Multiple Domains of MCIP1 Contribute to Inhibition of Calcineurin Activity*

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Calcineurin is a serine/threonine protein phosphatase that plays a critical role in many physiologic processes such as T-cell activation, apoptosis, skeletal myocyte differentiation, and cardiac hypertrophy. Calcineurin-dependent signals are transduced to the nucleus by nuclear factor of activated T-cells (NFAT) transcription factors that undergo nuclear translocation upon dephosphorylation and promote transcriptional activation of target genes. Several endogenous proteins are capable of inhibiting the catalytic activity of calcineurin. Modulatory calcineurin-interacting protein 1 (MCIP1) is unique among these proteins on the basis of its pattern of expression and its function in a negative feedback loop to regulate calcineurin activity. Here we show that MCIP1 can be phosphorylated by MAPK and glyogen synthase kinase-3 and that phosphorylated MCIP1 is a substrate for calcineurin. Peptides corresponding to the substrate domain competitively inhibit calcineurin activity in vitro. However, a detailed structure/function analysis of MCIP1 reveals that either of two additional domains of MCIP1 is sufficient for binding to calcineurin in vitro and for inhibition of calcineurin activity in vivo. We conclude that MCIP1 inhibits calcineurin through mechanisms that include, but are not limited to, competition with other substrates such as nuclear factor of activated T-cells.

Calcineurin is a serine/threonine protein phosphatase originally isolated from brain extracts (1). The most thoroughly characterized calcineurin substrate proteins are members of the nuclear factor of activated T-cells (NFAT) family of transcription factors (2). Calcineurin dephosphorylates multiple residues within the regulatory domain of NFAT, leading to its nuclear translocation and activation of target genes through co-operation with multiple partners including AP-1 (3), MEF2 (4, 5), and GATA (6, 7) proteins. The calcineurin/NFAT pathway was first described in the activation of T-cells upon antigen presentation and binding to the T-cell receptor (8). However, calcineurin is involved in numerous physiologic processes that include memory formation or apoptosis in neurons (9–11) and differentiation or remodeling of skeletal and cardiac myocytes (5–7, 12–16).

The calcineurin enzyme is composed of a catalytic A subunit (CnA) and a regulatory B subunit (CnB). The binding of CnB, an EF-hand Ca$_{2+}$-binding protein, is necessary but not sufficient for enzymatic activity of CnA. Protein phosphatase activity of calcineurin is activated upon binding of Ca$_{2+}$-bound calmodulin. Calmodulin binding displaces an autoinhibitory domain (AID) located at the C-terminal portion of calcineurin, which otherwise masks the active site. Constitutively active forms of calcineurin are generated by deletion of the calmodulin binding domain and AID (17). The immunosuppressant drugs FK506 and cyclosporin exert their effects by promoting the formation of complexes between CnA and FKBP12 or cyclophilin A, respectively (18). Several other cellular inhibitors of calcineurin have also been identified. AKAP79 is a scaffolding protein that co-localizes protein kinase A and calcineurin to specific locations within the cell and has been shown to inhibit the protein phosphatase activity of calcineurin (19). Cabin1/Cain was identified in a yeast two-hybrid screen as a calcineurin-binding protein and is capable of inhibiting nuclear translocation of NFAT and calcium-dependent activation of the IL-2 promoter in T-cells (20). Calcineurin B homology protein shares a high degree of similarity with the CnB subunit but inhibits calcineurin phosphatase activity in vitro and prevents NFAT translocation when overexpressed in Jurkat cells (21).

Modulatory calcineurin-interacting proteins (MCIPs) represent a new class of endogenous calcineurin inhibitors (22–25). Three mammalian MCIP genes have been identified: MCIP1/DSCR1, MCIP2/DSCR1L1, and MCIP3/DSCR1L2. Orthologues of MCIP genes are present in numerous organisms, including unicellular eukaryotes (26). All members of this family examined to date are capable of binding and inhibiting calcineurin, but each of the MCIP mammalian genes exhibit distinctive patterns of gene regulation. For example, expression of one particular splice variant of the MCIP1 gene is activated potently by calcineurin due to the presence of multiple consensus NFAT binding sites within an internal promoter region (27). Transcription of other splice variants of MCIP1 is initiated from different promoters and is not responsive to calcineurin activation. Likewise, expression of the MCIP2 gene
is not activated by calcineurin but responds to thyroid hormone (27). MCIP1 is unique, therefore, among known calcineurin inhibitory proteins because it can function as an endogenous feedback inhibitor.

MCIP proteins share a structural motif with NFAT proteins termed the SP repeat domain. Peptides derived from the SP repeat domain of human MCIP1 were shown previously to inhibit calcineurin activity (23), but here we examine the calcineurin inhibitory properties of MCIP1 protein in greater detail. MCIP1 itself can serve as a substrate for protein phosphatase activity of calcineurin, thereby providing a mechanism for competitive inhibition of the enzyme. Although the SP repeat of MCIP is sufficient for competitive inhibition of calcineurin activity in vitro, this region is neither necessary nor sufficient for either binding or inhibition of calcineurin in vivo. At least two additional and discrete domains from the MCIP1 protein are capable of inhibiting calcineurin in vivo in the absence of the SP repeat domain and are sufficient to bind calcineurin and to block NFAT binding in vitro. These findings support a competitive antagonist function for MCIP1 with respect to calcineurin action on NFAT proteins but suggest complex modes of interaction that may include additional inhibitory mechanisms.

EXPERIMENTAL PROCEDURES

Tissue Culture, Cell Transfection, and Reporter Gene Assays—C2C12 myoblasts were grown and maintained in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum. Reporter gene assays were performed on myoblasts plated on 12-well culture dishes transfected with 0.5 μg of each plasmid unless noted otherwise using LipofectAMINE 2000 (Invitrogen). Cells were harvested ~40 h after transfection in reporter lysis buffer (Promega). Luciferase activity was measured using the Luciferase Assay System (Promega). β-galactosidase activity was measured using the Galacto-light Plus system (Tropix, Inc.) and used to normalize the reporter assay results. All transfection data are presented as means (with standard errors of mean) of two or three separate transfection experiments each done in triplicate.

Plasmid Constructs—The mouse MCIP1 deletions were generated by PCR using the following primers: MCIP1 forward, 5'-ATGATTGTATTTA-GGACATTTT-3'; MCIP1 reverse, 5'-TCAGCTAAGGTGGATCGGTGT-3'; MCIP1102 forward, 5'-TGCCACCCCCGTCATAAATTAC-3'; MCIP1 reverse, 5'-TCAGCTAAGGTGGATCGGTGT-3'; MCIP1124 reverse, 5'-TCACAGTTGGGACCACCACT-3'; MCIP1152 reverse, 5'-TTAGTCCGGATTTGGGGGAGC-3'. Each MCIP1 deletion DNA fragment was ligated to the C terminus of green fluorescent protein (GFP) by subcloning into the EcoRI site of the pEGFP-C1 vector (CLONTECH). The mammalian expression vector pEGFP-C1 encoding constitutively active c-Jun N-terminal kinase (c-Jun*-A) (13), NRE-Luc (NFAT-responsive element-luciferase) (28), and the pCMV-lacZ reporter (24) constructs were described previously. The vector encoding full-length rat calcineurin subunit (a gift from Diane Barber, University of California San Francisco) was described previously (21).

The bacterial expression vector GST-MCIP1 was constructed by subcloning a cDNA fragment encoding the mouse MCIP1 protein into the EcoRI site of the pGEX vector (29). Site-directed mutagenesis was performed using the QuikChange kit (Stratagene). GST-NFAT contains the first 306 amino acid residues of the human NFAT-c2 protein in the pGEX vector (13).

For yeast two-hybrid experiments, a DNA fragment encoding amino acids 1–388 of the calcineurin A subunit (CaN*) was ligated into the EcoRI site of the pBridget vector (CLONTECH), placing it in-frame with the Gal4 DNA binding domain (CaN*-BD). A DNA fragment encoding the mouse MCIP1 protein linked in-frame with a nuclear localization sequence was ligated into the NolI and BglII sites of pBridget CaN*-BD vector, creating pBridget CaN*-BD/MCIP (–Met) plasmid. This pBridget plasmid construction places MCIP1 expression under the control of the Pmec promoter, thereby expressing nuclear-localized MCIP1 in the absence of methionine (–Met). In addition, a cDNA fragment encoding full-length MCIP1 was ligated into the pACT2 vector (CLONTECH) in-frame with the Gal4 activation domain (MCIP-AD). The VIIVT peptide (30) was fused to the Gal4 activation domain (VIVIT-AD) by annealing the oligonucleotides 5'-CATGGCCGGACACACCGCCGCCG-3' and 5'-AATCTCCTGGTTGACTATTACCACACCGGGG-3' and ligating to the pACT2 vector.

Calmodulin Pull-down Assay—C2C12 myoblasts were transfected with mammalian expression constructs encoding full-length calcineurin A and GFP or each of the GFP-MCIP fusion constructs. Forty h after transfection, cells were lysed in 500 μl of pull-down buffer (50 mM Tris (pH 7.4), 100 mM NaCl, 0.2% Triton X-100, and 0.5 mg/ml bovine serum albumin) supplemented with Complete protease inhibitors (Roche Molecular Biochemicals) for 30 min at 4 °C. The lysates were centrifuged at 13,000 × g for 10 min. Twenty-five μl of calmodulin-agarose (Sigma) equilibrated with pull-down buffer was added to the cleared lysate, and the sample was rotated at 4 °C for 1 h. The calmodulin-agarose beads were washed three times with 500 μl of pull-down buffer, resuspended in an equal volume of 2× SDS-PAGE loading buffer, and boiled for 3 min. The proteins were resolved on SDS-PAGE and transferred to Hybond membranes (Amersham Biosciences) for Western blot analysis. The membrane was probed with monoclonal antibodies recognizing GFP (CLONTECH) or c-Myc (Roche Molecular Biochemicals). SuperSignal reagent (Pierce) was used for detection.

Alkaline Phosphatase Treatment—C2C12 myoblasts were transfected with an expression construct encoding human MCIP1 with an N-terminal c-Myc affinity tag. Cells were lysed 18 h after transfection in phosphate-buffered saline plus 0.1% Triton X-100 and Complete Protease Inhibitors (Roche Molecular Biochemicals). Lysates were cleared by centrifugation at 13,000 × g for 1 min and treated with 1 unit of calf alkaline phosphatase (Roche Molecular Biochemicals) per 100 μl of cleared lysate at room temperature for 30 min. Samples were processed for Western blot analysis as described above.

In Vitro Kinase and Calcineurin Assays—Recombinant GST-MCIP1 and GST-NFAT proteins were purified with glutathione-Sepharose beads per the manufacturer's instructions (Amersham Biosciences). GST fusion protein bound to glutathione-Sepharose beads was phosphorylated in kinase buffer (50 mM Tris, pH 7.4, 10 mM NaCl) with [γ-32P]ATP (Amersham Biosciences) with p42/44 mitogen-activated protein kinase MAPK (Sigma) or protein kinase A (Sigma) at 30 °C for 30 min. Phosphorylation by GSK-3 (Calbiochem) was performed by first phosphorylating GST-MCIP1 or GST-NFAT fusion protein with either GST (26) or GST-MCIP1 protein kinase (GST-NFAT) in the presence of non-labeled ATP. The GST fusion protein was then washed three times in kinase buffer and phosphorylated with GSK-3 in the presence of [γ-32P]ATP as described above. After phosphorylation, the beads were washed three times and then resuspended in calcineurin assay buffer (100 mM HEPES, pH 7.4, 100 mM NaCl, 20 mM potassium acetate, 2 mM magnesium acetate, 2 mM CaCl2, 2 mM MnCl2). Calmodulin was added immediately after addition of NFAT fusion protein with either GST (26) or GST-MCIP1 protein kinase (GST-NFAT). Cells were incubated at 30 °C for 1 h. The beads were spun down, resuspended in an equal volume of 2× SDS-PAGE loading buffer, and boiled for 3 min. The proteins were resolved on SDS-PAGE, and protein phosphorylation state was detected by autoradiography.

A portion of the MCIP1 protein corresponding to the SP repeat peptide, HLAAPPNDPDKLQISSLAPPVGVK, was synthesized at the Biopolymer Facility, University of Texas Southwestern Medical Center. The calcineurin AID peptide was purchased from Biomet. A dose-response assay was performed in calcineurin assay buffer containing 200 μM RII peptide substrate (Biomol), 2 μM calmodulin, and 1 unit of calcineurin (Sigma) in the presence of varying concentrations of inhibitor peptide at 30 °C for 10 min. Phosphatase activity was detected by the addition of Malachite green reagent (Biomol) and measured at 620 μM.

Lineweaver-Burk analysis of the SP repeat peptide was determined using the phosphorylase assay performed as described above except with 50, 100, 150, or 200 μM RII peptide substrate with either no inhibitor or a fixed 0.5 μM SP peptide concentration.

Phosphorylation and Dephosphorylation of SP Repeat Domain of MCIP1—Phosphorylation of residues within the SP repeat domain of calcineurin in vivo for either binding or inhibition of calcineurin was investigated. Subsequent dephosphorylation of these sites by calcineurin leads to nuclear translocation of NFAT and
activation of target gene transcription. Consensus phosphorylation sites for p42/44 MAPK and GSK-3 are present in the SP repeat of MCIP1 (A). The putative phosphorylated serine residues are highlighted in bold. Recombinant MCIP1 protein was phosphorylated in vitro with [γ-32P]ATP by purified MAPK and/or GSK-3 (B). For phosphorylation by GSK-3, MCIP1 protein was first phosphorylated by MAPK and unlabeled ATP followed by phosphorylation by GSK-3 and [γ-32P]ATP. MCIP1 protein phosphorylated by either MAPK or GSK-3 was incubated with increasing amounts of purified calcineurin enzyme (C). NFAT protein phosphorylated by protein kinase A (PKA) or GSK-3 is shown as a positive control for calcineurin activity.

Because the phosphorylated SP repeats of NFAT serve as calcineurin substrates, we sought to determine whether the same was true of MCIP1 (Fig. 1C). MCIP1 was phosphorylated on serine 112 and 108 by MAPK and GSK-3, respectively. In addition, recombinant NFAT was phosphorylated by protein kinase A or GSK-3 to serve as a positive control. Increasing amounts of calcineurin were used to dephosphorylate the proteins. Although calcineurin efficiently dephosphorylated NFAT labeled by either protein kinase A or GSK-3, only the form of MCIP1 labeled by GSK-3 at serine 108 was dephosphorylated by calcineurin. These data suggest that MCIP can serve as a calcineurin substrate and may possibly act as a competitive inhibitor of calcineurin with respect to NFAT and other substrates, such as MEF-2 transcription factors (5).

**MCIP1 Inhibition of Calcineurin Activity in Vitro**—Previously, it has been shown that peptides corresponding to the SP repeat of MCIP1 or the AID of calcineurin were compared for their ability to inhibit calcineurin catalytic activity toward the RII phosphopeptide (A). Increasing concentrations of each inhibitory peptide were used to obtain a dose-response curve. Lineweaver-Burk analysis was used to examine the inhibitory mechanism of the SP repeat peptide (B). The SP repeat concentration was fixed (50 mM) against differing substrate concentrations. This was compared against results with no inhibitor present.

**FIG. 1.** MCIP1 is a substrate for calcineurin in vitro. Consensus phosphorylation sites for MAPK and GSK-3 are present in the SP repeat of MCIP1 (A). In vitro kinase reactions were performed with recombinant MCIP1 proteins, either wild-type or mutated forms (S108A or S112A), to determine whether MCIP1 could be phosphorylated by MAPK or GSK-3 at these positions. As shown in Fig. 1B, MAPK was capable of phosphorylating MCIP1 uniquely at serine 112. GSK-3 phosphorylates serine or threonine residues 4 amino acids N-terminal to another phosphorylated serine or threonine. Thus, a potential GSK-3 phosphorylation site is created at serine 108 after phosphorylation of serine 112 by MAPK. Following phosphorylation by MAPK in the presence of unlabeled ATP, MCIP1 was labeled by GSK-3 and [γ-32P]ATP. Mutational analysis confirmed that this phosphorylation event occurs at serine 108 since MCIP S108A was not phosphorylated by GSK-3. As expected, mutation of the MAPK phosphorylation site, serine 112, also prevented subsequent phosphorylation by GSK-3. Simultaneous phosphorylation of MCIP1 by MAPK and GSK-3 with labeled ATP confirmed these observations, and mutation of serine 112 prevented phosphorylation of MCIP1 by either kinase.

**FIG. 2.** The SP repeat is a competitive inhibitor of calcineurin activity. Peptides corresponding to the SP repeat of MCIP1 or the AID of calcineurin were compared for their ability to inhibit calcineurin catalytic activity toward the RII phosphopeptide (A). An assessment of the inhibitory mechanism of each inhibitory peptide was made using an in vitro calcineurin assay with the RII peptide substrate. The SP repeat peptide, a 24-mer peptide spanning the SP repeat domain of MCIP1, was compared with a peptide derived from the AID of calcineurin, which is known to inhibit calcineurin activity (25, 32). A dose-response curve based on increasing concentrations of peptide demonstrated that the SP repeat from MCIP1 inhibited calcineurin activity ($K_i = 91.5 \mu M$), although at a somewhat lesser efficiency than the AID peptide ($K_i = 36 \mu M$) (Fig. 2A). An assessment of the inhibitory mechanism of the SP repeat peptide was made using varying substrate concentrations against a fixed concentration of inhibitor peptide. The results shown in Fig. 2B (no change in the $V_{max}$ but an increase in the $K_m$) are consistent with the SP repeat peptide acting as a competitive inhibitor of calcineurin.
Fig. 3. Identification of MCIP1 domains necessary for inhibition of calcineurin activity. A, schematic diagram illustrating full-length MCIP1 or various deletions of the MCIP1 coding region fused to GFP. The hatched box represents the SP repeat domain. B, C2C12 myoblasts co-transfected with a calcineurin-responsive reporter construct, NRE-Luc, an expression vector containing CnA* with either GFP alone or each of the GFP-MCIP1 fusion constructs (shown in panel A). Data are expressed relative to the luciferase activity observed with CnA* without MCIP1 and represent mean values (± S.E.) from three independent experiments performed in triplicate. All results are corrected for variations in transfection efficiency by normalization to expression of a co-transfected pCMV-lacZ plasmid. C, a dose-response curve obtained using decreasing amounts of either full-length MCIP1 fused to GFP or two MCIP1 deletions comprising either the N-terminal half (MCIP192) or C-terminal half (MCIP124-198). Reporter activity is determined as percent of calcineurin activity using promoter activity (NRE-Luc) expressed with CnA* (without MCIP1) as 100% activity. Data are represented as mean (± S.E.) and corrected for differences in transfection efficiency by normalization against a co-transfected pCMV-lacZ plasmid. Data shown are the result of two or three independent experiments performed in triplicate.
MCIP1 Inhibition of Calcineurin Activity

We have demonstrated previously, using in vitro binding assays, that MCIP is capable of binding calcineurin directly (24). Here, physical associations with calcineurin were tested by in vitro binding assays using full-length and truncated forms of GFP-MCIP constructs (as shown in Fig. 3) and an expression vector encoding for the full-length calcineurin (B). GFP or GFP-MCIP1 was detected in the input or pull-down fractions by immunoblot analysis using an antibody directed against GFP.

**Fig. 4.** The SP repeat of MCIP is not necessary for inhibition of calcineurin activity. The serine-proline repeats of the SP repeat of MCIP1 were mutated to alanine-alanine (A). The phosphorylation state of MCIP1 was assessed using an antibody to c-Myc on an immunoblot containing an extract of C2C12 myoblasts transfected with an expression vector containing Myc-MCIP1, Myc-MCIP1 S108A, or Myc-MCIP1 S112A (B). Lysates were treated (+AP) or untreated (–AP) with alkaline phosphatase. C2C12 myoblasts were co-transfected with expression vectors encoding GFP fused to wild-type MCIP1 or the MCIP1-SP/AA mutant with constitutively active CnA* and a calcineurin-responsive promoter, NRE-Luc (C). Fold induction is relative to promoter activity without CnA*. Data shown are the result of two independent experiments performed in triplicate.

Multiple Domains of MCIP1 Are Sufficient to Inhibit Calcineurin Activity—Previous studies have suggested that the C terminus of the MCIP1 protein is critical for binding to and inhibition of calcineurin (22), but more precise definition of domains within MCIP1 that participate in its inhibitory activity has been lacking. Truncated or mutated forms of MCIP1 protein were fused to GFP (as illustrated in Fig. 3A) and tested for their ability to inhibit calcineurin. The GFP moiety was added to stabilize the MCIP1 fragments so as to achieve comparable expression of each variant, which was confirmed by immunoblot experiments using anti-GFP antibody (an example is shown as Input in Fig. 5B). Each of these GFP-MCIP constructs was co-transfected with CnA* and a luciferase reporter gene controlled by a calcineurin-responsive promoter, NRE-Luc. As shown in Fig. 3B, fusion of GFP to full-length MCIP did not impair its ability to inhibit calcineurin. Surprisingly, removal of the C-terminal half of MCIP (GFP-MCIP1–102), including the SP repeat, did not significantly alter its ability to inhibit calcineurin. In addition, removal of the N-terminal portion of MCIP (GFP-MCIP97–198, GFP-MCIP124–198) did not affect its inhibitory function. However, MCIP1 function is abolished when amino acids from both the N terminus and C terminus of the protein are deleted (GFP-MCIP97–161). These data suggest that domains in either the N-terminal half or the C terminus of the protein are sufficient to inhibit calcineurin activity. However, removal of both regions results in loss of MCIP inhibitory function. These differences in inhibitory potency among truncated forms of MCIP1 were confirmed by examination of dose-response relationships (Fig. 3C). As the ratio of MCIP to CnA* decreased, truncated forms of MCIP1 lacking the SP domain, MCIP102 and MCIP124–198, did not inhibit calcineurin activity to the same degree as the full-length MCIP161. Transfection assays performed with the MCIP1 exon 4 promoter region, another target of calcineurin (27), as the reporter construct instead of the NRE-Luc showed identical results to the NRE-Luc (data not shown).

The ability of MCIP1 to serve as a substrate for calcineurin (Fig. 1) is not essential for its function as an inhibitor of calcineurin. In vivo studies in C2C12 myoblasts show mutated forms of MCIP1 in which serine 108 or serine 112 are converted to alanines (Fig. 4A) migrate faster by Western blot analysis as compared with wild-type MCIP1 (Fig. 4B). The faster migrating bands were confirmed as dephosphorylated forms of MCIP1 by alkaline phosphate (AP) treatment of the transfected cell lysates. A mutated form of MCIP1 with substitutions of the serines and prolines at amino acid positions 108, 109, 112, and 113 to alanine SP/AA retains full calcineurin inhibitory activity (Fig. 4C).

Multiple Domains of MCIP1 Bind Calcineurin—We have demonstrated previously, using in vitro binding assays, that MCIP is capable of binding calcineurin directly (24). Here, physical associations with calcineurin were tested by in vitro binding assays using full-length and truncated forms of GFP-MCIP and calmodulin covalently linked to agarose beads. As
shown in Fig. 5A, MCIP protein was readily detectable in the calmodulin/calcineurin complex. The presence of MCIP in the complex did not decrease the amount of bound calcineurin, demonstrating that MCIP does not interfere with calmodulin binding to calcineurin. MCIP1 protein does not bind directly to calmodulin-agarose since MCIP1 protein was not detected when calcineurin was omitted from the assay (data not shown).

All GFP-MCIP deletions were detected in the calmodulin/cal-cineurin complex except for GFP-MCIP97–163 (Fig. 5B). These data demonstrate that the SP repeat region is neither necessary nor sufficient for binding to calcineurin in cell homogenates. Therefore, MCIP1 has at least two calcineurin binding domains lying outside the SP repeat in both the N-terminal half of the protein and the more distal C-terminal region of the protein. High concentrations of a peptide based on the SP domain of MCIP1 are capable of inhibiting purified calcineurin (Fig. 2), and its presence may contribute to the inhibitory potency of the full-length protein in vivo (Fig. 3).

MCIP1 Interferes with Interaction of Calcineurin and VIVIT Peptide—MCIP has been shown to prevent calcineurin-mediated nuclear translocation of NFAT (22). However, it has not been established whether this is solely a function of inhibition of protein phosphatase activity of calcineurin or whether MCIP also prevents binding of NFAT to calcineurin. Recently, a calcineurin docking domain of NFAT was localized to a small peptide motif (SPRIET) of the protein, and this sequence was optimized using a combinatorial peptide library to VIVIT (30).

A yeast two-hybrid system was established to determine whether MCIP1 interferes with the interaction of CnA* and this calcineurin-binding VIVIT peptide (based on the NFAT sequence). As shown in Fig. 6, physical interactions between CnA* (fused to the Gal4 transcriptional binding domain) and the VIVIT peptide sequence (fused to the Gal4 DNA activation domain) were inhibited by concomitant expression of native MCIP1. The potency with which native MCIP1 blocked binding of VIVIT to CnA* was comparable with that at which native MCIP1 blocked binding of an MCIP1-Gal4 activation domain fusion protein to CnA*. This finding suggests that MCIP1 interferes with the interaction of calcineurin and the calcineurin docking domain of NFAT.

**DISCUSSION**

Among a number of proteins known to function as endoge-nous inhibitors of calcineurin, MCIP proteins are distinctive in several respects. Unlike the intracellular targets for immunosuppressive drugs (cyclophilin and FKBP12), no exogenous molecules are required to promote their interaction with calcineurin. Unlike AKAP79 or cabin/cain, MCIP genes are ex-pressed abundantly in heart and skeletal muscles, as well as in brain. Only MCIP1 is known to be transcriptionally up-regul-ated by calcineurin activity, thereby establishing a feedback inhibition loop. Insight into the mechanisms by which MCIP proteins inhibit calcineurin is important to understanding calcineurin signaling in the context of development, plasticity, and disease.

Previous studies have shown MCIP to be a potent inhibitor of calcineurin signaling (22–25). MCIP inhibits calcineurin-mediated nuclear translocation of NFAT and activation of reporter gene constructs. MCIP forms a physical complex with the calcineurin A catalytic subunit in a manner that does not require the calmodulin binding domain of calcineurin (22, 24). In addition, MCIP proteins or peptides containing the highly conserved FLISPPASP sequence of the SP repeat domain were shown to inhibit calcineurin catalytic activity in vitro (23, 25). MCIP also blocks physiologic or pathologic changes produced by unrestrained calcineurin activity in intact animals. Transgenic mice expressing a constitutively active form of cal-

cineurin in the heart develop profound cardiac hypertrophy that progresses to dilated cardiomyopathy (6). Expression of MCIP1 blocks cardiac hypertrophy and heart failure in this transgenic model (33).

The results presented here advance our understanding of MCIP:calcineurin interactions in several important ways. New data show that MCIP1 is itself a substrate for the protein phosphatase activity of calcineurin. A specific serine residue within the conserved SP repeat domain was phosphorylated by GSK-3 and dephosphorylated by calcineurin in vitro. Studies of CBP1, the *Cryptococcus neoformans* orthologue of MCIP, sug-gest that CBP1 also is phosphorylated in vivo and is a substrate for calcineurin (25). A peptide based on this region of MCIP1 functions as a competitive inhibitor of calcineurin activity in vitro. The functional consequences of MCIP1 phosphorylation at serine 108 are unknown at this time, but non-phosphoryl-able forms of MCIP1 retain full inhibitory activity, suggesting that phosphorylation/dephosphorylation of MCIP1 has little bearing on its ability to inhibit calcineurin. Partitioning of MCIP1 between nuclear and cytoplasmic compartments of the cell likewise appears to be unaffected by the phosphorylation state of MCIP1 at serine 108 (data not shown). Interestingly, SP repeat phosphorylation mutants of CBP-1 are unstable when compared with the wild-type protein (25). We speculate...
that the phosphorylation state of mammalian MCIP proteins also may control protein stability or influence binding to proteins other than calcineurin within the cell, and experiments are underway to test these hypotheses.

Not surprisingly, we observed a direct correlation between the ability of truncated forms of MCIP1 to bind calcineurin in complex with calmodulin and their respective inhibitory potencies. An unexpected result, however, was the finding that both binding and inhibiting calcineurin, even in the absence of other regions of the protein. When MCIP1 associates with calcineurin, binding of a peptide based on the calcineurin docking domain of NFAT is blocked, but binding of calmodulin to calcineurin is unimpaired.

Taken together, these data reveal complex mechanisms of calcineurin inhibition by MCIP1. At least three discrete domains of MCIP1 participate in inhibiting calcineurin in vitro and in vivo. MCIP proteins are likely to function as competitive antagonists of calcineurin activity with respect to NFAT proteins and other calcineurin substrates. However, the data presented here raise the possibility of allosteric regulation of calcineurin activity as well. Future studies of these mechanisms would be most effectively guided by structural information based on x-ray crystallography of MCIP-calcineurin complexes.

Inhibition of calcineurin activity by FK506 and cyclosporin A has been instrumental in the success of modern transplantation medicine in humans. However, systemic toxicity precludes the administration of current pharmacological antagonists of calcineurin in doses sufficient for the prevention of cardiac diseases, the pathobiology of which calcineurin-dependent signaling events have been shown to contribute to (34). An increased understanding of the molecular mechanisms by which endogenous proteins such as MCIP1 inhibit calcineurin may open new opportunities for preventive or therapeutic interventions by rational drug design.

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