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The C2 Domain of PKCδ Is a Phosphotyrosine Binding Domain

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Summary

In eukaryotic cells, the SH2 and PTB domains mediate protein-protein interactions by recognizing phosphotyrosine residues on target proteins. Here we make the unexpected finding that the C2 domain of PKCδ directly binds to phosphotyrosine peptides in a sequence-specific manner. We provide evidence that this domain mediates PKCδ interaction with a Src binding glycoprotein, CDCP1. The crystal structure of the PKCδ C2 domain in complex with an optimal phosphopeptide reveals a new mode of phosphotyrosine binding in which the phosphotyrosine moiety forms a ring-stacking interaction with a histidine residue of the C2 domain. This is also the first example of a protein Ser/Thr kinase containing a domain that binds phosphotyrosine.

Introduction

Fifteen years ago, the discovery of Src homology 2 (SH2) domain interactions with phosphotyrosine proteins established a new paradigm for signal transduction in which protein phosphorylation on tyrosine leads to acute formation of multiprotein complexes. More than 10 years ago, the discovery of a second family of phosphotyrosine binding (PTB) domains unrelated to SH2 domains revealed an independent evolution of this important mechanism of signal transduction. Up to now these two domains were the only known phosphotyrosine binding domains.

The C2 domains (C2 stands for conserved domain 2) are eight β strand modules of about 120 amino acids that are found in a large number of eukaryotic proteins (Nalefski and Falke, 1996). C2 domains are found in all eukaryotes, including yeast, but are not found in prokaryotes. Originally identified in classical/conventional protein kinases C (PKC), they were found to mediate calcium-dependent lipid binding. However, the residues required for calcium binding in classical PKCs and other proteins are not found in all the C2 domains. Nonetheless, for several of these proteins the C2 domain is still known to bind phospholipids (Rizo and Sudhof, 1998). Several C2 domains have also been shown to serve as protein-protein interaction domains (Dekker and Parker, 1997; Gray et al., 1997; Ron et al., 1995). Interestingly, in coordination with lipid binding this could permit recruitment of the C2 domain-containing protein to specific membrane compartments (Mellor and Parker, 1998). In the case of classical PKCs, binding of the C2 domain to the membrane is also important to release the pseudosubstrate from the catalytic site (Newton and Johnson, 1998).

The regulatory domain of the novel protein kinases C (PKCδ, -ϵ, -θ, and -η) contains a tandem repeated C1 domain and a C2 domain that does not bind calcium. Very recently it was shown that the C2 domain of PKCδ does not bind to phospholipids (Stahelin et al., 2004). PKCδ is known to bind to several proteins, including actin, GAP-43, and possibly SRBC, via its C2 domain (Dekker and Parker, 1997; Izumi et al., 1997; Lopez-Lluch et al., 2001). PKCδ is also known to be regulated by tyrosine phosphorylation downstream of Src (Benes and Soltoff, 2001; Blake et al., 1999; Shanmugam et al., 1998; Zang et al., 1997). Although several studies have reported an interaction between Src and PKCδ (Shanmugam et al., 1998; Zang et al., 1997), the mechanism underlying this interaction remains unclear.

The activity of Src is regulated by phosphorylation of a tyrosine residue near the C terminus. This residue binds to the SH2 domain of the same Src molecule, thereby constraining the catalytic domain in an inactive conformation (Sicheri et al., 1997; Xu et al., 1997). Phosphorylation and engagement of the Src SH2 domain thus provide both a means for enzyme activation and the targeting of the enzyme to a specific location in the cell. Several multiprotein complexes containing Src and other kinases have been identified, including the PDGF receptor and focal adhesion kinase (FAK) (DeMali et al., 1999; Hanks et al., 2003). CDCP1, a newly identified transmembrane protein that is overexpressed in colon cancer (Hooper et al., 2003; Scherl-Mostageer et al., 2001), possesses several binding sites for the Src SH2 domain. In the majority of colon cancers, Src activity is upregulated, either by increases in its specific activity or levels of expression (Irby et al., 1997; Irby et al., 1999; Malek et al., 2002; Mao et al., 1997). Therefore it was of interest that we found that CDCP1 was phosphorylated on tyrosine residues in a Src-dependent manner, and this prompted us to examine this finding in more detail.

Here we show that the C2 domain of PKCδ is a phosphotyrosine binding domain. We determine its specificity by peptide library screens, its affinity by isothermal titration calorimetry, and the structural basis for phosphotyrosine binding by X-ray diffraction of its crystal structure. Our data indicate that Src phosphorylates and binds to CDCP1 by its SH2 domain and the C2 domain of PKCδ binds to CDCP1, thus forming a multiprotein complex that includes two important kinases.

Results

Several studies, including ours, have previously shown that PKCδ is tyrosine phosphorylated and that its ki-

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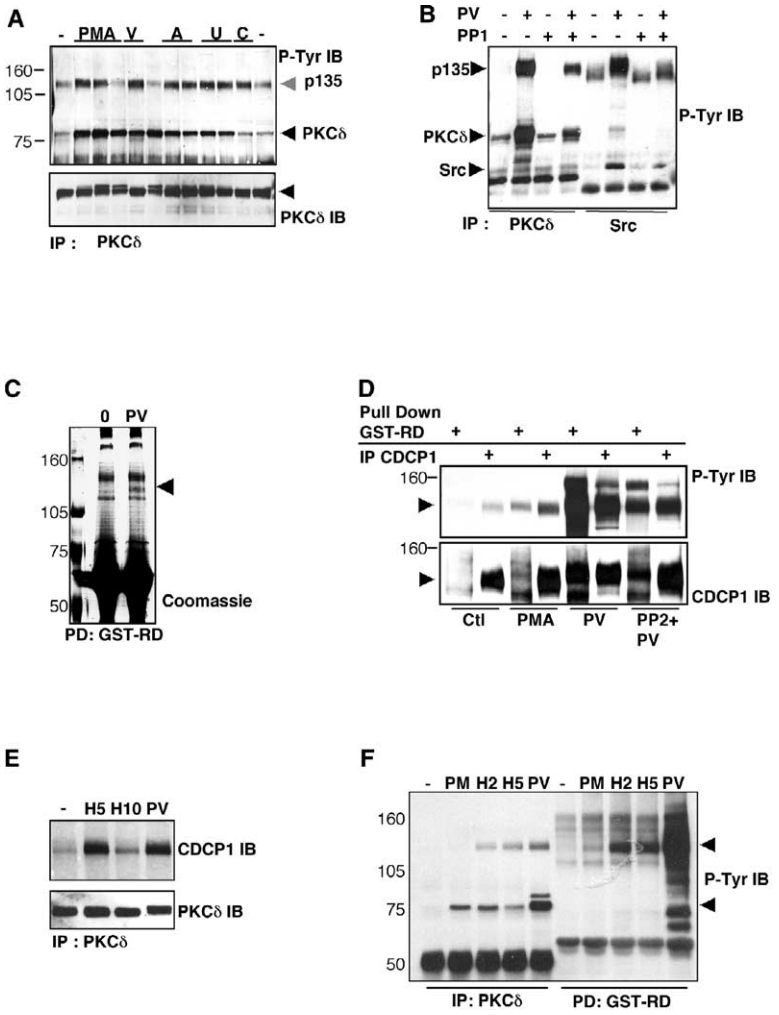

Figure 1. Identification of a Phosphotyrosine Protein Directly Binding to the C2 Domain of PKCδ

Immunoblot analysis of proteins immunoprecipitated from cell lysates and subjected to SDS-PAGE.

(A) RPG1 (rat parotid gland 1) cells were left untreated in growing medium or were treated with PMA (PMA, 100 μM for 2, 5, or 15 min, respectively), pervanadate (V, 100 μM, 5 min), ATP (A, 100 μM, 2 and 5 min), UTP (U, 100 μM, 2 and 5 min), and carbachol (C, 100 μM, 5 min).

(B) RPG1 cells were left untreated or treated with pervanadate (PV, 100 μM, 5 min) and/or PP1 (PP1, 5 μM, 30 min prior to PV).

(C) The 135 kDa protein that associates with the regulatory domain of PKCδ is CDCP1. Lysates from 10^8 A431 cells untreated or treated with pervanadate (PV, 100 μM, 15 min) were incubated with 50 μg of GST-RD PKCδ fusion protein, and bound proteins were revealed by Coomassie staining. P135 was subsequently identified as CDCP1 using mass spectrometry analysis on tryptic peptides extracted from the gel.

(D) A431 cells were stimulated as indicated and the lysates were either immunoprecipitated with an anti-CDCP1 antibody or subjected to a pull-down with GST-RD PKCδ fusion protein.

(E and F) HCT116 cells were treated with hydrogen peroxide (H, 5 mM, 2, 5, or 10 min), pervanadate (PV, 100 μM, 5 min), and PMA (PM, 100 nM, 5 min). For all blots, proteins were immunoprecipitated and immunoblotted as indicated.

nase activity is regulated by Src in various cell types (Benes and Soltoff, 2001; Blake et al., 1999; Denning et al., 1996; Soltoff and Toker, 1995). In the hope of understanding the mechanisms of this regulation, we sought to identify a 135 kDa tyrosine-phosphorylated protein that we found associated with PKCδ and Src in various cell lines upon stimulation with phorbol ester (PMA, phorbol-12-myristate-13-acetate) and addition of pervanadate, as well as in response to more physiologically relevant conditions, such as muscarinic and purinergic receptor stimulation (Figure 1A). The use of Src-family kinase inhibitors (PP1, PP2) suggested that p135 tyrosine phosphorylation was controlled by a Src family member (Figure 1B). Since we also found that the regulatory domain (RD) of PKCδ (residues 1–332, fused to glutathione S-transferase) bound to a tyrosine-phosphorylated 135 kDa protein when cells were treated with PMA or pervanadate, we used this domain (GST-RD PKCδ) as an affinity reagent to isolate p135 from lysates of cells treated with pervanadate (Figure 1C). Using mass spectrometry we identified p135 as CDCP1, a recently described transmembrane glycosylated protein overexpressed in colon cancer (Scherl-Mostageer et al., 2001). The same protein has also been identified as SIMA135 (subtractive immunization M(+)HEp3-associated 135 kDa protein) by subtractive immunization using a highly metastatic tumor cell line (Hooper et al., 2003). Although the predicted size of CDCP1 is 92 kDa, glycosylation increases its apparent molecular weight in SDS-PAGE to approximately 135 kDa (Hooper et al., 2003; Scherl-Mostageer et al., 2001; data not shown). By performing immunoprecipitation and pull-down in parallel, we determined that there is a correlation between the tyrosine phosphorylation of CDCP1 and its ability to interact with the regulatory domain of PKCδ (Figure 1D).

Since the treatment of several cell types with hydrogen peroxide (H_2O_2) initiates signaling events relayed in part by PKCδ and involving tyrosine phosphorylation (Storz et al., 2004), we utilized H_2O_2 as an agent to examine the relationship between CDCP1 and PKCδ. In fact, H_2O_2 induced the association of PKCδ with CDCP1 (Figure 1E). This association of endogenous proteins correlates with the tyrosine phosphorylation of CDCP1, as seen in PKCδ immunoprecipitates and in pull-downs using GST-RD (Figure 1F). Thus, the tyrosine phosphorylation of CDCP1 by several stimuli known to affect PKCδ and Src activation (or Src associ-

Table 1. Phosphotyrosine Peptide Motif Selection by the Regulatory Domain of PKCδ

–3	–2	–1		+1	+2	+3	+4
Y (1.4) F (1.3)	S (1.6) A (1.3)	V (1.3) I (1.2)	pY	Q (1.4) R (1.4) M (1.4) V (1.3)	X	*Y (2.8)* *F (1.8)* *W (1.7)*	V (1.2)
Y (1.4) F (1.2)	S (1.7) A (1.7)	I (1.5) V (1.4) L (1.2) M (1.2)	pY	Q (1.8) R (1.8) I (1.5) V (1.5) M (1.4)	P (1.3)	Y	V (1.4) I (1.4) F (1.3) L (1.2)

A GST fusion of the regulatory domain (residues 1–332) of PKCδ was screened for binding to phosphopeptide libraries containing the sequences MAXXXXpYXXXXAKKK and MAXXXXpYXXYXXAKKK, where X indicates all amino acids except Cys and pY denotes phosphotyrosine. Residues showing strong enrichment are in italics.

ation with PKCδ) leads to the association of PKCδ with CDCP1.

The correlation between the tyrosine phosphorylation of CDCP1 and an apparently direct association with the regulatory domain of PKCδ suggested that the regulatory domain could contain a phosphotyrosine binding domain. To test this possibility, a completely degenerated phosphotyrosine peptide library (XXXXpYXXXX, where pY designates the phosphorylated tyrosine) was screened against the GST-RD PKCδ fusion protein. The results of this screen strongly suggested that the regulatory domain of PKCδ indeed bound specifically to tyrosine-phosphorylated peptides (Table 1). A subset of peptides containing a tyrosine or phenylalanine residue three residues C-terminal to the phosphorylated tyrosine was preferentially selected. We then screened a second library containing a fixed tyrosine residue at +3 (XXXXpYXXYX) to obtain more detailed amino acid preferences at other positions. The result of these library screens characterized the regulatory domain of PKCδ as containing a phosphotyrosine binding domain that has a strong preference for aromatic residues three amino acids C-terminal to the phosphorylated tyrosine, with the following consensus sequence (Table 1): (Y/F)-(S/A)-(V/I)-pY-(Q/R)-X-(Y/F)-X.

Since several reports indicated that the PKCδ C2 domain was responsible for protein-protein interactions, we investigated whether the C2 domain of PKCδ was sufficient for the binding to CDCP1. Notably, the C2 domain alone (the folding unit encompassing residues 1 to 123, as defined by the previously solved crystal structure of the free C2 domain [Pappa et al., 1998]) was able to pull down CDCP1 expressed in U2-OS cells (Figure 2A).

To confirm that the C2 domain of PKCδ itself is a phosphotyrosine binding domain that can pull down CDCP1, a biotinylated version of the optimal peptide defined by the library screens (Biotin-GGALYSIpYQ PYVFAKKK) was used as bait for in vitro-translated fragments of PKCδ. We found that the C2 domain of PKCδ bound strongly to the phosphorylated version of the optimal peptide but only very weakly to the non-phosphorylated version (Figure 2B). When an XXXpYX XYX biotinylated library was used, the same phospho-dependency was shown. As a control, we showed that an incomplete C2 domain (denoted C2*) that misses a β strand essential to fold properly (Pappa et al., 1998) does not bind to the optimal phosphopeptide. Under the same conditions, a short version of the C2 domain (residues 1–123) also binds to the phosphopeptide (data not shown).

Competition experiments showed that the phosphorylated version of the optimal peptide could block the interaction of the PKCδ C2 domain with multiple phosphotyrosine proteins, including CDCP1, with an apparent IC_{50} of about 500 nM (Figure 2C). The affinity of the C2 domain for the optimal peptide was determined using isothermal titration calorimetry. These experiments showed that the dissociation constant for the optimal phosphopeptide is less than 250 nM (the highest value from three independent determinations) and the stoichiometry of the interaction is 1 mole phosphopeptide per mole of C2 domain (Figure 2D). This affinity is comparable to affinities reported for binding of optimal phosphopeptides to SH2 and PTB domains (Pawson et al., 2001), the only two phosphotyrosine binding domains previously described.

To give insight into the structural basis of the phosphotyrosine binding property of the C2 domain, the structure of the C2 domain from human PKCδ in complex with the high-affinity phosphotyrosine peptide (MALYSIpYQPYVFAKKK) was solved to a 1.7 Å resolution (R_{cryst} = 17.6%, R_{free} = 22.1%; see data collection and refinement statistics, Table 2). The overall C2 structure is very similar to that of the rat PKCδ C2 domain (Pappa et al., 1998), with an rmsd of 0.49 Å from the 106 C_α atom. Thus, no significant difference in protein structure is observed upon peptide binding (except for deviations in the loop regions between β strand 1 and 2), analogous to what has been observed for the binding of phosphotyrosine peptides to SH2 and PTB domains (Eck et al., 1996; Waksman et al., 1993). Electron density for the associated phosphopeptide is clear from Met–6 to Ala+6 (– positions are N-terminal to the phosphotyrosine, Figure 3B). The peptide binds in an extended conformation across one end of a β sheet (strands 4, 1, 8, and 7), reaching the other β sheet (strands 2, 5, and 6) (Figure 3A).

Most of the specificity of the peptide recognition comes from the phosphotyrosine (pTyr) binding. It is held in a deep pocket with extensive hydrogen bonds (His62 side chain and backbone NH), electrostatic interactions (Arg67 and Lys48 side chains), and van der Waals interactions (ring stacking with His62) (Figures

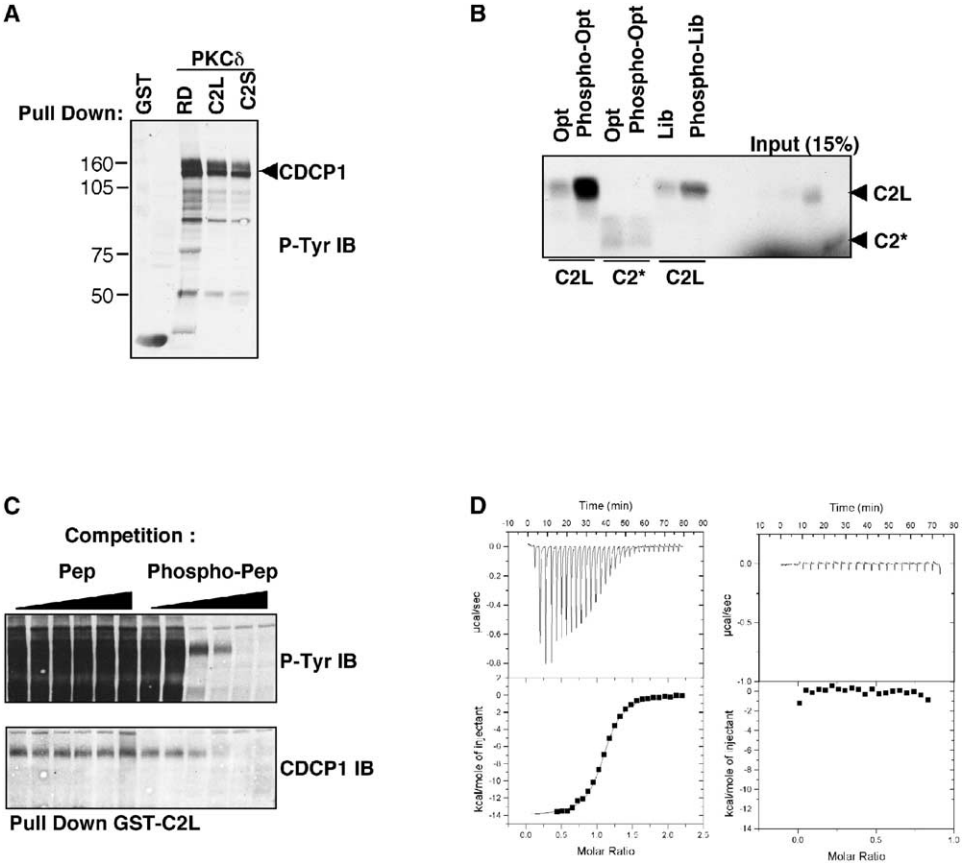

Figure 2. The C2 Domain of PKCδ Is a High-Affinity Phosphopeptide Binding Module

(A) U2-OS cells transfected with Src and CDCP1 were treated with pervanadate (100 μM, 15 min) and the indicated pull-downs were performed. RD: regulatory domain (residues 1–332); C2L: C2 domain long form (residues 1–160); C2S: C2 domain short form (residues 1–123).

(B) The C2 domain of PKCδ (C2L residues 1–160) was in vitro transcribed and translated in the presence of ^{35}S Met and incubated with the phosphorylated or unphosphorylated biotinylated optimal peptide (Opt) (GGALYSIpYQPYVFAKKK) or the XXXpYXXYX biotinylated peptide library (Lib) bound to avidin beads. Bound radioactive material was revealed by autoradiography.

(C) HCT116 cells were stimulated with pervanadate (100 μM) for 15 min and lysates were incubated with a GST-C2 fusion protein that had been previously incubated with increasing concentration of phosphorylated or nonphosphorylated optimal peptide (0.01, 0.1, 0.5, 1, 5, 10 μM).

(D) The affinity of the optimal phosphopeptide (GGALYSIpYQPYVFAKKK) for binding to the C2 domain of PKCδ was determined by isothermal titration calorimetry using the GST-C2L PKCδ (1–160) fusion protein. Left, phosphopeptide; right, nonphosphopeptide. Fitting of the experimental data with a one-type-of-site binding model gave a binding stoichiometry of 1.08 with dissociation constant (K_D) of 240 nM at 25°C ($\Delta H = -1.4 \times 10^4$, $\Delta S = -16.8$, $X^2 = 26{,}631$).

3C and 3D). In addition, the two backbone amides around the pTyr have direct H bonds to two conserved residues in PKCδ, Asp60 and Glu123, helping to position the pTyr into the positive pocket. Notably, a phosphoserine or phosphothreonine side chain would likely be too short to reach Arg67 at the bottom of this pocket.

The rest of the peptide forms several backbone contacts with the protein either directly or indirectly through water molecules, as well as side chain interactions that explain some of the peptide library results (Figure 3D). There are two direct peptide side chain recognitions by the protein at Ser–2 and Tyr+3. The Ser–2 side chain forms a hydrogen bond with the Arg6 guanidine head group of the C2 domain, and the Tyr+3 OH interacts with the backbone amide of Tyr52. In addition, the Tyr+3 aromatic ring stacks onto Met51 and interacts with Phe+5. Together they form a small hydrophobic cluster that acts as a continuation of the hydrophobic core in between the two β sheets. This structure explains the peptide library data where the +3 position showed strong preference for aromatic residues, particularly tyrosine. Although the peptide library showed some selection for Gln at +1, there is no prominent reason for this preference suggested by the structure. The Gln side chain points into the solvent region and interacts with Thr58 indirectly through water molecules. Compared to the other two known phosphotyrosine binding domains, SH2 and PTB, which primarily recognize residues either C-terminal or N-terminal to pTyr, respectively, the PKCδ C2 domain interacts significantly with both ends of the peptide.

Phosphorylation on Tyr64 has been suggested to regulate PKCδ (Joseloff et al., 2002). Interestingly, this residue sits just outside of the positive binding pocket for pTyr. Its OH is 5.46 Å from Arg67, compared to 5.20 Å

Table 2. Data Collection and Model Refinement Statistics

Data Collection	
Space group	$P2_12_12_1$
Resolution (Å)	1.7 (1.76-1.70)[a]
Total reflections	67,012
Unique reflections	15,254
Completeness (%)	97.9 (92.9)
R_{sym} (%)	7.7 (18.6)
I/σ	18.9 (5.8)
Refinement	
Non-H atoms	1229
R_{cryst} (%)	17.6
R_{free} (%)	22.1
Mean B factor (Å^2)	12.9
Rmsd bond lengths (Å)	0.010
Rmsd bond angles (°)	1.5
Rmsd B value (bonds) (Å^2)	1.24
Rmsd B value (angles) (Å^2)	1.65

$R_{sym} = \Sigma_{hkl}\Sigma_i|I_i - \langle I\rangle|/\Sigma_{hkl}\Sigma_i\langle I\rangle$.
$R_{cryst} = \Sigma_{hkl}|F_{obs} - F_{calc}|/F_{obs}$.
R_{free} was calculated over the 6.8% of reflections not used for model refinement.
[a] Values for the highest resolution shell are in parentheses.

in the case of the equivalent OH on pTyr in the bound peptide. When phosphorylated, Tyr64 would likely interact electrostatically with Arg67, preventing the binding of phosphopeptides.

Coexpression experiments were used to determine the mechanism of interaction between CDCP1, PKCδ, and Src. Experiments using HEK293 or U2-OS cells showed that PKCδ and Src associated robustly with CDCP1 when the two kinases were coexpressed (Figure 4A). Moreover, the kinase activity of Src was necessary for the interactions between Src and CDCP1 and between PKCδ and CDCP1 to occur. When CDCP1 was mutated at Tyr734, which is the most likely SH2 domain binding site for Src (Yaffe et al., 2001) (http://scansite.mit.edu), Src and PKCδ were also impaired in their ability to interact with CDCP1 (Figures 4B and 4C). Thus, tyrosine phosphorylation of CDCP1 by Src appears to promote the formation of a complex between CDCP1, Src, and PKCδ.

The sequence surrounding Tyr762 of CDCP1 (VDTp**YRPF**) is in good agreement with the motif predicted to bind to the C2 domain of PKCδ and is also predicted to be a good Src phosphorylation site (Songyang and Cantley, 1995a; Yaffe et al., 2001) (http://scansite.mit.edu). We mutated Tyr762 to phenylalanine and assessed the effects on the association of CDCP1 with Src and PKCδ. Coimmunoprecipitation and pull-down experiments showed that the Y762F mutant of CDCP1 is impaired in its association with PKCδ but not with Src (Figures 5A and 5B). The regulatory domain of PKCδ was able to pull down wild-type CDCP1 from cells cotransfected with CDCP1, Src, and PKCδ (Figure 5B), but mutation of Tyr762 to phenylalanine led to a clear decrease in CDCP1 pull-down, showing that Tyr762 is likely to be the primary binding site of the PKCδ C2 domain on CDCP1. A GST fusion protein of the regulatory domain (SH2-SH3) of Src pulled down wild-type CDCP1 but not the CDCP1 Y734F mutant (Figure 5B), suggesting that the interaction between Src and CDCP1 is stabilized mainly through an SH2-pY734 link. Although CDCP1 also contains a polyproline-rich sequence susceptible to binding SH3 domains, the same result was obtained using the Src SH2 domain alone (data not shown). Another tyrosine residue (Tyr743) is found in a favorable amino acid context to be phosphorylated by Src (Yaffe et al., 2001). Using phosphospecific antibodies (data not shown), we have determined that this site is indeed phosphorylated in these experiments, but this site does not appear to play a significant role in the interaction of CDCP1 with either Src or PKCδ (Figure 5B). Overall our data support a model in which Src initiates phosphorylation of CDCP1 at Tyr734, associates through its SH2 domain at that site, and promotes further phosphorylation at Tyr743 and Tyr762. Phosphorylation of Tyr762 results in the recruitment of PKCδ to CDCP1. It is also possible that direct contact between Src and PKCδ participates in the formation of the complex since PKCδ wt or kinase dead (KD) has a positive effect on the recruitment of Src to CDCP1.

To confirm that in the context of the full-length protein the C2 domain is necessary for the interaction between PKCδ and CDCP1, a truncated version of PKCδ was tested for its ability to interact with CDCP1. As shown in Figure 5C, the mutant of PKCδ missing the C2 domain was unable to interact with CDCP1, but Src still bound to CDCP1. Since His62 in the C2 domain seems to play a major role in the interaction between the C2 domain and the phosphopeptide, we created a mutant of PKCδ in which His62 was mutated into an aspartate. We also mutated the Arg67 that appears to be crucial for phosphate coordination. Mutation of either of these residues was shown to abolish the interaction of PKCδ with an optimal phosphopeptide (data not shown) or with a phosphopeptide library linked to beads (Figure 5D). The lack of binding to a partially degenerate library (XXXpYXXYXX) shows that the mutation of the residue critical for tyrosine binding (His62) in the C2 domain does not change the specificity of the C2 domain while preserving phosphotyrosine binding. Importantly, in the context of coexpression in cells, the mutant proteins H62D PKCδ as well as R67S PKCδ are unable to interact with CDCP1 (Figure 5E).

Discussion

Our studies show that PKCδ, a Ser/Thr protein kinase identified more than 15 years ago, contains an unexpected phosphotyrosine binding domain. This domain mediates the association of PKCδ with a transmembrane protein, CDCP1. The activity of the protooncogene Src promotes this interaction by phosphorylating key residues on CDCP1, and this leads to the formation of a CDCP1-Src-PKCδ complex. In some instances these results may explain how the previously observed complex between PKCδ and Src occurs. It is also possible that direct interaction between the two kinases is involved in complex formation or that cells that do not express CDCP1 express analogous scaffold proteins. The structure of the C2 domain of PKCδ in complex with an optimal binding peptide reveals that the coordi-

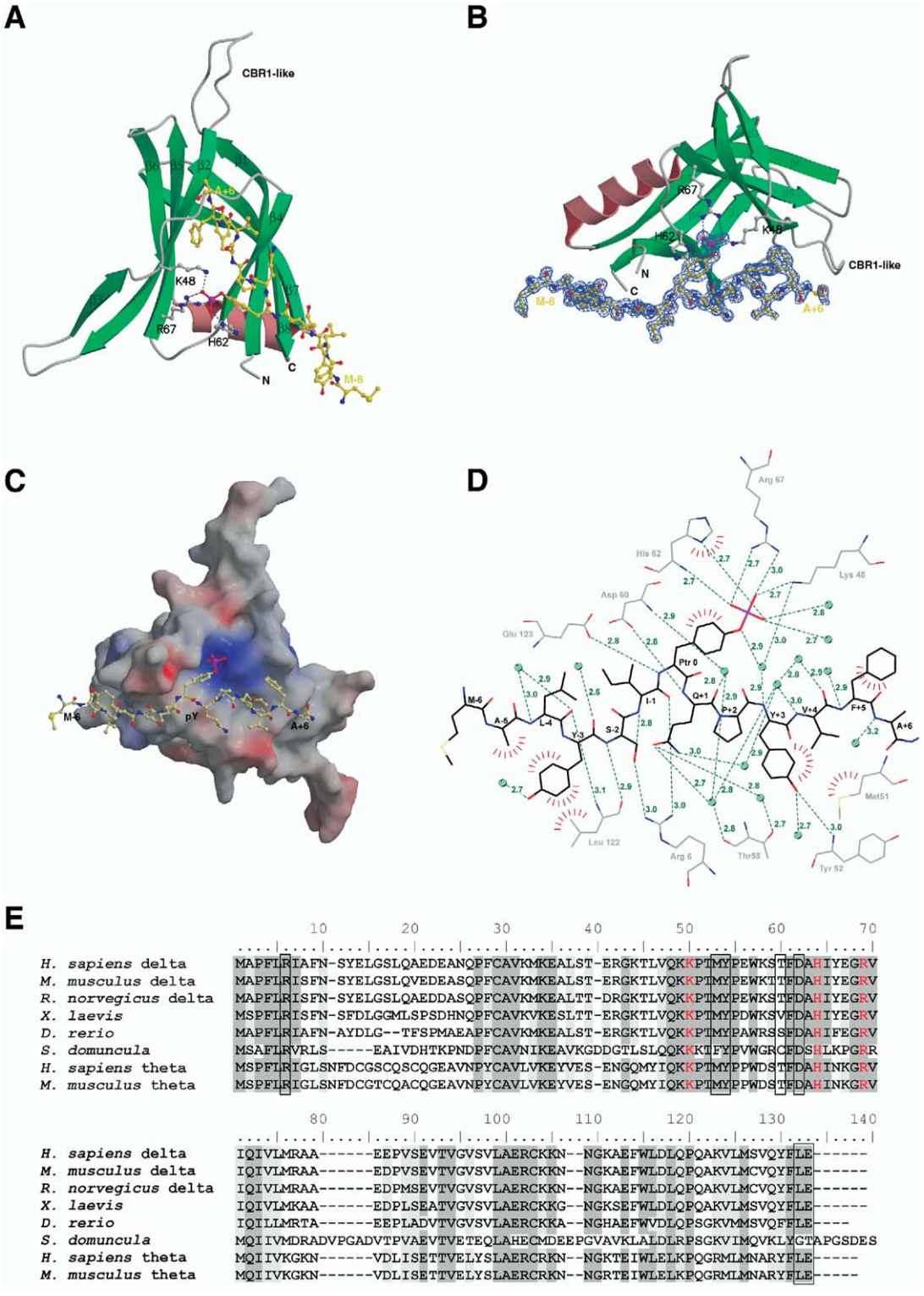

Figure 3. Crystal Structure of the C2 Domain of PKCδ in Complex with the Optimal Phosphopeptide

(A) Overall structure of the C2 domain of PKCδ bound to the phosphotyrosine peptide.

(B) Bottom view of the complex structure with composite-omit $2F_o - F_c$ map contoured at 1.2 σ around the peptide.

(C) Surface potential drawing of the C2 domain illustrating the deep positively charged pocket (blue) formed by Arg67 and Lys48 for phosphate binding.

(D) Schematic representation of interactions between the peptide, C2 domain, and water molecules (green spheres) at the interface. Peptide backbone is in black and protein residues are in gray.

(E) Multiple sequence alignment of C2 domains from PKCδ (showing the conservation throughout evolution) and PKCθ (showing its potential phosphotyrosine binding site). The residues that interact with the phosphate are highlighted in red and the residues that make contact with the peptide are boxed.

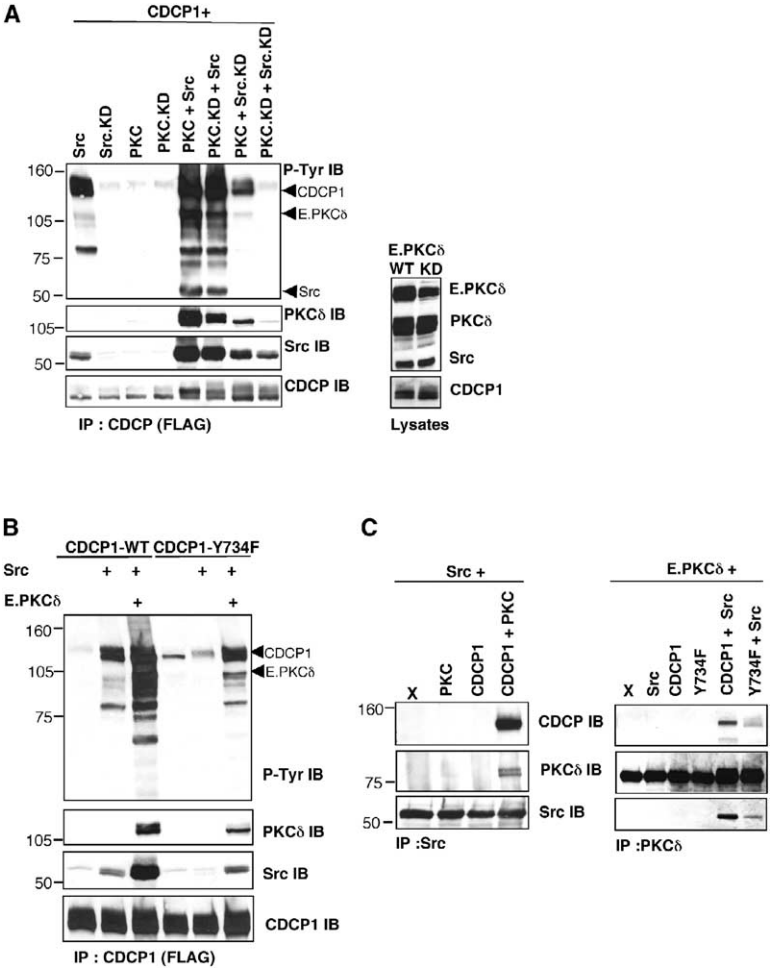

Figure 4. Mechanism of the Association of PKCδ, Src, and CDCP1

(A) U2-OS cells were transfected with plasmids encoding for FLAG-tagged CDCP1 wt, EGFP-PKCδ wt or kinase dead (KD), and Src wt or KD, as indicated. Phosphotyrosine proteins as well as EGFP-PKCδ and Src were detected in anti-FLAG immunoprecipitates as indicated. (E.PKCδ: EGFP-PKCδ; similar results were obtained using untagged PKCδ). Right panel: total cell lysates corresponding to lanes 5 and 6 of the left panel showing lower expression of EGFP-PKCδ KD compared to EGFP-PKCδ wt.

(B) U2-OS cells were transfected with plasmids encoding for FLAG-tagged CDCP1 wt or CDCP1-Y734F mutant, EGFP-PKCδ, and Src, as indicated. Phosphotyrosine proteins as well as EGFP-PKCδ and Src were detected in anti-FLAG immunoprecipitates as indicated.

(C) U2-OS cells were transfected with plasmids encoding for FLAG-tagged CDCP1 wt or CDCP1-Y734F mutant, EGFP-PKCδ, and Src, as indicated. (E.PKCδ: EGFP-PKCδ; similar results were obtained using untagged PKCδ). Immunoprecipitations were performed using anti-Src antibody (left) or anti-PKCδ antibody (right).

nation of the phosphate is achieved mainly through an arginine residue, as in PTB and SH2 domains. Notably, the interaction with the tyrosine is maintained by stacking against a histidine residue, a mode of binding that has not been observed in phosphotyrosine binding domains. Importantly, the affinity of the peptide for the C2 domain is comparable to the affinities reported for SH2 and PTB domains with cognate peptides. Interestingly, the binding site is close to the N and C termini of the C2 domain; therefore, binding could potentially alter the position of the domain relative to the rest of the protein. Because the C2 domain is followed by the pseudosubstrate region, binding of phosphotyrosine to the C2 domain could participate in activation of PKCδ as shown for classical PKC when the C2 domain binds to lipids. (For classical PKCs this event has to be followed by binding of DAG to the C1 domain.) Numerous C2 domains lack the amino acids residues necessary for calcium binding. However, these calcium-insensitive C2 domains are largely considered to be lipid binding modules, although it has only been demonstrated for a few of them (Pepio and Sossin, 2001). The crystal structures of the C2 domains that have been solved show, for the most part, a very similar fold. In that respect it is interesting to note that the C2 domain of PKCδ is significantly divergent from the other C2 domains only in the region in which we observed phosphotyrosine binding situated on the opposite side of where calcium and phospholipids bind in the classical C2 domains.

This is the first evidence of the presence of a phosphotyrosine binding domain in the structure of a protein Ser/Thr kinase, thus directly linking tyrosine phosphorylation with serine/threonine phosphorylation. Our findings raise the question of whether other C2 domains are phosphotyrosine binding domains. In terms of the PKC family, the level of homology between all the classical and novel PKCs is around 70% for the C1 domains and the kinase domains. On the other hand, the homology level for the C2 domain is far less, about 45%, even between PKCδ and PKCε, another member of the novel subfamily. The only close homolog of the C2 domain of PKCδ is the C2 domain of PKCθ, another nPKC family member, which has a homology of 70%. All the critical residues that contact the optimal binding peptide are conserved in PKCθ (Figure 3), and preliminary results indicate that PKCθ indeed contains a phosphotyrosine binding domain.

Among the proteins known to associate with PKCδ, only a subset of them contains potential binding sites for the C2 domain (i.e., sequences that contain the consensus defined by the peptide library screens). Among

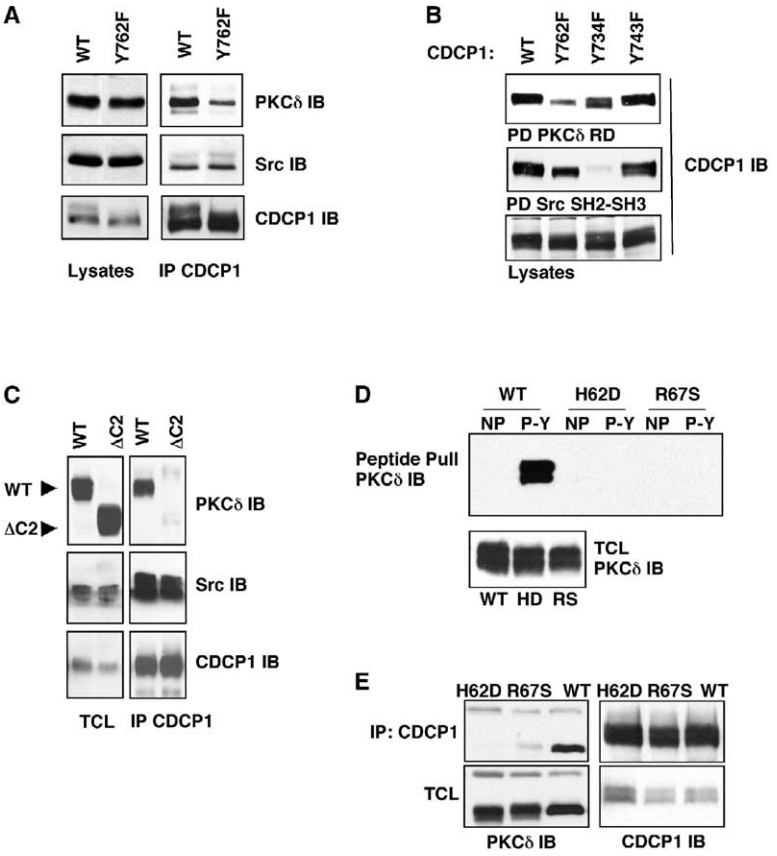

Figure 5. Point Mutations in CDCP1 and PKCδ Disrupt Their Phosphotyrosine-Dependent Interaction

(A) Src and PKCδ were coexpressed with either FLAG-tagged CDCP1 wt or CDCP1-Y762F in U2-OS cells, and an anti-FLAG antibody was used for IP. Bound proteins were detected with the indicated antibodies.

(B) Src, PKCδ, and wt CDCP1 or CDCP1 mutated at the indicated sites were expressed in U2-OS cells. Pull-downs were done using GST-RD PKCδ or GST-SH2-SH3 Src fusion proteins, and the bound CDCP1 was revealed by immunoblot.

(C) 293 cells were transfected with plasmids encoding for FLAG-tagged CDCP1 wt, Src, and PKCδ wt or PKCδ lacking the C2 domain (ΔC2). Immunoprecipitations were performed using anti-FLAG antibodies. TCL: total cell lysates.

(D) PKCδ wt or mutated versions of PKCδ (H62D and R67S) were expressed in COS-7 cells and tested for their ability to bind a phosphotyrosine library linked to beads. NP: nonphosphorylated library (XXXYXXYX); P-Y: phosphorylated library (XXXpYXXYX).

(E) PKCδ wt, H62D, or R67S were coexpressed with FLAG-tagged CDCP1 and Src, and an anti-FLAG immunoprecipitation was performed. Bound PKCδ was detected by immunoblot.

them is MUC1, a marker of transformed cells and a scaffold for PKCδ and Src that assembles a multiprotein complex in a way strikingly similar to CDCP1. Another potential binder is PKD, which was recently shown to bind to and be a substrate for PKCδ (Storz et al., 2004). In addition, the proteins Abl, hnRNPK, and PLD2, which all have also been shown to associate with PKCδ, also contain a potential binding site. However, it is unlikely that the C2 domain mediates all the interaction of PKCδ with other proteins.

These studies demonstrate that a C2 domain within a Ser/Thr kinase constitutes a third phosphotyrosine binding domain, following the prior identifications of the SH2 and the PTB domains. This likely reveals an interesting evolution from a widespread calcium/phospholipid binding domain into a different binding module and illustrates the well-documented modification of common ancestral folds evolving to a variety of functions. CDCP1, the first identified target of this new domain, has been proposed to serve as a marker for a variety of malignant cells (Buhring et al., 2004; Hooper et al., 2003; Scherl-Mostageer et al., 2001), and Src also has a well-known involvement in the development of tumors. This provides a high potential for relevance in the biological implications of complex formations between this newly identified tyrosine binding domain and these signaling molecules. Our current efforts are directed toward understanding the involvement of the PKCδ C2 domain (and the one in PKCθ) in biological events.

Experimental Procedures

Cell Lines

RPG1 (rat parotid salivary gland cell line) (Soltoff et al., 1998), A431, HCT116, HEK293, U2-OS, and other cells were grown in standard culture conditions.

Immunoprecipitation and Immunoblot Analysis

Anti-CDCP1 was obtained by rabbit immunization using the cytosolic domain of CDCP1 fused to GST (Cocalico Biologicals, Reamstown, Pennsylvania). Anti-PKCδ monoclonal (BD Biosciences) and polyclonal (Santa Cruz Biotechnology) antibodies were used for immunoprecipitation and blotting, respectively. Anti-Src (SC-18) antibody was from Santa Cruz Biotechnology. Cells were lysed and subjected to SDS-PAGE as previously described (Benes and Soltoff, 2001; Blake et al., 1999; Shanmugam et al., 1998; Zang et al., 1997).

Identification of CDCP1

GST-RD PKCδ was used to affinity purify a 135 kDa protein (as seen on SDS-PAGE after Coomassie staining) from A431 cells treated with pervanadate for 15 min in growing medium. The band was not seen in the GST control or when using untreated cells. Mass spectrometry analysis of tryptic digest identified the protein as an unknown protein, AK023834, later on shown by Scherl-Mostageer et al. (Scherl-Mostageer et al., 2001) to be an incomplete version of CDCP1. Full-length CDCP1 was assembled using the cDNA AK023834 (Takara Biomedicals, Japan) and the product of a reverse transcriptase-PCR (Hot Star Taq, Roche) corresponding from

the start ATG to a unique SacI site in AK023834, using mRNA from A549 cells (Rneasy, QIAGEN).

Plasmids and Transfections

CDCP1-FLAG plasmid was constructed by PCR amplification of full-length CDCP1. The CDCP1 mutants Y734F, Y743F, and Y762F were obtained by PCR-based mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). GST-RD of human PKCδ was a kind gift of Dr. Donald Kufe (Dana Farber Cancer Institute, Boston). GST-C2L (1–160) and GST-C2S (1–123) from PKCδ were obtained by standard PCR methods using the GST-RD plasmid as a template and inserted into pGEX vectors (Amersham). The Src (wt and R295K mutant) and EGFP-PKCδ (wt and R378K mutant) encoding plasmids were generously given by Dr. Joan Brugge (Harvard Medical School, Boston) and Dr. Pradip Majumder (Dana Farber Cancer Institute).

Peptide Library Screening

The peptide libraries were generated by standard methods and quality was assessed by mass spectrometry (Michael Burn, Tufts University, Boston). The peptide libraries were screened as described elsewhere (Songyang and Cantley, 1995b) using the regulatory domain (residues 1–332) of PKCδ fused to GST.

Isothermal Titration Calorimetry

ITC was performed on a VP-ITC apparatus (MicroCal, Northampton, Massachusetts) according to manufacturer instructions. The C2 domain of PKCδ (residues 1–160) fused to GST was prepared by affinity purification on glutathione beads. The fusion protein was eluted from the beads using 30 mM reduced glutathione in MES (pH 6.4), NaCl 150 mM buffer, and the eluted material was dialyzed against MES, NaCl buffer overnight. The final concentration was adjusted to 0.015 mM. Nonphosphorylated and phosphorylated versions of the optimal PKCδ peptide (GGALYSIpYQPYVFAKKK) were synthesized based on peptide library screening results and purified by HPLC. The peptides were used at 0.15 mM. Analysis of the data was done using Origin software.

Crystal Structure Determination

Human PKCδ (residues 1–123) was cloned into pET28 vector (Novagen), expressed in *E. coli*, and purified using Ni-NTA agarose (QIAGEN). Five mg/ml protein containing 1 mM peptide was combined in a 1:1 ratio with the reservoir solution as the hanging drop. Crystals were obtained with 14% PEG4K, 100 mM MES (pH 6.0), 8% PEG200, 200 mM potassium acetate, and 10% glycerol as the reservoir solution. Data were collected at CHESS F1 station and processed with DENZO and SCALEPACK (Otwinowski and Minor, 1997). The structure was solved by molecular replacement using CNS (Brunger et al., 1998) and 1BDY as the model. Refinement was performed with CNS.

In Vitro Transcription and Translation

The C2 domain of PKCδ encompassing the pseudosubstrate region (residues 1–160) or the first 100 residues corresponding to an incomplete C2 domain (Pappa et al., 1998) were in vitro transcribed and translated from pcDNA 3.1(+)-based plasmids (Invitrogen) using the TNT T7 system (Promega) in the presence of ^{35}S methionine. The optimal phospho- and nonphosphopeptides synthesized and coupled to biotin were immobilized on Ultralink streptavidin beads (Pierce) and incubated with the products of in vitro translation. After washes in PBS 1% NP40, the bound proteins were subjected to SDS-PAGE and revealed by autoradiography.

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Accession Numbers

The Protein Data Bank ID code for the human PKCδ C2 domain in complex with the optimal phophotyrosine peptide is 1YRK.