [(**A**) and (**B**), right panels]. The distribution of PKC-C2 was determined by indirect immunofluorescence staining with anti-HA primary antibody and fluorescein-conjugated goat anti-(mouse IgG) secondary antibody. (**C**) COS1 cells were transfected with a plasmid containing HA-HRas. Afterwards they were incubated for 24 h in DMEM containing 5% (v/v) FCS; before being fixed they were treated as described above. Shown are the distributions of HRas protein by indirect immunofluorescence staining with anti-HA primary antibody of cells incubated in DMEM containing 5% (v/v) FCS (left panel) or treated with 5 mM EGTA/3 μM A23187 (right panel).

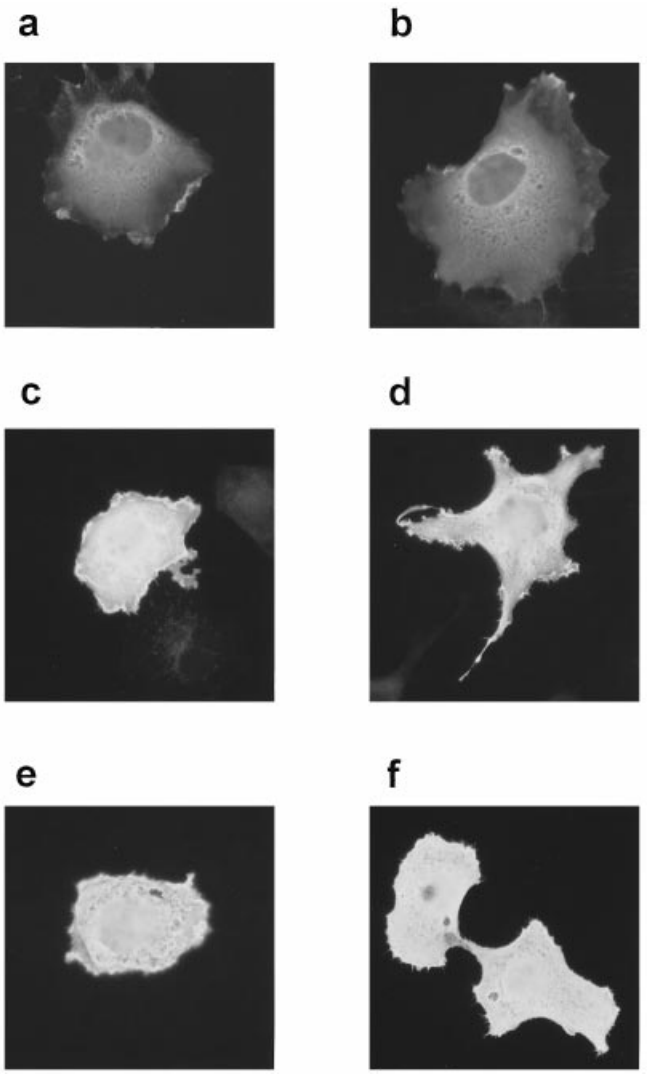

Figure 3 Ca^{2+} titration *in vivo* with EGTA as a clamper

COS1 cells were transfected with a plasmid containing PKC-C2; they were then incubated for 24 h in medium containing DMEM and 5% (v/v) FCS. They were incubated for 2 min in a medium containing Ca^{2+}-free DPBS medium supplemented by 20 mM Hepes (pH 7.3)/1 mM EGTA/3 μM A23187. Different concentrations of total Ca^{2+} were added to obtain the following free μM Ca^{2+} concentrations: 0.01 (**a**), 0.1 (**b**), 0.32 (**c**), 1 (**d**), 10 (**e**) and 100 (**f**). The distribution of PKC-C2 was determined by indirect immunofluorescence staining with anti-HA primary antibody and fluorescein-conjugated goat anti-(mouse IgG) secondary antibody.

this PKC-C2 domain is positively co-operative, suggesting more than one binding site. As a control, Ca^{2+}-dependent phospholipid binding to GST alone was performed under the same conditions as above: no phospholipid binding was found (Figure 1B).

We were also interested in examining the dependence of PKC-C2 binding on phosphatidylserine. Table 1 shows the binding of PKC-C2 to phospholipid vesicles containing phosphatidylcholine and different proportions of phosphatidylserine (0–40%) in the presence of saturating Ca^{2+} concentration (100 μM). The binding results showed that 20% phosphatidylserine was required for maximal binding activity.

To test the specificity of phospholipid binding by the single PKC-C2 domain, the Ca^{2+}-dependent binding of liposomes containing different phospholipid compositions was investigated (Table 2). These experiments revealed that liposomes containing

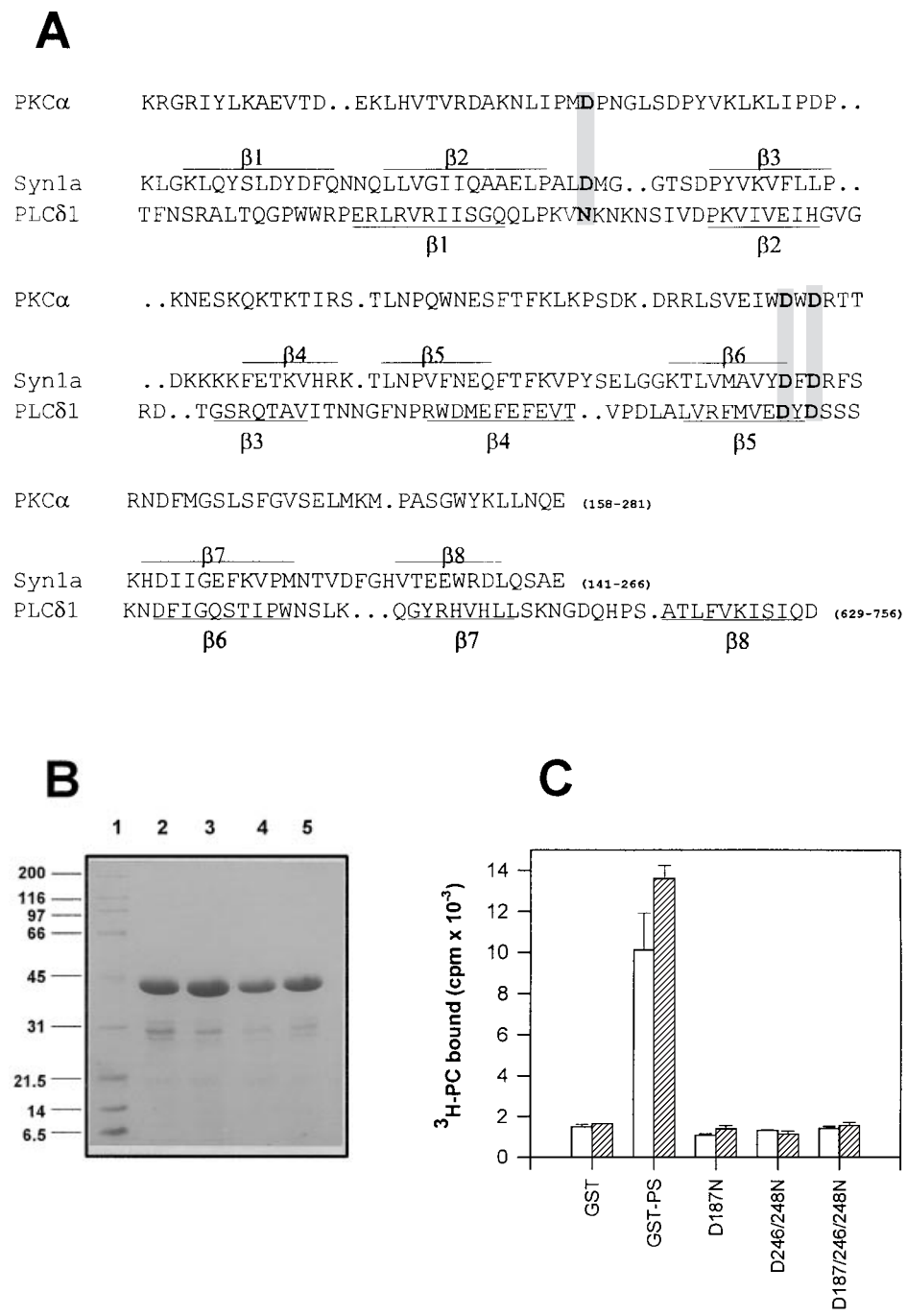

Figure 4 Map of the Ca^{2+}-binding sites

(**A**) Sequence alignment and secondary structure comparison of PKC-C2 with two C2 domains with known structures. Synaptotagmin I is representative of the type I conformation and PLCδ is representative of the type II conformation. Conserved elements of secondary structure (β-sheets) are represented by a line located on the primary sequence for synaptotagmin I or under the primary sequence for PLCδ. The positions of mutations in PKC-C2 and their counterparts are labelled with shadowed patterns. (**B**) SDS/PAGE analysis of purified wild-type PKC-C2 (lane 2), D187N (lane 3), D246/248N (lane 4), and D187/246/248N (lane 5). All of them were fused to GST. Lane 1 was loaded with molecular mass markers whose molecular masses are indicated (in kDa) at the left. (**C**) Lipid binding assay was performed as described in the Experimental section. Basically, wild-type PKC-C2 and the D187N, D246/248N and D187/246/248N mutants were assayed for binding to vesicles containing phosphatidylcholine and phosphatidylserine (3:1, mol/mol) in the presence of 10 μM $CaCl_2$ (open columns) or 100 μM $CaCl_2$ (hatched columns).

70% phosphatidylcholine in combination with 30% phosphatidylserine or 30% phosphatidic acid bound to the isolated PKC-C2 domain at the same extension and maximal binding activity in the presence of 100 μM $CaCl_2$. In contrast, liposomes containing phosphatidylcholine alone or phosphatidylcholine in combination with 30% phosphatidylethanolamine or 30% diacylglycerol showed no significant binding to that domain. These results demonstrate that, as with full-length PKCα [27,28], in the absence of diacylglycerol, the negative charge of the phospholipid is the principal factor in determining the selectivity of PKC-C2.

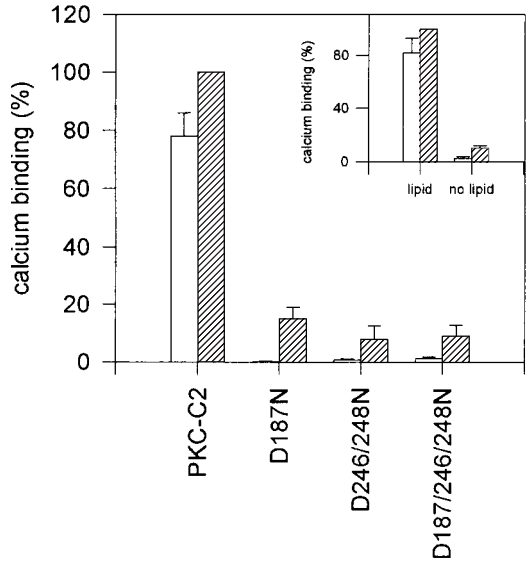

Figure 5 Ca^{2+} binding overlay assay

PKC-C2, D187N, D246/248N, D187/246/248N and BSA (5 μg of each protein) were immobilized on nitrocellulose membrane in a Bio-Dot SF microfiltration apparatus. The $^{45}Ca^{2+}$ overlay was performed by incubating the filters for 30 min at room temperature at 10 μM $CaCl_2$ (open columns) or 100 μM $CaCl_2$ (hatched columns) in the presence of lipid vesicles containing phosphatidylcholine and phosphatidylserine (3:1, mol/mol). BSA was used as a background control for the $^{45}Ca^{2+}$ bound to the lipids; these results were subtracted from those obtained in the presence of PKC-C2 and each mutant. The inset shows $^{45}Ca^{2+}$ binding to PKC-C2 in the presence and the absence of lipid vesicles. Quantitative analysis was performed by densitometry. Results are expressed as percentages of the maximum binding at 100 μM $CaCl_2$. Error bars indicate the S.E.M. for determinations in triplicate. The experiments shown are representative of three independent experiments.

PKC-C2 is localized at the plasma membrane

Although the C2 domain of PKC is believed to be involved in its Ca^{2+}-dependent phospholipid-binding properties [16,29,30], it is not clear whether Ca^{2+} binding is sufficient to determine the functionality of this domain. To study this, we cloned PKC-C2 into a mammalian expression vector (pCGN) containing an HA epitope at the N-terminus. When transfected into COS1 cells, the expression plasmid gave rise to a polypeptide of approx. 17 kDa as determined by immunoblotting with anti-HA antibody (see Figure 6A, lane 2). To investigate the subcellular localization of PKC-C2, both COS1 and HeLa cells were transiently transfected and protein expression was detected by immunofluorescence with anti-HA antibody. The staining showed clearly that PKC-C2 was associated with the plasma membrane (Figures 2A and 2B, left panels) in both cell lines in which immunofluorescence was detected overlaying the cell surface and thus impeded a clear appearance of the shape of the nucleus. As a control for this, we performed transfection with HRas, which is a classical anchored-membrane protein [31] that does not depend on Ca^{2+} for its localization. COS1 cells were transiently transfected with an HA-tagged vector containing HRas; the cells were then treated under the same conditions as above and finally protein expression was detected by immunofluorescence by using an anti-HA antibody. In Figure 2(C) (left panel) the immunofluorescence pattern is very similar to that displayed by COS1 cells transfected with PKC-C2. It appears as a fluorescent layer covering the whole cell and increased fluorescence at certain levels that can be attributed to the assembly of actin filaments at the cell periphery, which implies a membrane folding that produces an intensified fluorescence signal and consequently a spiky staining.

We also wished to study whether this localization depended on the cytosolic Ca^{2+} concentration. An efficient way of depleting intracellular Ca^{2+} is by adding ionophore in the presence of EGTA in the culture media [32,33]. Cells were transfected with PKC-C2 plasmid; they were then incubated for 1 h with Ca^{2+}-free media and for 2 min with 3 μM ionophore A23187 and 5 mM EGTA before being fixed. The immunofluorescence image shown in Figures 2(A) and 2(B) (right panels) revealed a general cytoplasmic localization of the expressed protein in COS1 and HeLa cells. As a control for Ca^{2+} dependence, we also transfected HA-HRas into COS1 cells and treated them under the same conditions as above before they were fixed. Figure 2(C) (right panel) shows that this protein localized at the plasma membrane independently of Ca^{2+} concentration and EGTA treatment. In contrast, the location of PKC-C2 depended on the Ca^{2+} concentration.

The number of transfected cells in which the expressed domain (PKC-C2) showed a preference to localize at the membrane in the presence of intracellular Ca^{2+} was 83% and 85% for COS1 and HeLa cells respectively. In contrast, when intracellular Ca^{2+} was depleted, only 19% and 15% of transfected COS1 and HeLa cells showed membrane localization. Taken together, these results suggest that cytosolic Ca^{2+} is involved in translocating PKC-C2 to the plasma membrane *in vivo*, but these experiments do not determine the intracellular Ca^{2+} concentration that is critical for localization of the domain at the plasma membrane. To answer this question we used an ionophore/EGTA/Ca^{2+} buffer system to control the intracellular Ca^{2+} concentration. COS1 cells were transfected with a plasmid containing PKC-C2; before being fixed they were incubated for 2 min in the buffer system described above at the following free Ca^{2+} concentrations: 0.01, 0.1, 0.32, 1, 10 and 100 μM (Figures 3a, 3b, 3c, 3d, 3e and 3f respectively). Immunofluorescence pictures demonstrated that the critical intracellular Ca^{2+} concentration was between 0.1 μM (Figure 3b) and 0.32 μM (Figure 3c), where the domain translocated from the cytosol to the membrane.

Mapping of the Ca^{2+}-binding sites critical for the membrane association of PKC-C2

To investigate whether the Ca^{2+}/lipid-binding activity of PKC-C2 has a role in its membrane association, we generated several mutants of this domain in which aspartic residues were mutated to asparagines, to neutralize their negative charges. The first mutation affected Asp-187 (D187N), the second affected simultaneously Asp-246 and Asp-248 (D246/248N) and the third consisted of a triple mutation of Asp-187, Asp-246 and Asp-248 (D187/246/248N) (Figure 4A). On the basis of the alignment with another C2 domain of known structure, i.e. that of synaptotagmin I, these negatively charged residues are predicted to be located in a concave depression formed at the edge of the β-sandwich between the β2–β3 and β6–β7 loops [14].

Mutants were generated by PCR-directed mutagenesis and fused to GST. The Coomassie Blue staining in Figure 4(B) shows the three mutants after their purification (lanes 3, 4 and 5). They were expressed in *E. coli* at comparable levels to that of PKC-C2 (lane 2). A good indirect signal to correlate with non-conformational change is that the mutants were as soluble as the wild-type domain when they were expressed in bacterial system. To determine whether the mutation of the PKC-C2 domain had any effect on its interaction with phospholipid vesicles in the presence of Ca^{2+}, we performed a lipid-binding assay at free Ca^{2+} concentrations of 10 and 100 μM. It is apparent that the D187N,

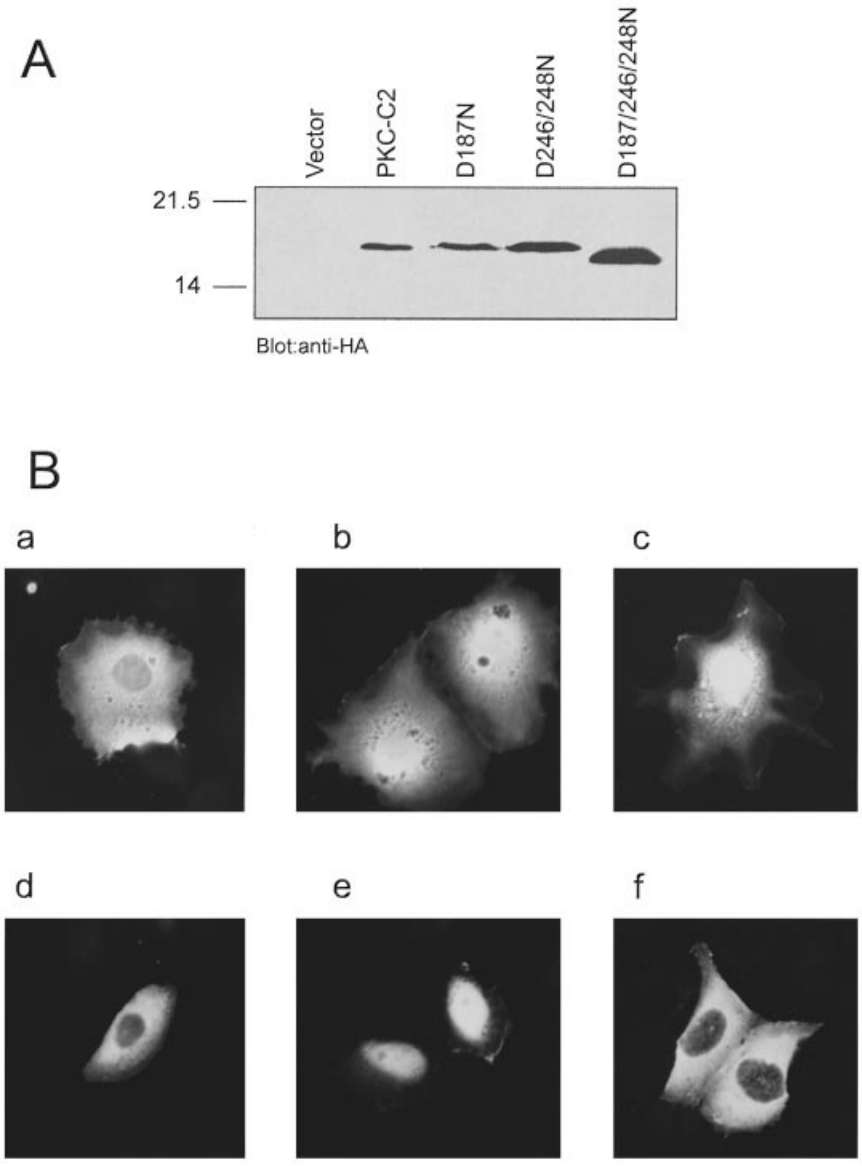

Figure 6 Expression and localization of PKC-C2 and mutants

(**A**) Expression of wild-type PKC-C2 and the D187N, D246/248N and D187/246/248N mutants. COS1 cells were transfected with expression vectors encoding HA-tagged PKC-C2 constructs. Cell lysates were separated by SDS/PAGE [15% (w/v) gel] and subsequently analysed by immunoblotting with anti-HA antibody and developed by enhanced chemiluminescence. The positions of marker proteins are indicated (in kDa) at the left. (**B**) Localization of the D187N, D246/248N and D187/246/248N mutants. COS1 cells (**a**–**c**) and HeLa cells (**d**–**f**) were transfected with the various mutants; the distribution of the expressed protein was examined by indirect immunofluorescence staining as described in the legend to Figure 2. Fluorescence images were examined on a DMRD-Leitz fluorescence microscope.

D246/248N and D187/246/248N mutants behaved in the same way, and none of them was able to bind to lipid vesicles containing 25% (mol/mol) phosphatidylserine at saturating Ca^{2+} concentration (Figure 4C).

Up to this point, we had demonstrated that mutations affected lipid binding but we could not be sure that this effect was due to Ca^{2+} binding. Thus we were also interested in studying the ability of these mutants to bind Ca^{2+}. The $^{45}Ca^{2+}$ overlay assay showed that PKC-C2 only bound $^{45}Ca^{2+}$ at 10 and 100 μM free Ca^{2+} in the presence of lipid vesicles containing 25% (mol/mol) phosphatidylserine (Figure 5, inset), suggesting that the affinity of the PKC-C2 domain for Ca^{2+} was increased in the presence of lipids. In contrast, the domains containing D187N, D246/248N and D187/246/248N mutations were unable to bind Ca^{2+} under these conditions (Figure 5). Our results suggest that the abolition of either the carboxylate group of Asp-187 or the carboxylate groups of Asp-246/248 produce a huge decrease in the affinity of the domain for Ca^{2+}. We cannot discard the possibility that the protein itself would enhance Ca^{2+}–lipid binding, but in any case the mutants are not able to produce this effect.

Ca^{2+}-binding sites are also critical for the binding of PKC-C2 to the cell membrane *in vivo*

To investigate the significance of these Ca^{2+}-binding sites in the localization of the PKC-C2 domain *in vivo*, we cloned D187N,

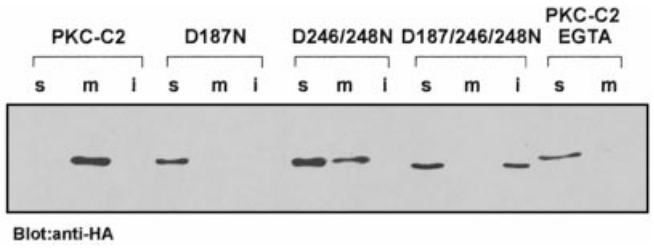

Figure 7 Subcellular distribution of wild-type PKC-C2 and the D187N, D246/248N and D187/246/248N mutants

The distribution of these domains, after transient transfection of COS1 cells, was analysed by Western blotting with anti-HA antibody. Lanes containing fractions corresponding to the cytosol are labelled s, those with extracts obtained from the particulate fraction with 1% (v/v) Triton X-100 are labelled m, and those containing insoluble pellets remaining after extraction with Triton X-100 are labelled i.

D246/248N and D187/246/248N mutants into a pCGN vector as described above for PKC-C2. COS1 cells were transiently transfected with plasmids encoding each one of the mutants. As shown in Figure 6(A), all of them were expressed approximately at the same level under the conditions adjusted. It is noteworthy that the D187/246/248N mutant was expressed at a higher level than the other constructs. It appears to have a lower molecular mass than wild-type PKC-C2 or the D187N or D246/248N mutants when the protein is separated by SDS/PAGE, which could have been due to a change in the net charge of the domain, going from two electropositive charges in PKC-C2 to five electropositive charges in D187/246/248N mutant. We observed the same behaviour when using the constructs expressed in bacteria after thrombin cleavage (results not shown).

In each case the expressed mutant proteins were diffusely distributed in the cytoplasm of both cell types used (COS1 and HeLa cells), indicating that these residues at the Ca^{2+}-binding site of the domain were necessary for their membrane localization (Figure 6B). It also shows that some of the mutants were localized at the nucleus, notably D246/248N expressed in COS and HeLa cells. D187/246/248N expressed in COS cells also showed a clear nuclear localization, but not when the mutant was expressed in HeLa cells.

It is difficult to evaluate the relative amounts of PKC-C2 present in the cytosol or bound to the membrane by using immunofluorescence analysis; the results are therefore mainly qualitative. Within this limitation we also studied its localization by subcellular fractionation of the cell lysates (Figure 7). For that purpose, COS1 cells were transfected with each construct; the post-nuclear supernatant was used to obtain particulate and cytosolic fractions. Afterwards, the particulate fraction was solubilized by extraction with 1% (v/v) Triton X-100, and membranes were found to be solubilized, although the cytoskeletal components remained insoluble. Figure 7 shows that PKC-C2 partitioned in the soluble membrane fraction (more than 98%); in contrast, the D187N mutant produced translocation of the C2 domain to the cytosol (more than 98%). The D246/248N mutant was present mainly in the cytosolic fraction (approx. 80%), although a small percentage (20%) was located at the membrane. The triple mutant (D187/246/248N) partitioned in the cytosolic (60%) and insoluble membrane fractions (approx. 40%). We also performed, as a control, a subcellular fractionation from COS1 cells transfected with PKC-C2 containing 10 mM EGTA in the lysis buffer. Most of the protein was located in the cytosolic fraction when Ca^{2+} was not present in the lysis buffer. These results also support that obtained by immunofluorescence, suggesting once more that the mutated residues are critical for the domain's localization.

DISCUSSION

In this study we have attempted to shed light on the biological role of the C2 domain of PKCα by generating a recombinant protein corresponding to the C2 domain fused to GST. Biochemically, the purified PKC-C2 displays characteristics that are similar to other C2 domains, such as that it binds with very high affinity to phosphatidylserine-containing vesicles in a Ca^{2+}-dependent manner. It is noteworthy that the interaction of PKC-C2 (half-maximal binding at 0.6 μM) is stronger than that seen in studies with other C2 domains such as those of synaptotagmin (5.8 μM), PKCβ (4.2 μM) or cPLA2 (3.1 μM). It is also very similar to the affinity described for full-length PKCα (0.4–0.7 μM) [7,14,17,33–36]. NMR studies have suggested a side-chain co-ordination model in which two Ca^{2+} ions bind to a C2 domain. Thus this surface contains a cluster of acidic residues that seem to mediate the binding to Ca^{2+} ions [14]. Although the three-dimensional structure of the C2 domain of PKC is not known, our finding that Ca^{2+}–membrane binding *in vitro* depends on acidic residues located at the presumptive negatively charged surface suggests that the folding of the C2 domain of PKCα might be very similar to that of other C2 domains. An example is shown in Figure 8, in which we have taken the model proposed by Shao et al. [14] and have replaced residues from synaptotagmin by their counterparts in PKCα, demonstrating that they might form a very similar crevice.

After characterization of the C2 domain *in vitro*, we studied its function *in vivo*. Our strategy was to transfect PKC-C2 into living cells and to determine its subcellular distribution by immunofluorescence, which showed that the domain localizes at the plasma membrane in the presence of cytosolic Ca^{2+}. The demonstration that the membrane localization of the C2 domain could be prevented by the pretreatment of cells with EGTA and Ca^{2+}-ionophore indicates that the interaction is regulated by Ca^{2+} in a manner consistent with that determined *in vitro*. We have established that the critical intracytosolic Ca^{2+} concentration lies in the range 0.1–0.32 μM (Figures 3b and 3c). It is noteworthy that this range of Ca^{2+} concentrations (0.1–0.3 μM) does not correlate well with that obtained in the experiments performed *in vitro* (Figure 1B), in which lipid binding was not

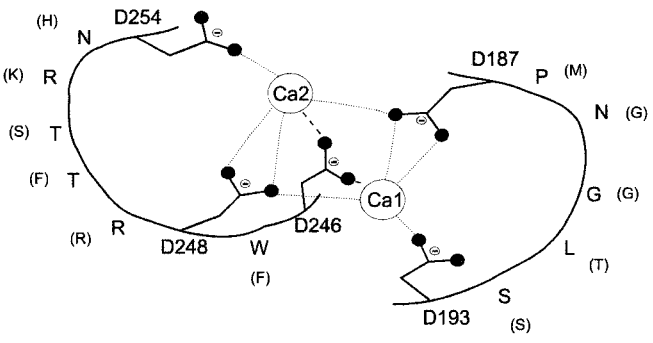

Figure 8 Model for Ca^{2+} co-ordination by the C2 motif

The model is based on that proposed by Shao et al. [14]. Aspartic residues mutated to asparagines in this study are located in grey rectangles. Amino acid residues have been replaced by their counterparts in PKCα. Amino acids shown in brackets correspond to those in synaptotagmin.

supported with the same Ca^{2+} concentration range. An explanation of this might be that in conditions *in vivo* other co-operative factors exist that could not be supplied *in vitro*. We have also found that mutations in the C2 domain that abolish Ca^{2+} and phospholipid binding *in vitro* interfere with the ability of the protein to target itself to the plasma membrane. Thus it seems that Ca^{2+} binding is necessary for the anchoring of PKC-C2 to the plasma membrane *in vivo* and that these aspartic residues are critical for this interaction.

It is interesting to note that these results for PKC-C2 are in contrast with the model proposed for full-length PKCα, which is localized in the cytosol and needs the co-operation of Ca^{2+} and diacylglycerol to translocate to the membrane [30]. This fact could be explained because in the PKC-C2 domain no other regulatory fragment of full-length protein is present (i.e. the C1 and catalytic domains). Consequently the folding of the full-length protein could impede the total exposure of the C2 domain to the cytosolic medium. Thus PKC would need a conformational change before activation that allows the C2 domain to interact with the plasma membrane.

With respect to the mechanism of Ca^{2+} regulation, several models can be put forward. Because phosphatidylserine bears a negative charge at physiological pH, it is tempting to think that charge alterations, after Ca^{2+} binding, mediate its interaction with the membrane. This has been suggested for the first C2 domain of synaptotagmin [12]. In this way Ca^{2+} activates an electrostatic switch to promote synaptotagmin–syntaxin interaction. However, this model did not account for the results obtained for PKC βII by Edwards and Newton [17]. They demonstrated that the mutation of two aspartic residues, residing in a basic surface at the Ca^{2+}-binding site, to arginines, with the aim of conferring an electropositive character on the Ca^{2+}-binding site, was not enough to promote the binding of PKC to anionic membranes in the absence of Ca^{2+}. Hence this model was not adequate for the C2 domain of this protein.

With the use of NMR, structural studies of the second C2 domain of synaptotagmin gave place to another model [14]. These three-dimensional studies revealed that two Ca^{2+} ions bind to this domain; they also showed an incomplete co-ordination of one of these ions, suggesting that sites for interaction with other molecules are available at this Ca^{2+} ion. These results permit a very attractive model implying that Ca^{2+} would serve as a bridge between the protein and the lipid surface, in a way similar to that previously suggested for annexins [37]. The results presented in this paper fit well with this model because they show clearly that there is mutual co-operativity between Ca^{2+} and phosphatidylserine in their binding to the domain. Furthermore the fact that the replacement of only one aspartic residue with asparagine, at the Ca^{2+}-binding site (Figure 8), inhibits both Ca^{2+} and phospholipid binding also gives strong support to this co-ordination model.

Nevertheless the results presented here do not allow us to rule out the possibility that the binding of Ca^{2+} might give rise to subtle conformational changes in the C2 domain, so that the binding of phospholipids could be facilitated.

In summary, our results strongly suggest a direct co-operation between Ca^{2+} and phospholipids to produce the translocation of the C2 domain to the plasma membrane. Our mutagenesis study has revealed at least three important sites for Ca^{2+} binding that are involved, directly or indirectly, in membrane interaction. Further studies on the three-dimensional structure of this C2 domain of PKCα and on the effect of these mutations in the context of the full-length protein, which are in progress, will help us to obtain a better understanding of the mechanism by which Ca^{2+} ions regulate the binding of this protein to the plasma membrane.

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