Independent Signals Control Expression of the Calcineurin Inhibitory Proteins MCIP1 and MCIP2 in Striated Muscles

John Yang, Beverly Rothermel, Rick B. Vega, Norbert Frey, Timothy A. McKinsey, Eric N. Olson, Rhonda Bassel-Duby, R. Sanders Williams

Abstract—Calcineurin, a calcium/calmodulin-regulated protein phosphatase, modulates gene expression in cardiac and skeletal muscles during development and in remodeling responses such as cardiac hypertrophy that are evoked by environmental stresses or disease. Recently, we identified two genes encoding proteins (MCIP1 and MCIP2) that are enriched in striated muscles and that interact with calcineurin to inhibit its enzymatic activity. In the present study, we show that expression of MCIP1 is regulated by calcineurin activity in hearts of mice with cardiac hypertrophy, as well as in cultured skeletal myotubes. In contrast, expression of MCIP2 in the heart is not altered by activated calcineurin but responds to thyroid hormone, which has no effect on MCIP1. A 900-bp intragenic segment located between exons 3 and 4 of the MCIP1 gene functions as an alternative promoter that responds to calcineurin. This region includes a dense cluster of 15 consensus binding sites for NF-AT transcription factors. Because MCIP proteins can inhibit calcineurin, these results suggest that MCIP1 participates in a negative feedback circuit to diminish potentially deleterious effects of unrestrained calcineurin activity in cardiac and skeletal myocytes. Inhibitory effects of MCIP2 on calcineurin activity may be pertinent to gene switching events driven by thyroid hormone in striated muscles. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2000;87:e61-e68.)

Key Words: calcineurin • hypertrophy • gene transcription • thyroid hormone

Changes in intracellular calcium concentrations control gene expression in many cell types by calmodulin-dependent activation of calcineurin, a serine-threonine protein phosphatase.1,2 Signaling pathways controlled by calcineurin have been most extensively characterized in lymphocytes, where binding of antigen to cell surface receptors triggers calcium entry in a pattern that activates calcineurin, which then removes phosphate groups from transcriptional regulatory proteins of the NF-AT family. Dephosphorylated NF-AT proteins translocate from the cytoplasm to the nucleus, where they bind cognate recognition elements within target genes, in association with other transcription factors such as AP-1. Downstream genes required for T-cell activation (eg, interleukin-2) are induced in this manner. The pharmacological actions of immunosuppressive drugs such as cyclosporin A and FK-506 are based on inhibition of calcineurin activity in immune effector cells.

Recent studies also have revealed roles for calcineurin-dependent signaling pathways in cardiac and skeletal muscles. Transgenic mice that express constitutively active forms of calcineurin or NF-AT3 in the heart develop massive cardiac hypertrophy that progresses to dilated cardiomyopathy.3 Administration of calcineurin antagonist drugs prevents cardiac hypertrophy induced by a calcineurin transgene and also blocks hypertrophic responses to other stimuli in some, but not all, models.4–7 In skeletal muscles, calcineurin signaling has been implicated in both the hypertrophic response to insulin-like growth factor-18,9 and the remodeling of myofiber phenotypes in response to motor neuron activity.10–13 Calcineurin also stimulates differentiation of myogenic precursor cells.14 and animals lacking NF-AT2 fail to develop normal cardiac valves,15 indicating that calcineurin signaling is pertinent to development of heart and skeletal muscles as well.

A number of different proteins have been shown to bind to the catalytic subunit of calcineurin (calcineurin A) and to regulate its enzymatic activity. The holoenzyme includes calcineurin A and a regulatory subunit, calcineurin B. This complex is activated upon binding of calcium/calmodulin.16 CHP is a calcineurin B homologue that inhibits calcineurin activity by hindering the formation of the calcineurin/calmodulin/calcineurin B heterotrimer.17 Cyclosporin A and FK506 when bound to their respective binding proteins, cyclophilin A and FKBP12, form oligomeric complexes with calcineurin and inhibit its activity.18 Other proteins act to localize calcineurin within the cell. FKBP12, in the absence of FK506 ligand, anchors calcineurin to IP3 and ryanodine receptors,19 and AKAP79 is a scaffolding protein that binds calcineurin, protein kinase A, and protein kinase C.20 Forced expression of a ubiqui-
tously expressed calcineurin-binding protein called Cabin/Cain inhibits calcineurin signaling in cultured cells and can prevent hypertrophic responses in rat cardiomyocytes.21–23 We have recently described a family of proteins—MCIP1 and MCIP2—that are highly expressed in striated muscles and that inhibit calcineurin through a direct physical interaction.24 The human gene encoding MCIP1 resides on chromosome 21 within the Down syndrome critical region (termed DSCR1) and was shown independently by other laboratories to function as an inhibitor of calcineurin.25,26 Two other human genes annotated as ZAKI-4/DSCR1L1 and DSCR1L2 encode closely related proteins that we term MCIP2 and MCIP3, respectively.27,28 The MCIP gene family includes a yeast protein Rcn1p capable of inhibiting calcineurin.25 MCIP proteins differ from previously described inhibitors of calcineurin in several important respects. Unlike immunophilin and FKBP, no exogenous molecules are required for the ability of MCIPs to inhibit calcineurin. MCIP proteins bind directly to calcineurin A using different binding surfaces compared with the larger AKAP79 or Cabin/Cain proteins. Finally, MCIP1 and MCIP2 are expressed most abundantly in striated muscles, compared with the ubiquitous expression of AKAP79, Cabin/Cain, and CHP.

In the present study, we report that the genes encoding MCIP1 and MCIP2 are subject to distinctive mechanisms of regulation. Specifically, expression of MCIP1 is induced by calcineurin activity, whereas the MCIP2 gene fails to respond to this stimulus. Conversely, MCIP2 expression is regulated by thyroid hormone, which has no discernible effects on MCIP1. An intragenic region of the MCIP1 gene located 5' to exon 4 contains a dense cluster of 15 NF-AT binding motifs within a ~900-bp segment and functions as an alternative calcineurin-responsive promoter. These results identify independent mechanisms by which different MCIP proteins are induced, presumably to protect the cell from otherwise deleterious effects of unrestrained calcineurin activity in different contexts.

Materials and Methods

Plasmid Constructions

The segment of intron 3 from the human MCIP1 (DSCR1) gene was isolated by polymerase chain reaction (PCR) using human genomic DNA as template and primers based on sequence information from the human chromosome 21 database.29 This ~900-bp fragment was subcloned into a pGL3 luciferase reporter vector (Promega). Other plasmids were previously described.10,24

Tissue Culture, Cell Transfection or Infection, and Reporter Gene Assays

C2C12 myoblasts and myotubes were cultured as previously described.10 Ionomycin (2 μmol/L) and cyclosporin A (50 to 200 nmol/L) were added 4 hours before harvesting the cells. When included, cycloheximide (25 μmol/L) was added 15 minutes before ionomycin. Transient transfection with plasmids or infection with recombinant, replication-defective adenoviruses and luciferase assays were performed as previously reported.10,24,31

Animal Experiments

Lines of transgenic mice in which the α-myosin heavy chain promoter is used to drive expression of a constitutively active form of calcineurin selectively in the heart were generated and described previously.3 Wild-type male C57Bl/6 mice were injected intraperi-

toneally with 3,5,3'–triiodothyronine (T3) (0.1 μg/g body weight) or an equal volume of 0.9% saline once a day for 10 days.32 All experiments involving animals were conducted using IACRAC-approved protocols.

RNA Isolation and Northern Blot Analysis

Total RNA was prepared from mouse tissues or C2C12 cells using TriPure (Boehringer Mannheim, Inc) following the manufacturer’s protocol. Northern blot analysis was performed with 20 μg of total RNA in each lane and probed in UltraHyb (Ambion) with complementary sequences representing the 3' untranslated region (UTR) of MCIP1 (common to all known splicing variants), exon 1 of MCIP1, exon 4 of MCIP1, or ORF segments of MCIP2 or GAPDH cDNA. Probes were generated by PCR and labeled as described previously.24 Signals from Northern blots were detected on a Storm PhosphorImager (Molecular Dynamics) and quantified using ImageQuant (version 1.2).

cDNA Microarray Analysis of Calcineurin-Transgenic Mice

RNA was isolated from two calcineurin-transgenic mice3 at 10 weeks of age and from a wild-type littermate. One of the transgenic mice was determined to be in heart failure, on the basis of anasarca with massive ascites, whereas the other appeared grossly normal. Both transgenic mice showed >100% increase in heart weight relative to the wild-type control (250 and 240 mg versus 96 mg, respectively). Total body weights of the wild-type (26 g) and nonfailing calcineurin transgenic mice (27.5 g) were comparable, whereas the calcineurin-transgenic mouse in heart failure had a greater body weight (35.6 g), reflecting the edematous state. Microarray analyses were conducted by Incyte Genomics as described elsewhere.33 Briefly, polyA+ RNA was labeled with Cy3/Cy5 fluorescent dyes and hybridized with a mouse cDNA microarray (mouse GEM 1.14) containing 8734 elements each (7832 unique genes: 3336 annotated/4496 unannotated) genes. Differential expression was calculated as the ratio of fluorescent signals after subtraction of background.

Results

Calcineurin Induces Expression of MCIP1 but Not MCIP2

Gene expression profiling by microarray analysis was conducted to identify genes that are differentially regulated in hearts of transgenic mice engineered to express a constitutively active form of calcineurin compared with normal controls. Calcineurin-transgenic animals (αMHC-CnA*) develop massive cardiac hypertrophy that progresses to dilated cardiomyopathy.3 This analysis identified MCIP1 as a gene that is potently upregulated in this model (Figure 1A). Other genes known to be controlled by hypertrophic signals (eg, atrial natriuretic factor) also were identified by this analysis and were induced to a comparable extent (~3-fold) as MCIP1 in hypertrophic, nonfailing hearts of animals at 10 weeks of age compared with a wild-type littermate (Figure 1A, top). Both MCIP1 and calcineurin A transcripts were elevated further in hearts of age-matched animals that had progressed to overt heart failure compared with levels noted in hypertrophic, nonfailing hearts (Figure 1A, bottom). This induction of MCIP1 gene expression within the intact myocardium by activated calcineurin was confirmed by Northern blot analysis (Figure 1B). Quantitative estimates of the extent of MCIP1 induction in αMHC-CnA* hearts (Table 1A) varied among individual animals of comparable age (3–to 17-fold), perhaps reflecting different stages in disease progression.

Downloaded from http://circres.ahajournals.org/ by guest on May 10, 2017
Northern blots (Figure 1B) also revealed that the MCIP2 gene was not similarly regulated. In contrast to the upregulation of MCIP1 mRNA in this model, there was no detectable change in MCIP2 mRNA (Figure 1B and Table 1A).

We also assessed the ability of calcineurin to stimulate expression of MCIP1 in C2C12 cells that differentiate into skeletal myotubes in cell culture. Increased intracellular calcium concentrations evoked by administration of the calcium ionophore ionomycin led rapidly (~4 hours) to an increased abundance of MCIP1 mRNA in C2C12 cells (Figure 2A). MCIP1 mRNA is increased during differentiation of C2C12 cells (reported previously,24 not shown here), but a 2-fold induction of MCIP1 by ionomycin was evident irrespective of the stage of differentiation (myoblasts or myotubes) and the correspondingly lower or higher initial levels of MCIP1 mRNA. In contrast, expression of MCIP2 mRNA was unaffected by ionomycin (Figure 2A and Table 1A). To determine whether the induction of MCIP1 gene expression by calcium ionophore was attributable to calcineurin activity, as opposed to other calcium-regulated signaling events, we assessed the effects of cyclosporin A on this response. In a dose-dependent manner, cyclosporin A blocked the ability of ionomycin to upregulate MCIP1 transcript levels in C2C12 cells (Figure 2B) and even reduced MCIP1 transcripts below the levels observed in cells untreated with either drug, presumably by blocking basal as well as ionophore-stimulated calcineurin activity. Compared with cells treated with the highest dose of cyclosporin A, MCIP1 mRNA was increased 4-fold by ionomycin in the absence of cyclosporin A (Figure 2B).

Figure 1. Differential regulation of MCIP1 and MCIP2 mRNA by calcineurin in hearts from wild-type and transgenic mice expressing constitutively active calcineurin (α-MHC-CnA*). A, Gene expression profiling by microarray analysis (Incyte Genomics). Listed are the 8 genes found to be potently upregulated during compensated hypertrophy (top) and in the transition to overt heart failure (bottom) that are characteristic of this model,3 along with accession numbers and estimated fold change in mRNA abundance. Calcineurin A is the transgene product in this model, and upregulation of atrial natriuretic factor gene expression is a well-defined marker of cardiac hypertrophy. MCIP1 (arrows) is one of the genes most strongly activated in hypertrophic, nonfailing hearts of α-MHC-CnA* animals and is upregulated further in hearts of animals that have progressed to overt cardiac failure. B, Northern blot analysis of RNA extracted from hearts of wild-type and transgenic mice. Each lane was loaded with 20 μg total RNA from a single mouse heart, and blots were hybridized with probes complementary to MCIP1, MCIP2, and GAPDH (loading control). This experiment was repeated 3 times with comparable results.
protein (Figure 2C). Thus, experimental strategies based on loss-of-function and gain-of-function approaches in cultured myocytes support the conclusion that MCIP1 gene expression, but not that of MCIP2, is regulated by calcineurin signaling.

**Induction of MCIP1 Expression by Calcineurin Does Not Require New Protein Synthesis**

In the presence of cycloheximide, an inhibitor of protein synthesis, activation of calcineurin by ionomycin continued to upregulate MCIP1 mRNA in C2C12 cells, indicating that this induction is not dependent on the generation of new proteins (Figure 2D). The magnitude of ionomycin-stimulated upregulation of MCIP1 in the presence of cycloheximide (~10-fold) was greater than that observed when cycloheximide was absent (~2-fold). We interpret these data to support the hypothesis that calcineurin stimulates MCIP1 gene transcription by posttranslational modification of a preexisting pool of NF-AT proteins (and possibly other transcription factors). The greater magnitude of MCIP1 induction by calcineurin when new protein synthesis is blocked is potentially attributable to abrogation of calcineurin-dependent induction of endogenous MCIP1 synthesis, thereby eliminating negative feedback that otherwise would restrain calcineurin activity.

**Thyroid Hormone Induces Expression of MCIP2 but Not MCIP1**

The gene encoding MCIP2 was identified originally in a subtractive cloning experiment designed to identify genes that are upregulated by thyroid hormone in cultured human fibroblasts. To determine whether MCIP genes are regulated by thyroid hormone in hearts of intact animals, hyperthyroidism was induced in wild-type mice by intraperitoneal injection of T3 for 10 days. As noted previously, T3-treated hearts were uniformly hypertrophic (mean heart weight = 180 mg versus 130 mg; mean heart weight/body weight ratio = 7.2 mg/g versus 4.9 mg/g; n = 4 animals in each group). In contrast to the effects of activated calcineurin in the murine heart (Figure 1), the expression of MCIP1 was unaltered in hyperthyroid hearts (Figure 3). However, MCIP2 transcript levels were increased ~2-fold in both heart and soleus skeletal muscles of T3-treated mice (Figure 3 and Table 1B). It remains to be determined whether the effects of T3 are a direct consequence of nuclear receptor binding to regulatory elements of the MCIP2 gene or a result of indirect mechanisms.

**An Intragenic Region Located 5’ to Exon 4 of the MCIP1 Gene Is Sufficient to Promote a Transcriptional Response to Calcineurin**

The human MCIP1 gene (annotated initially as DSCR1) was reported to express four variant mRNAs with each of four alternative exons incorporated selectively at the 5’ terminus of the expressed transcripts. The majority of these transcripts were identified to represent isoforms that include
sequences encoded either by exon 1 or exon 4. These variants have unique 5' UTR regions and encode proteins that differ within the first 29 amino acids. The remaining 168 residues of MCIP1, encoded by exons 5 to 7, are identical in all MCIP1 variants (Figure 4A). In our experiments on hearts of transgenic mice, we determined that expression of the exon 4 variant of MCIP1 mRNA was particularly sensitive to calcineurin activity. The increased abundance of MCIP1 mRNA detected by a probe complementary to the 3' UTR, which is included within all variants of MCIP1 (Figure 4A), was mirrored by the increase detected with a probe complementary only to unique exon 4 sequences (Figure 4B). In contrast, MCIP1 transcripts that include exon 1 sequences were present only at the limit of detection in wild-type murine hearts and were not induced by the activated calcineurin transgene (not shown).

The selectively increased expression of the exon 4 variant of MCIP1 mRNA suggested the possibility of alternative promoter use as a function of calcineurin activation, and we sought to determine whether transcriptional regulatory elements involved in transducing this signal reside in proximity to exon 4 of the MCIP1 gene. Accordingly, we isolated a ~900-bp genomic segment from this position (−874 to +30 relative to the first nucleotide of exon 4). This region was found to contain a remarkably dense cluster of consensus NF-AT binding motifs (T/AGGAAANA/T/C) (Figure 4A). A reporter plasmid was constructed to link this MCIP1 genomic region to a luciferase reporter gene (Figure 4C), and this construct was tested for its ability to respond to calcineurin after transfection into C2C12 cells. Like the endogenous MCIP1 gene, expression of this transgene is increased by activated calcineurin (Figure 4D), which has no effect on
a control plasmid (minimal TATA plus luciferase; not shown). Inhibition of calcineurin activity by concomitant overexpression of MCIP1 represses this response (not shown). Luciferase reporter plasmids controlled by shorter segments of this genomic region 5' to exon 4 (-231 to +30 or -163 to +30; Figure 4C) retain basal activity equivalent to the -874 to +30 segment but progressively lose calcineurin responsiveness as the number of NF-AT binding sites is reduced (Figure 4D).

**Discussion**

A major finding of this study is that the genes encoding the calcineurin-interacting proteins MCIP1 and MCIP2 are regulated selectively in skeletal and cardiac myocytes by calcineurin and thyroid hormone, respectively. Both genes are expressed in striated myocytes, and both proteins are capable of inhibiting the enzymatic activity of calcineurin. However, only MCIP1 is induced by calcineurin and only MCIP2 by thyroid hormone. Regulatory responses of this nature have not been reported for genes encoding other proteins (Cabin/Cain, CHP) that function as endogenous inhibitors of calcineurin.

The induction of MCIP1 expression by calcineurin in striated myocytes is rapid and robust. An increased abundance of MCIP1 mRNA is detected within 4 hours after activation of calcineurin by calcium influx into cultured C2C12 myoblasts or myotubes. A microarray analysis capable of screening 7832 independent genes identified MCIP1 as one of the genes most markedly upregulated in hearts of transgenic mice engineered to express a constitutively active form of calcineurin. Among several variants of MCIP1 mRNA that arise by alternative promoter use and/or alternative splicing, transcripts including sequences encoded by exon 4 were found to be induced by calcineurin. This response was recapitulated by a plasmid construct that linked a ~900-bp intragenic region located 5' to exon 4 to a luciferase reporter gene. It is possible that other regions of the MCIP1 gene contribute to its transcriptional regulation, but the exceptionally dense clustering of NF-AT binding motifs upstream of exon 4 is likely to mediate the potent response to calcineurin signaling. Serial deletions of this promoter region lose responsiveness to calcineurin in proportion to the number of NF-AT binding sites that are removed.

These findings with respect to MCIP1 gene regulation by calcineurin, in concert with the ability of MCIP1 to inhibit the enzymatic activity of calcineurin, support the concept that MCIP1 functions in a negative feedback circuit (Figure 5) to limit potentially deleterious consequences of otherwise unhindered calcineurin signaling, such as apoptosis. Our experiments using cycloheximide show that new protein synthesis is not required for induction of MCIP1 transcription by calcineurin, in keeping with a mechanism based on posttranslational modification of NF-AT (Figure 5). Moreover, the magnitude of MCIP1 induction by calcineurin is increased in the presence of cycloheximide, as would be predicted if newly synthesized MCIP1 negatively regulates calcineurin activity. Findings recently reported by other laboratories also are consistent with the notion of negative feedback on calcineurin by MCIP proteins. It remains to be determined, however, whether inhibition of calcineurin is the sole function of MCIP1 in mammalian cells. The MCIP orthologue of *Saccharomyces cerevisiae* (Rcn1p) inhibits calcineurin signaling at high concentrations, but calcineurin signaling is impaired if Rcn1p is absent. This latter observation suggests that basal levels of Rcn1p function in some manner to facilitate calcineurin signaling. Loss-of-function experiments based on targeted disruption of each of the three MCIP genes will be critical to determine whether this is also true for MCIP proteins in mammalian cells.

The responsiveness of endogenous MCIP1 gene expression to calcineurin activity in cultured cells and in tissues of intact animals, and the ability of the exon 4 flanking region to recapitulate this response when placed into a reporter plasmid, potentially can be exploited as sensitive indicators of calcineurin activity. As noted in recent reviews, current methods used to assess the activation state of calcineurin in vivo are subject to problematical artifacts. It may be possible to redress this deficiency by designing assays based on the peculiar responsiveness of MCIP1 to calcineurin activation. Such assays could aid in the interpretation of experiments that seek to modify calcineurin signaling in animal models or be used in high-throughput screens for discovery of new chemical agents that alter calcineurin signaling.

The ability of T3 to induce expression of MCIP2 in mammalian striated muscles was anticipated on the basis of experiments in which a cDNA encoded by the human ZAKI-4 gene (here termed MCIP2) was identified in a screen for transcripts upregulated by treatment of human fibroblasts with thyroid hormone. In the present study, we confirm that
this response occurs in skeletal and cardiac muscles, and we provide new information to show that such regulation by T3 does not extend to the MCIP1 gene. In addition, we demonstrate that, unlike MCIP1, MCIP2 is not subject to regulation by calcineurin signaling. It will be important in future studies to ascertain whether the induction of MCIP2 by thyroid hormone and the ensuing inhibition of calcineurin activity that should result from this response are pertinent to any of the consequences of hyperthyroidism that affect skeletal and cardiac muscles. For example, inhibition of calcineurin activity in skeletal muscles by cyclosporin A promotes transformation of slow myofibers to the fast fiber phenotype.10,13 Excess T3 can induce a similar transformation of myofiber subtypes, as well as myosin isoform switching in the heart.38,39 It is plausible to propose that T3-induced accumulation of the calcineurin inhibitory protein MCIP2 may contribute to these effects.

Our observation that, unlike MCIP2, expression of MCIP1 is not induced in hearts of thyrotoxic mice has additional implications. Unchanged levels of MCIP1 mRNA in hypertrophic hearts of T3-treated animals suggests that the induction of MCIP1 produced by expression of an activated calcineurin transgene is a direct consequence of calcineurin activity, rather than a uniform feature of all forms of cardiac hypertrophy. Moreover, on the premise that expression of MCIP1 provides an indicator of the state of activation of the calcineurin signaling pathway, normal levels of MCIP1 in hypertrophic hearts of T3-treated animals suggests that the induc-

Control of MCIP1 and MCIP2 Expression

References


Acknowledgments

This work was supported by grants from the National Institutes of Health (NIH) (to E.N.O and R.S.W.), the Robert A. Welch Foundation (to E.N.O.), and Myogen, Inc (to E.N.O.). N.F. was supported by a fellowship of the Deutsche Forschungsgemeinschaft, T.A.M. is a Pfizer Fellow of the Life Sciences Research Foundation, J.Y. and R.V. were supported by an NIH training grant. We are grateful to John Shelton for preparing images of primary data for publication.


Independent Signals Control Expression of the Calcineurin Inhibitory Proteins MCIP1 and MCIP2 in Striated Muscles

John Yang, Beverly Rothermel, Rick B. Vega, Norbert Frey, Timothy A. McKinsey, Eric N. Olson, Rhonda Bassel-Duby and R. Sanders Williams

_Circ Res._ 2000;87:e61-e68
doi: 10.1161/01.RES.87.12.e61

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/87/12/e61

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/