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Characterization of the Interaction of Phorbol Esters with the C1 Domain of MRCK (Myotonic Dystrophy Kinase-related Cdc42 Binding Kinase) α/β*[S]

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C1 domains mediate the recognition and subsequent signaling response to diacylglycerol and phorbol esters by protein kinase C (PKC) and by several other families of signal-transducing proteins such as the chimerins or RasGRP. MRCK (myotonic dystrophy kinase-related Cdc42 binding kinase), a member of the dystrophia myotonica protein kinase family that functions downstream of Cdc42, contains a C1 domain with substantial homology to that of the diacylglycerol/phorbol ester-responsive C1 domains and has been reported to bind phorbol ester. We have characterized here the interaction of the C1 domains of the two MRCK isoforms α and β with phorbol ester. The MRCK C1 domains bind [20-^{3}H]phorbol 12,13-dibutyrate with K_d values of 10 and 17 nM, respectively, reflecting 60–90-fold weaker affinity compared with the protein kinase C δ C1b domain. In contrast to binding by the C1b domain of PKCδ, the binding by the C1 domains of MRCK α and β was fully dependent on the presence of phosphatidylserine. Comparison of ligand binding selectivity showed resemblance to that by the C1b domain of PKCα and marked contrast to that of the C1b domain of PKCδ. In intact cells, as in the binding assays, the MRCK C1 domains required 50–100-fold higher concentrations of phorbol ester for induction of membrane translocation. We conclude that additional structural elements within the MRCK structure are necessary if the C1 domains of MRCK are to respond to phorbol ester at concentrations comparable with those that modulate PKC.

sn-1,2-Diacylglycerol (DAG)[2] is a central second messenger in cells, generated through the receptor-mediated activation of phosphatidylinositol 4,5-bisphosphate-specific phospholipase C isoforms and indirectly through the action of phospholipase D (1). The major recognition motif for DAG is a zinc finger structure called a C1 domain (2–4). The C1 domains were first shown to be responsible for the recognition of DAG and phorbol ester by the classical and novel protein kinase C (PKC) isoforms. Subsequently, homologous domains have been identified in some 50 different mammalian proteins.

Among these proteins, five families of proteins have been shown to have C1 domains that in fact bind phorbol esters and DAG (5). The protein kinase D family are kinases most closely related to the myosin light chain kinases (6, 7). The chimerins function as GTPase-activating proteins for Rac (8, 9). The RasGRP family members function as guanyl nucleotide exchange proteins for Ras and Rap1 (10). The Munc-13 family promote vesicle fusion with membranes, enhancing synaptic transmission (11, 12). The DAG kinases phosphorylate DAG, terminating DAG signaling (13, 14).

Many other proteins have been described with C1 domains that do not appear to respond to phorbol ester or DAG. Structural analysis suggests that these non-responsive C1 domains can be divided into two groups. X-ray crystallographic and NMR analysis, together with molecular modeling, have provided a detailed understanding of the interaction of phorbol esters and DAG with C1 domains (15). The phorbol ester binds in a hydrophilic cleft in an otherwise hydrophobic surface at the top of the C1 domain. By occupying this cleft, the phorbol ester both completes the hydrophobic surface and contributes further variable hydrophobic elements. The major class of phorbol ester non-responsive C1 domains, exemplified by that of Raf, shows modifications that disrupt this binding cleft. The C1 domains of the atypical PKCs, in contrast, appear to maintain the structure of the binding cleft but have introduced residues that destabilize membrane insertion and can interpose themselves in the binding cleft, competing for occupancy (16).

A C1 domain has been described in the MRCK isoforms α and β (17). The MRCK (myotonic dystrophy kinase-related Cdc42 binding kinase) isoforms are downstream effectors of Cdc42, structurally related to the dystrophia myotonica protein kinase (DMPK) family, and function in actin cytoskeletal reorganization. Tan *et al.* (18) have described that [20-^{3}H]phorbol 12,13-dibutyrate (60 nM) was able to bind to MRCKα and that treatment of HeLa cells with phorbol 12-myristate 13-acetate

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[2] The abbreviations used are: DAG, *sn*-1,2-diacylglycerol; PKC, protein kinase C; MRCK, myotonic dystrophy kinase-related Cdc42 binding kinase; PDBu, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; GST, glutathione *S*-transferase; GFP, green fluorescent protein.

(300 nM) followed by immune precipitation of the endogenous MRCKα enhanced the kinase activity of the immune precipitated MRCKα for myosin light chain 2. In the present report, we wished to characterize the phorbol ester responsiveness of the C1 domains of MRCK α and β in more detail and to compare them with the C1b domain of PKCδ, which is perhaps the C1 domain whose interactions with phorbol ester have been evaluated most extensively. We confirm that the C1 domains of MRCK α and β recognize phorbol ester but their affinities both when reconstituted in phospholipid vesicles and in intact cells are substantially weaker than that of the C1b domain of PKCδ.

EXPERIMENTAL PROCEDURES

Materials—[20-^{3}H]Phorbol 12,13-dibutyrate ([^{3}H]PDBu) (17 Ci/mmol) was purchased from PerkinElmer. 1,2-Dioctanoyl glycerol was purchased from Sigma. The DAG lactones HK-434 and 130C045 were synthesized as described previously (26, 27). PDBu and phorbol 12-myristate 13-acetate (PMA) were purchased from LC Laboratories (Woburn, MA). Phosphatidyl-L-serine was purchased from Avanti Polar Lipids (Alabaster, AL). Reagents for expression and purification of glutathione *S*-transferase (GST) fusion proteins were obtained from Pierce Biotechnology. Cell culture medium, reagents, and all DNA primers were obtained from Invitrogen. Mouse monoclonal anti-GFP antibody was purchased from Roche Applied Science. Rabbit polyclonal anti-PKCδ antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-mouse IgG was purchased from Bio-Rad.

Construction of GST- and GFP-fused C1 Domains of MRCKα and MRCKβ—The C1 domains of MRCK α and β were generated by PCR using the Platinum® *Pfx* DNA polymerase (Invitrogen). The full-length cDNA clones of *Homo sapiens* myotonic dystrophy kinase-related CDC42-binding protein kinase α (DMPK-like) (MRCKα) and *H. sapiens* myotonic dystrophy kinase-related CDC42-binding protein kinase β (DMPK-like) (MRCKβ) were used as the templates. The following oligonucleotides were used as the PCR primers to pull out the targeted C1 domains: (i) forward and reverse primers for the C1 domain of MRCKα were 5′-CGG GAT CCA GCC TGG TTC AAC TGG CTT T-3′ and 5′-CGG TCG ACG TCA TGT TCC TAT TCC TTT CTG-3′ (3905–3953); (ii) forward and reverse primers for MRCKβ were 5′-AT G AA T TC AGG CCC TGG CTC TGG C-3′ and 5′-AT GTC GAC T GCA CGT CCA CGC CCA-3′ (3330–3561). The C1 domains of MRCK α and β were subcloned into the pGEX vector to produce GST fusion proteins in *Escherichia coli* strain BL21. To produce a construct suitable for mammalian expression and confocal studies, the cDNAs of the C1 domains of MRCK α and β were amplified by PCR and subcloned into the pEGFP vector (Clontech, Palo Alto, CA). The DNA sequence of each construct was confirmed by sequencing analysis (DNA Minicore, Center for Cancer Research, NCI, National Institutes of Health).

Expression in E. coli and Purification of the C1 Domains of MRCK α and β—The recombinant plasmids of the individual C1 domains of MRCK α and β were transformed into BL-21-Gold (DB3) *E. coli* strain BL21. The expression of the GST fusion proteins was induced with 0.5 mM isopropyl-*O*-D-thiogalactopyranoside that was added when the optical density of the Luria Bertani medium containing bacteria (Quality Biological, Inc., Gaithersburg, MD) reached 0.5–0.7. The bacteria were harvested after 4 h of induction at 37 °C. The expressed GST-tagged C1 protein was purified using a B-PER GST spin purification kit (Pierce). The purified GST-C1 proteins were stored in 30% glycerol at −70 °C.

Measurement of [^{3}H]PDBu Binding—[^{3}H]PDBu binding to the C1 domains of MRCKα/β and PKCα/δ was measured using the polyethylene glycol precipitation assay developed in our laboratory (19) with minor modifications. Briefly, the assay mixture (250 μl) containing 50 mM Tris-Cl, pH 7.4, 100 μg/ml phosphatidylserine, 4 mg/ml bovine immunoglobulin G, 0.1 mM $CaCl_2$, and various concentrations of [^{3}H]PDBu was incubated at 18 °C for 10 min in 1.5-ml microfuge tubes. Samples were then chilled on ice for 7 min, 200 μl of 35% polyethylene glycol in 50 mM Tris-Cl, pH 7.4, was added, and the samples were mixed and incubated on ice for an additional 10 min. The tubes were then centrifuged in a Beckman Allegra 21R centrifuge at 4 °C (12,200 rpm, 15 min). A 100-μl aliquot of the supernatant from each tube was removed for determination of the free concentration of [^{3}H]PDBu; the remaining supernatant was discarded, and the pellet was carefully dried. The tip of the centrifuge tube containing the pellet was cut off and transferred to a scintillation vial for the determination of the total bound [^{3}H]PDBu. Radioactivity was determined by scintillation counting. Nonspecific binding was determined in the presence of 30 μM non-radioactive PDBu. Specific binding was calculated as the difference between total and nonspecific binding. To measure competition of [^{3}H]PDBu binding by different compounds, assays were performed under similar conditions but using a fixed concentration of [^{3}H]PDBu and increasing concentrations of the nonradioactive ligand. In a typical competition assay, six to eight different concentrations of the competing ligand were used. Standard Scatchard analysis was performed to determine the dissociation constants (K_d) of the individual C1 domains, and the inhibitory dissociation constants (K_i) were calculated using our standard method as described previously (19). Binding data of 1,2-dioctanoyl glycerol to the C1 domain of PKCα were analyzed by the modified Hill equation. Values of specific binding were determined in triplicate at each ligand concentration in each experiment. All binding experiments were performed at least three times unless otherwise specified.

Expression and Imaging of GFP-tagged C1 Domains in Living Cells—LNCaP cells (obtained from ATCC, Manassas, VA) were cultured at 37 °C in RPMI 1640 containing 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (0.05 mg/ml) in a 5% CO_2 atmosphere. The plasmid DNAs of the GFP-fused individual C1 domain of MRCKα and the C1b domain of PKCδ were transfected into LNCaP cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The translocation of C1 domains was determined 24 h after transfection. Cells were examined with a Zeiss LSM 510 confocal microscope (Carl Zeiss Inc., Thornwood, NY) with an Axiovert 100 M inverted microscope operating with a 25-milliwatt argon laser tuned to 488 nm. Cells were imaged with a 63 × 1.4 NA Zeiss Plan-Apochromat oil immersion objective and with varying zooms (1.4–2). Time lapse images were col-

lected before and after administration of PMA every 30 s using the Zeiss AIM software, in which the green emission was collected in a photomultiplier with an LP 505 filter.

Preparation of Cell Fractions and Immunoblot Analysis of Membrane Translocation of the C1 Domain of MRCKα and the C1b Domain of PKCδ in LNCaP Cells—LNCaP cells (obtained from ATCC) were seeded in 60-mm Petri dishes. 24 h after transfection with the C1 domain of MRCKα or the C1b domain of PKCδ, the cells were treated with PMA (0.001–30 μM) for 30 min. The cells were washed with ice-cold Dulbecco's phosphate-buffered saline (KD Medical, Inc., Columbia, MD) and then harvested with 120 μl of 50 mM Tris-HCl, pH 7.4, plus protease inhibitor mixture (Roche Applied Science). Cells were frozen for 1 min in a dry ice-ethanol mixture and subsequently thawed for 1 min in a 37 °C water bath; the process was repeated eight times. 100 μl of the cell lysate was transferred to a Beckman ultracentrifuge tube and centrifuged at 200,000 × *g* for 2 h to separate the cytosolic and membrane fractions; the remaining 20 μl was used as the total cell fraction. After ultracentrifugation, the supernatant was designated as the cytosolic fraction. The pellet was resuspended in 100 μl of lysis buffer (50 mM Tris-Cl, pH 7.4, protease inhibitor mixture, and 1% Nonidet P-40) (Calbiochem) and incubated on ice for 1 h. This mixture was again subjected to centrifugation (100,000 × *g* for 1 h). This supernatant was designated as the membrane fraction. Each sample were diluted in 2× SDS buffer and boiled 10 min on 100 °C. An equal volume of sample (18 μl) for each fraction was subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After the membranes were blocked with 5% nonfat milk in phosphate-buffered saline for 1 h, the blots were probed with anti-GFP monoclonal antibody (Roche Applied Science) and the appropriate secondary antibody. The signal was developed by enhanced chemiluminescence (ECL) (Amersham Biosciences) and imaged on BioMax MAX films (Kodak). Bands were quantified densitometrically using ImageJ software (NIH Image). In a typical membrane translocation assay, ten concentrations of ligand were used.

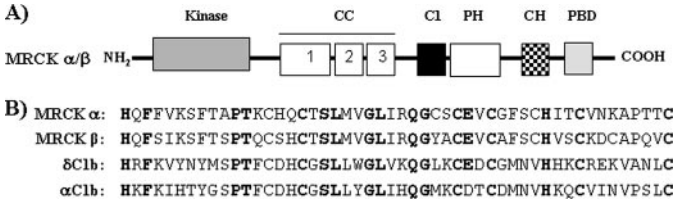

FIGURE 1. **Structures of MRCK α and β.** *A*, schematic structures of MRCK α and β. *CC*, coiled-coil; *C1*, cysteine-rich domain; *PH*, pleckstrin homology domain; *CH*, citron homology domain; *PBD*, p21 GTPase binding domain. *B*, comparison of the C1 domains of MRCK α and β and the C1b domains of PKC α and δ.

RESULTS

Binding of Phorbol Esters to the Recombinant C1 Domains of MRCKα and MRCKβ—The structures of MRCK α and β (20) are shown schematically in Fig. 1*A*. Both MRCK isoforms contain C1 domains that show high sequence homology with phorbol ester binding C1 domains in PKC isoforms (Fig. 1*B*), and indeed the C1 domain of MRCKα was shown directly to bind phorbol ester although it was not further evaluated (18). We wished to determine the binding characteristics of the C1 domains of these proteins for direct comparison with the well characterized domains of protein kinase C isoforms.

The isolated C1 domains of MRCK α and β were expressed as GST fusion proteins in *E. coli* and partially purified as described under "Experimental Procedures." [^{3}H]PDBu binding was measured in the presence of 100 μg/ml of phosphatidylserine. As is the case for the C1 domains of protein kinase C, [^{3}H]PDBu (20 nM) bound rapidly to the C1 domains of MRCK α and β; a maximal level of binding was obtained between 10 and 15 min (data not shown) at 18 °C and slowly decreased thereafter. A similar level of binding was observed at 0 °C (a single experiment), whereas binding at 37 °C was substantially less. For subsequent analysis, we therefore used a 10-min incubation time at 18 °C.

Under these conditions, Scatchard analysis revealed that C1 domains of MRCK α and β bound [^{3}H]PDBu with dissociation constants (K_d values) of 10.3 ± 2.0 and 17 ± 1.2 nM, respectively (mean ± S.E., n = 3 experiments), in the presence of 0.1 mM

TABLE 1

Structure-activity analysis of binding to the C1 domain of MRCK α/β, PKCα C1b, and PKCδ C1b

The K_d values for PDBu binding were measured directly with [^{3}H]PDBu. The ID_{50} values for the other compounds were determined from competition curves for inhibition of [^{3}H]PDBu binding, and the corresponding K_i values were calculated as indicated under "Experimental Procedures." Values represent the mean ± S.E. of three experiments per group.[1], from Ref. 26. *, calculated using the modified Hill equation.

	Phorbol ester (PDBU)	1,2-dioctanoyl glycerol	DAG-lactone (HK-434)	DAG-lactone (130C045)	Sapintoxin D
MRCKα C1	10.3 ± 2.0 nM	2820 ± 260 nM	1033 ± 81 nM	22600 ± 1700 nM	5.9 ± 1.8 nM
MRCKβ C1	17 ± 1.2 nM	6960 ± 300 nM	1070 ± 110 nM	26000 ± 4500 nM	12.6 ± 2.1 nM
PKCα C1b	3.40 ± 0.12 nM[1]	6698 ± 1320 nM*	402 ± 5.4 nM	4200 ± 500 nM[1]	18.2 ± 1.8 nM
PKCδ C1b	0.177 ± 0.004 nM	6.8 ± 0.636 nM	1.16 ± 0.07 nM	1.54 ± 0.22 nM[1]	0.14 ± 0.02 nM

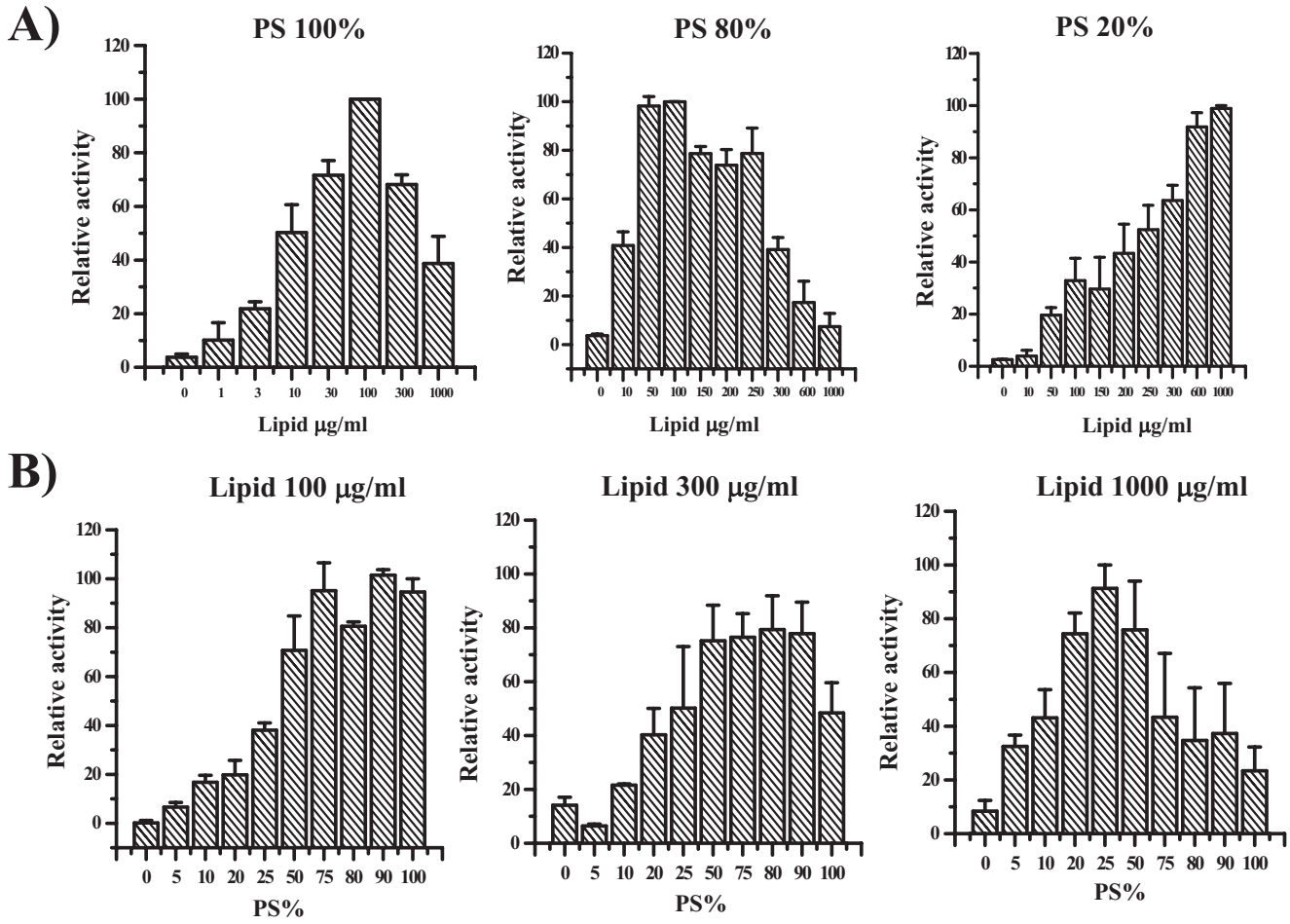

FIGURE 2. **Effect of phospholipid composition on [^{3}H]PDBu binding to the C1 domain of MRCKα.** Binding of specific [^{3}H]PDBu (9 nM) was performed in the presence of 0.1 mM $CaCl_2$ and increasing concentrations of PS:PC (100:0, 80:20, or 20:80, as indicated) (*A*) or increasing proportions of PS in PS:PC mixtures at total amounts of phospholipid of 100, 300, or 1000 μg/ml (*B*). Values represent the mean of four independent experiments. S.E. values are represented by the *error bars*. In each individual experiment, the maximal mean specific [^{3}H]PDBu binding value for a specific lipid condition was set to 100% and the mean specific [^{3}H]PDBu binding values under the other lipid conditions were normalized to that value. Because in the replicate experiments in *B* the same proportion of PS did not always represent the optimal binding condition, the maximal mean values were consequently not always 100%. *PS*, phosphatidylserine; *PC*, phosphatidylcholine.

$CaCl_2$. These values were 58- and 96-fold weaker than that for the C1b domain of PKCδ (K_d = 0.18 ± 0.004 nM) and 3- and 5-fold weaker than for the C1b domain of PKCα (K_d = 3.4 ± 0.12 nM) (mean ± S.E., n = 3, experiments performed in parallel for the C1 domains of PKCδ, MRCKα, and MRCKβ) (Table 1; supplemental Fig. S1). These results confirm that the C1 domains are able to bind phorbol ester but suggest that the binding affinities, at least of the isolated C1 domains, are appreciably weaker than those of the C1b domain of PKC δ or α.

PDBu Binding to the C1 Domains of MRCKα and MRCKβ as a Function of Phosphatidylserine Concentration and the Total Amount of Phospholipids—Acidic phospholipids are essential cofactors for the activation of PKC (21), with phosphatidylserine being the most effective phospholipid for supporting phorbol ester/DAG binding to PKC, n-chimerin, and Unc-13 (22–24). Because of the relatively low affinity binding of [^{3}H]PDBu to the C1 domains of MRCK α and β, we wished to determine whether some lipid conditions other than those selected would lead to enhanced affinities. In the first series of experiments, we determined the specific binding of [^{3}H]PDBu to the MRCKα C1 domain as a function of total phospholipid at various fixed proportions of phosphatidylserine (Fig. 2*A*). Under all conditions, the [^{3}H]PDBu binding was dependent on the presence of phospholipid. It also showed a marked dependence on phosphatidylserine and, at high proportions of phosphatidylserine, showed inhibition at excess amounts of total lipid. Under our standard conditions of 100% phosphatidylserine, the level of binding was optimal at 100 μg/ml phospholipid and decreased at higher concentrations. In contrast, at 20% phosphatidylserine maximal binding was only achieved at 1000 μg/ml total lipid.

A similar pattern was observed when the proportion of phosphatidylserine was varied at various fixed amounts of total phospholipid (Fig. 2*B*). At 100 μg/ml of total phospholipid, maximal binding was seen at 75% or more phosphatidylserine. In contrast, at 1000 μg/ml total phospholipid, optimal [^{3}H]PDBu binding was observed at 25% phosphatidylserine, with decreasing binding at either higher or lower proportions.

In single experiments, we obtained similar results for the C1 domain of MRCKβ, both for the optimal amount of total phospholipid at 20 and 80% phosphatidylserine and for the optimal % phosphatidylserine at 300 and 1000 μg/ml total phospholipid. We conclude that the requirements of the C1 domains of MRCK for phosphatidylserine are appreciably higher than that

of the PKCδ C1b domain, which retains 50% of maximal binding in the absence of phosphatidylserine at 100 μg/ml total phospholipid (16). Furthermore, our binding conditions appear appropriate and do not readily explain the relatively low affinity observed for the C1 domains of MRCK α and β.

Structure Activity Analysis of Binding to the C1 Domain of MRCKα/β—Different C1 domains show different patterns of ligand recognition (25, 26). For comparison of the ligand selectivity of the C1 domains of MRCK α and β with that of other C1 domains, we performed competition binding studies using several structurally diverse, high affinity ligands for other C1 domains (Table 1). Competition experiments with the DAG lactone HK-434 (27) revealed 1000-fold lower affinities for MRCK α and β than for the C1b domain of PKCδ but only 2.5-fold weaker affinity than for the C1b domain of PKCα (supplemental Fig. S2). The results for 1,2-dioctanoyl glycerol, for the DAG lactone 130C045 (26), and for sapintoxin D revealed a similar pattern. Both the C1 domains of MRCK α and β and the C1b domain of PKCα bound 1,2-dioctanoyl glycerol with 400–1000-fold weaker affinity than did the C1b domain of PKCδ. Of incidental note, the measurement of the binding affinity of 1,2-dioctanoyl glycerol to the C1b domain of PKCα should be considered approximate because of non-theoretical behavior, probably reflecting a secondary effect of enhanced PDBu affinity as 1,2-dioctanoyl glycerol partitions into the phosphatidylserine in the assay. The C1 domain of MRCK α and β displayed 15,000-fold weaker binding of 130C045 than did the C1 domain of PKCδ, compared with only 5–6-fold weaker binding relative to the C1b domain of PKCα. For sapintoxin D, the C1 domains of MRCK α and β indeed bound with modestly higher affinity than did the C1b domain of PKCα, whereas once again the C1b domain of PKCδ bound with 40–90-fold stronger affinity. We conclude that the C1 domains of MRCK α and β show ligand selectivity similar to the C1b domain of PKCα, at least for this series of ligands. Relative to the C1b domain of PKCδ, the C1 domains of MRCK α and β bind the other ligands examined not appreciably better than they do PDBu, and they bind diacylglycerol, the physiological ligand, markedly more poorly.

Comparison of Translocation to the Membrane of the C1 Domain of MRCKα and the C1b Domain of PKCδ in Response to PMA—We were concerned that the apparent weak affinity of the C1 domain of MRCKα for phorbol ester might reflect an inappropriate environment under our assay conditions, despite our efforts to optimize the lipid conditions. We therefore examined the ability of the C1 domain of MRCKα to undergo translocation from the cytosol to the membrane as a function of phorbol ester concentration and compared its sensitivity with that of the C1b domain of PKCδ. We prepared the C1 domain of MRCKα as a fusion construct with GPF and expressed it in the LNCaP human cell line. The transfected LNCaP cells were treated with various doses of PMA (0.001–30 μM) for 30 min, after which the cells were harvested, membrane and cytoplasmic fractions were prepared, and the distribution of the C1 domain of MRCKα between cytosolic and membrane fractions was determined by Western blotting using anti-GFP antibody (Fig. 3*A*). Translocation required 1–30 μM PMA, in contrast to a concentration of 0.01–0.03 μM PMA for the C1b domain of PKCδ (Fig. 3*B*). We conclude that, in intact cells as in the *in vitro* binding assays, the response of the C1 domain of MRCKα was ~100–1000-fold weaker than that of the C1b domain of PKCδ.

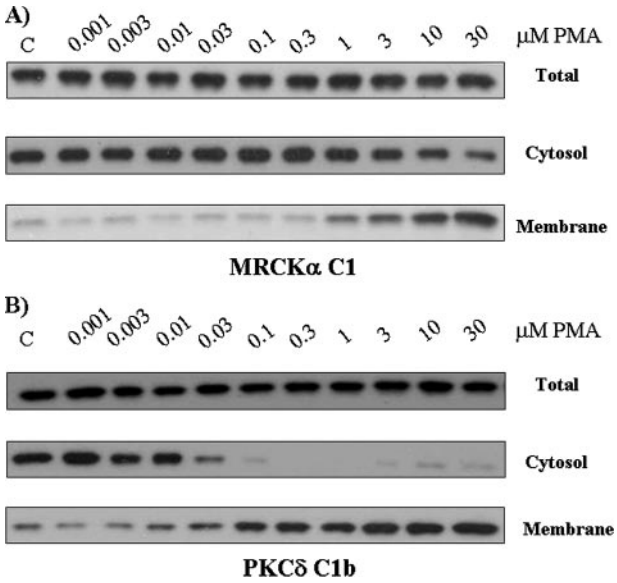

FIGURE 3. **Induction of translocation by PMA of the C1 domain of MRCKα and the C1b domain of PKCδ in LNCaP cells.** LNCaP cells were transfected with the GFP-tagged C1 domain of MRCKα (*A*) or the GFP-tagged C1b domain of PKCδ (*B*) and treated with the indicated concentrations of PMA (0.001–30 μM) for 30 min. Cells were then fractionated by ultracentrifugation into cytosolic and membrane fractions as described under "Experimental Procedures." Aliquots of the different fractions were subjected to electrophoresis on SDS-polyacrylamide gels and Western blotting with anti-GFP antibody. Similar results were obtained in four additional independent experiments.

Comparison of the Redistribution of the C1 Domain of MRCKα and the C1b Domain of PKCδ in LNCaP Cells after Phorbol Ester Treatment—Using the GFP fusion protein with the C1 domain of MRCKα, we also examined its subcellular distribution in living LNCaP cells as a function of time after treatment with various doses of PMA and compared its response with that of the C1b domain of PKCδ. The cells were maintained at room temperature and imaged with a Zeiss LSM 510 confocal microscope. Under control conditions, most of the C1 domain of MRCKα was present in the cytoplasm of the cells with lower amounts in the nucleus and perhaps a small amount associated with the plasma membrane (Fig. 4*A*). Addition of PMA led to a slow, concentration-dependent shift in the distribution of the MRCK C1 domain. Translocation only began to be observed at concentrations of PMA of 0.5 μM and higher. Translocation was to the plasma membrane, with loss of MRCK C1 domain from the nucleus also being evident. In contrast, the C1b domain of PKCδ showed slow translocation at 0.01 μM PMA and a more rapid response at higher concentrations of PMA (Fig. 4*B*). We conclude that the C1 domain of MRCKα was ~50–100-fold less responsive to PMA under these conditions in living cells. Similar results were obtained with measurements conducted at 37 °C (data not shown).

DISCUSSION

Our analysis confirms the findings of Tan *et al.* (18) that MRCK α and β represent the seventh class of proteins with phorbol ester-responsive C1 domains, along with PKC, protein kinase D, the chimerins, RasGRP, munc-13, and the DAG

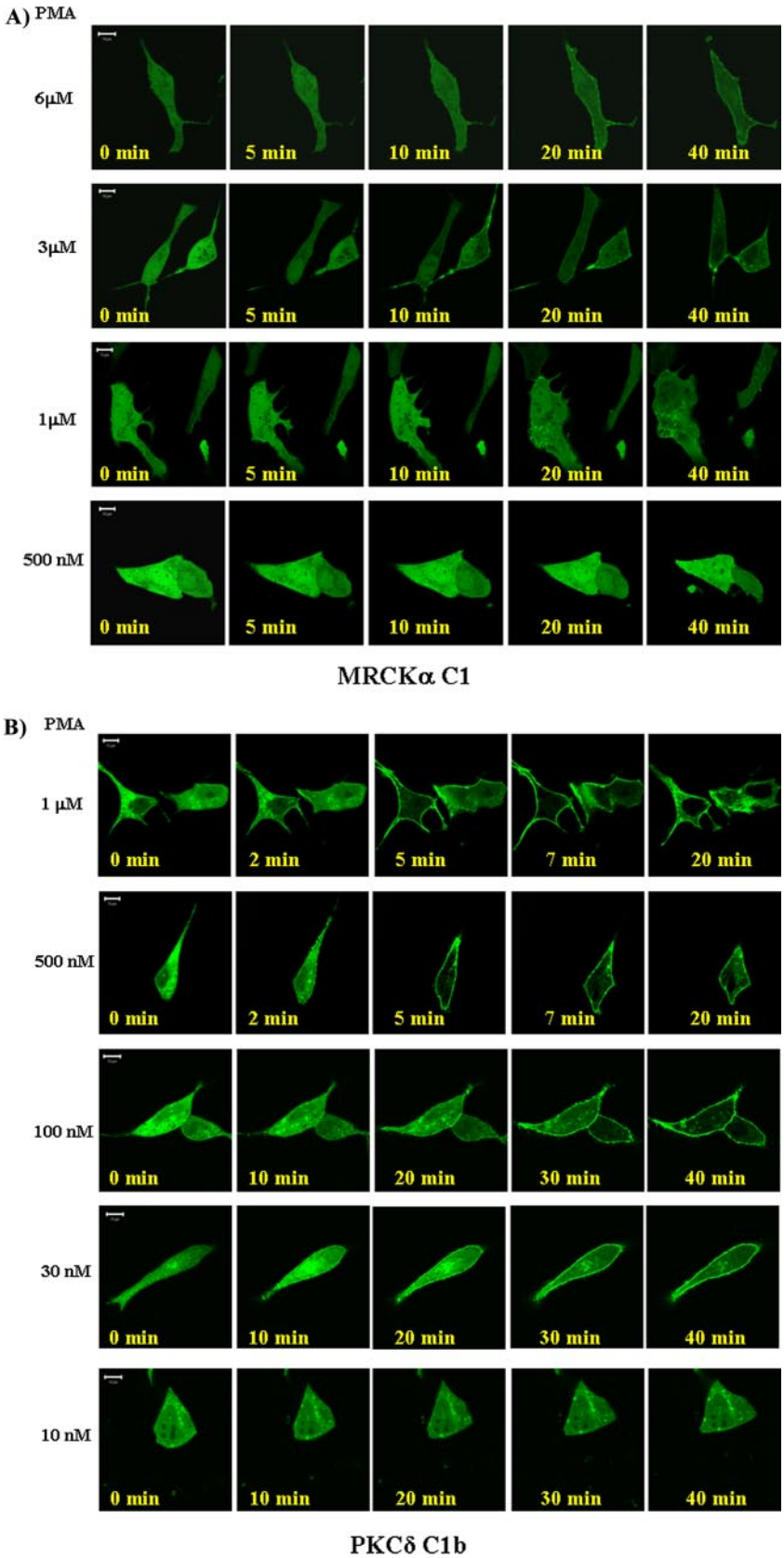

FIGURE 4. **Subcellular redistribution of the GFP-tagged C1 domain of MRCKα following PMA treatment in living LNCaP cells.** LNCaP cells were transiently transfected with the GFP-tagged C1 domain of MRCKα (*A*) or the GFP-tagged C1b domain of PKCδ (*B*). Live cells were treated for 20 or 40 min with different concentrations of PMA (0.01–6 μM), and the translocation of the green fluorescent proteins was recorded as a function of time after the addition of compound using a Zeiss LSM 510 laser-scanning confocal microscope as indicated under "Experimental Procedures" (*scale bar* on each figure represents 10 μm). The time in each panel represents the period after the drug administration. Images are from single, representative experiments. Each experiment was repeated at least three additional times with similar results.

kinases. MRCK thus provides yet another divergent pathway for phorbol ester action. The interactions of phorbol esters with the pathways involving the small GTPases are extensive. The chimerins function as GTPase-activating proteins for Rac, reducing Rac activity. The RasGRP proteins function as GTP exchange factors for Ras and Rap, enhancing their activity. PKC activates RasGRP1/3 by phosphorylation and has additional effects downstream of Ras. Finally, the MRCK proteins function downstream of Cdc42. One of the very early observations about the response of cultured cells to phorbol esters was their dramatic effect on cellular morphology (28). The identification of the multiple targets of phorbol ester action involved in aspects of morphology emphasizes the complexity underlying this observation.

The C1 domains of MRCK α and β provide further examples of the diversity of behavior within C1 domains. The differences for the binding affinity of PDBu are in general larger than those reported by Irie *et al.* (25) among the C1 domains from previously characterized families of phorbol ester receptors. As observed by us and others, different C1 domains differ in their ligand selectivities (25, 26). Thus, for example, 130C037 has a K_d of 1.8 nM for the C1b domain of PKCδ and a value of >10 μM for that of PKCα. Likewise, depending on the ligand, the difference in selectivity between the C1 domain of MRCKα and the C1b domain of PKCδ ranged from 42-fold for sapintoxin D to 15000-fold for 130C045. These results argue that it is not simply that the MRCK C1 domains have low affinity for ligands but rather that they show altered structure activity relations.

The context in which the C1 domains are present plays an important role in their function. Canacarajah *et al.* (29) have elegantly demonstrated with β2-chimerin that the C1 domain is shielded by other residues in the intact protein and that unfolding of the protein markedly enhances the potency of phorbol ester for inducing translocation. Less direct studies suggest a similar situation for the PKCs (30). For the PKCs, a mechanistic basis for such effects is the presence of other membrane- or ligand-interacting domains, for example the C2 domains of the classical PKCs that contribute, along with phorbol ester binding to the C1 domain, to the overall energetics of membrane association (31). Thus, whereas the C1b domain of PKCδ binds the selective constrained DAG lactone 130C037 with 300-fold higher affinity than does either of the two isolated C1 domains of PKCα, the intact PKCδ binds 130C037 with only 4-fold stronger affinity than does intact PKCα (26). Our present results with MRCK argue that other structural elements will be needed to drive a robust response to phorbol ester or diacylglycerol.

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