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Myocyte Enhancer Factor 2 Mediates Calcium-Dependent Transcription of the Interleukin-2 Gene in T Lymphocytes

--A calcium signaling module that is distinct from but collaborates with NFAT

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Running Title: MEF2 Mediates Calcium-Dependent IL-2 Transcription.

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SUMMARY

The second messenger calcium plays an essential role in the T cell receptor-mediated signal transduction pathways leading to transcription of the interleukin-2 gene. A key mechanism of calcium signaling has been shown to be mediated by calcineurin and NFAT. We report herein that the transcription factor myocyte enhancer factor (MEF)-2 is another calcium signal transducer involved in the regulation of the IL-2 promoter. A MEF2-binding site was identified in proximity to the TATA box of the IL-2 promoter. This site was shown to be bound by MEF2 in both resting and activated T cells. Overexpression of MEF2 enhanced, while overexpression of a dominant negative form of MEF2 or the MEF2-specific transcriptional corepressors Cabin1 and histone deacetylase 4 inhibited, the TCR-dependent activation of an IL-2 reporter gene. Downregulation of MEF2 by RNA interference in primary human T cells led to the inhibition of endogenous IL-2 transcription. These results suggest that MEF2 is required for the transcriptional activation of IL-2 and likely other cytokine genes in response to calcium signaling and may serve as a novel target for development of immunosuppressants.

INTRODUCTION

Activation of peripheral T cells through the engagement of TCR with MHC-peptide complexes on antigen presenting cells leads to the transcriptional activation of a number of cytokine genes that are involved in the orchestration of the cellular immune response. Prominent among the cytokines secreted by activated T cells is IL-2, which plays an essential role in subsequent T cell proliferation and homeostasis (1). The intracellular signal transduction pathway linking TCR and the IL-2 promoter has been used as a paradigm to elucidate mechanisms of signaling from cell surface to the nucleus. The second messenger calcium has been shown to be an essential mediator of the TCR signaling pathway. Through the use of the immunosuppressive natural products CsA and FK506, both of which inhibit a calcium-dependent signaling step leading to IL-2 transcription, a key downstream transducer of calcium signaling was identified as the calcium, and calmodulin-dependent protein phosphatase calcineurin (2-8) (also known as protein phosphatase 2B). The primary calcineurin substrates were revealed to be members of the nuclear factor of activated T cells (NFAT) protein family (9-11). NFAT exists in its latent form as a phosphoprotein in the cytosol in resting T cells. Upon activation of TCR, an increase in the cytosolic calcium concentration leads to the activation of calcineurin by calmodulin, which dephosphorylates NFAT, leading to its translocation into the nucleus where it binds to the IL-2 promoter and activates its transcription.

Another calcium signaling module, mediated by the transcription factor MEF2 (12), was recently identified and shown to be involved in the regulation of the pro-apoptotic gene Nur77 during thymocyte apoptosis (13-16). Unlike NFAT, MEF2 is constitutively bound to its cognate DNA-binding elements in the nucleus independent of the intracellular calcium concentration. In unactivated thymocytes, MEF2 recruits a family of functionally redundant transcriptional repressors including Cabin1 (also known as cain) (17,18), MEF2 interacting transcriptional repressor (MITR), and HDAC4, 5, & 7 (14,19,20). These MEF2-specific corepressors recruit histone deacetylases to MEF2-associated promoter region, which remodel the chromatin structure, silencing the promoter

activity. Upon calcium influx, these MEF2 repressors are removed from MEF2 by activated calmodulin, enabling it to bind to such coactivators as p300, turning on transcription of the target genes (14,21). The binding of some MEF2 transcriptional repressors has also been shown to be subject to regulation by calmodulin-dependent kinases, providing another mechanism of activation of MEF2 by calcium (22,23).

In an attempt to ascertain the role of Cabin1 in thymocyte apoptosis *in vivo*, we generated a partial Cabin1 knockout mouse (Cabin1ΔC) in which the C-terminal calcineurin- and MEF2-binding domains are deleted (24). The Cabin1ΔC mice are normal in thymocyte development despite the deletion of the MEF2-binding domain of Cabin1, likely reflecting the functional redundancy of Cabin1 and other MEF2 corepressors in thymocytes. However, the Cabin1ΔC mice exhibited upregulation of a number of cytokines including IL-2, IL-4 and Interferon-γ upon stimulation with TCR agonists. Examination of NFAT ruled out the possibility that this upregulation of cytokines was due to the enhanced calcineurin-NFAT pathway, suggesting that other factors such as MEF2 might play a role in regulating the expression of IL-2 and other cytokine genes.

We report herein that the MEF2 calcium signaling module is involved in the regulation of IL-2 transcription in response to TCR signaling. We demonstrate that MEF2 associates with the IL-2 promoter through the putative MEF2-binding site. Overexpression of wild type MEF2 caused upregulation of the IL-2 promoter while expression of a dominant negative MEF2 mutant lacking the transactivation domain or expression of the MEF2 transcriptional repressors Cabin1 or HDAC4, significantly diminished the activation of the IL-2 promoter. Finally, downregulation of endogenous MEF2 expression in primary human CD4 T cells by small interfering RNA (siRNA) abrogated the transcriptional activation of endogenous IL-2 gene in response to stimulation with anti-CD3 and anti-CD28 antibodies. Thus, MEF2 plays an important role in calcium-dependent chromatin remodeling of the IL-2 promoter during T cell activation.

EXPERIMENTAL PROCEDURES

Cell culture, transfection and reporter gene assay. Jurkat T cells were cultured and transfected as described previously (17). The Cabin1 deletion mutant (with the calcineurin-binding domain deleted) was subcloned into pSG5 mammalian expression vector (Stratagene) and the resultant plasmid was named as pSG5-Cabin1ΔCNBD. The mutant of pIL-2 luciferase (pIL2-Luc/Mut) reporter was made using a site-directed mutagenesis kit (Stratagene).

EMSA. Nuclear extracts from Jurkat T cells treated with PMA and ionomycin were prepared as described (17). The probe used was derived from the IL-2 promoter (-70 to -45) and contained the minimal putative MEF2-binding site (5'-ATTTTGACACCCCCATAATATTTTTC-3').

CHIP Assays. CHIP assays were carried out as previously described with slight modifications (15). The forward primer sequence was 5'-TTTCATACAGAAGGCGTTAATTGC-3' and the reverse primer sequence was 5'-ATGCAATTTATACTGTTAATTCTGG-3'.

Construction of siRNA expression vector. The procedure for plasmid construction is similar to that described previously with slight modifications (25). To generate pBS/U6-siMEF2D, two oligonucleotides were synthesized. Oligo1 (sense):
5’-GGCTTCAATGGCTGCGACAGttcaagagaCTGTCGCAGCCATTGAAGCCCTTTTTG-3’.
The first 20-nucleotide of Oligo 1 corresponds to 273-288 of human MEF2D cDNA sequence, followed by 9 nucleotides linker (small letter) and the inverted sequence containing five T’s.
Oligo2 (antisense): 5’-AATTCAAAAAGGGCTTCAATGGCTGCGACAGtctcttgaaCTGTCG
CAGCCATTGAAGCC-3’. The two oligonucleotides were annealed and cloned into pBS/U6 digested sequentially with *Apa*I (followed by blunting with T4 DNA polymerase) and *Eco*RI. Positive clones containing the appropriate inserts were confirmed by DNA sequencing.

Construction of Lentivirus Vector. To generate the lentiviral siRNA vector, pBS/U6-siMEF2D was digested with *BamH*I to release the U6 promoter along with the MEF2D siRNA targeting sequence. The *BamH*I fragment was subsequently blunt ended with Klenow fragment and inserted into the unique *Pac*I site FUGW vector, affording pFUP-siMEF2D (26). For the control, the *BamH*I fragment from pBS/U6 was inserted into FUGW to give pFUP. Of the two possible orientations, the plasmid in which the U6 promoter was in opposite orientation to the LTR was chosen, as it gives slightly higher viral titer.

Lentivirus production. Recombinant lentiviruses were generated using a three-plasmid system as previous described (27). Viruses were harvested at 48 and 72 h after transfection and titer was determined based on percentages of GFP-positive Jurkat T cells after transduction with serially diluted viral supernatant. The titer, calculated as transducing units (TU) per milliliter of supernatant, was in the range of 2×10^6 to 8×10^6 TU/ml. The virus-containing supernatant was concentrated using Amicon Ultra Concentrator (Millipore) and stored at –80 °C as previously described (27,28).

Isolation and culture of human primary T cell. Human peripheral blood mononuclear cells (PBMCs) were obtained from AllCells (Berkeley, CA). $CD4^+$ T cells were purified by positive selection with magnetic activated cell separation beads (Miltenyi Biotech). The purity of the $CD4^+$ T cells was more than 95%, as judged by FACS analysis. Cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum at a density of 1×10^6 cells/ml in the presence of human recombinant IL-7 at 5 ng/ml (29) (BD Pharmingen, San Diego, CA).

Transduction of human $CD4^+$ T cells. Cells (5×10^5) were mixed with viral supernatants in the presence of polybrene (8 μg/ml) in a 5-ml tube, followed by addition of 10 mM HEPES and centrifugation at $2000 \times g$ for 3 h at 37 °C (28). After incubation with viral supernatant for 10 h, cells were washed and incubated in fresh culture medium containing IL-7 for 12-16 h, followed by

another cycle of transduction. Cells were washed and plated at the density of 1×10^6 cells/ml in the presence of IL-7 as a T cell survival factor (29). A fraction of cells (1×10^5) from each group were analyzed by FACS to determine the efficiency of transduction (~80%) by monitoring the GFP expression 60 h after transduction. The remaining cells were stimulated with either plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (2 μg/ml, BD Pharmingen, CA) or control IgG for 6 h. Supernatants were collected for IL-2 ELISA assay. The cells were harvested for RT-PCR and Western Blot analyses.

RT-PCR. The Titan One Tube RT-PCR System (Roche Biochemicals, Indianaplis, IN) was used to detect IL-2 or GAPDH mRNA per manufacturer's instructions. The sequences of the primers are: 5'-CATTGCACTAATTCTTGCACTTGTCA-3' (IL-2, 5'–primer); 5'-CGTTGATATTGCTGATTAAGTCCCTG-3' (IL-2, 3'–primer); 5'-TCCACCACCCTGTTGCTGTA-3' (GAPDH, 5' –primer); 5'-ACCACAGTCCATGCCATCAC-3' (GAPDH, 3'- primer).

RESULTS AND DISCUSSION

A MEF2-binding site exists in close proximity to the TATA box of the IL-2 promoter--As deletion of the C-terminal MEF2-binding domain along with the calcineurin-binding domain in Cabin1 resulted in upregulation of the expression of IL-2 and other cytokine genes, we hypothesized that MEF2 may be involved in the regulation of not only Nur77 expression during thymocyte apoptosis, but also transcription of IL-2 and other cytokines during activation of peripheral T cells (24). Thus, we searched the IL-2 promoter region for a potential MEF2-binding site, and a contiguous AT-rich sequence composed of eleven A or T nucleotides was found between –46 and –55 in the proximal IL-2 promoter (Figure 1A). Although this AT-rich sequence deviates from the consensus MEF2-binding sequence [$CAT(A/T)_4TAG$] (30), the core AT nucleotides are conserved. The putative MEF2-binding site in the human IL-2 promoter differs from that in the murine IL-2 promoter in a

single nucleotide change from an "A" in the human sequence to a "T" in the mouse sequence, but this does not disrupt the contiguous "A/T" feature required for MEF2 binding.

To test whether the putative MEF2-binding site in the IL-2 promoter is capable of binding to MEF2, we prepared a [^{32}P]-labeled double-stranded DNA probe spanning the site, and performed electrophoretic gel mobility shift assay (EMSA). Using nuclear extracts of Jurkat T cells, we observed a protein-DNA complex (Figure 1B, Lane 2), which was sensitive to competition by an excess amount of cold probe (Figure 1B, Lane 3). When anti-MEF2 antibodies were included in the binding reaction mixture, the protein-DNA complex was supershifted, indicating that MEF2 was bound by the DNA probe. These results demonstrate that the putative MEF2-binding site in the IL-2 promoter can bind MEF2 *in vitro*.

MEF2 is bound to the IL-2 promoter in vivo--We next employed the chromatin immunoprecipitation-PCR (CHIP) assay to determine whether MEF2 is bound to the IL-2 promoter *in vivo*, and if so, whether the binding is mediated by the putative MEF2-binding site. We assessed the binding of MEF2 to the endogenous IL-2 promoter using the CHIP assay (31). Indeed, MEF2D, the most abundant isoform of MEF2 in T cells, is bound to the promoter of IL-2 (Figure 2A). Similar to its association with the Nur77 promoter in T cell hybridoma, the binding of MEF2D to the endogenous IL-2 promoter is independent of the activation state of T cells (Figure 2A, Lane 2 and 3). As a negative control, we also attempted to determine whether anti-MEF2 antibodies also pulled down the promoter of the house-keeping gene glyceraldehyde-6-phosphate dehydrogenase (GAPDH) using a pair of specific primers (32). Anti-MEF2D antibodies failed to pull down the GAPDH promoter (data not shown), suggesting that the binding of MEF2 to the IL-2 promoter was specific.

It has been previously shown that the MEF2-specific transcriptional co-repressors Cabin1 and HDAC4 are bound to MEF2 in a calcium-dependent fashion (14,21). These repressors are

released from MEF2 upon calcium signaling. We therefore determined if Cabin1 and HDAC4 could also associate with the IL-2 promoter via MEF2. Both Cabin1 and HDAC4 were found to be associated with the IL-2 promoter in non-stimulated T cells (Figure 2A, Lanes 4 and 6). Upon stimulation with PMA and ionomycin with the continued presence of calcium in the lysis buffer, Cabin1 and HDAC4 dissociate from MEF2 and the IL-2 promoter (Figure 2A, Lanes 5 and 7). These results suggest that MEF2 is likely to play a negative regulatory role in the transcription of the IL-2 promoter through recruitment of different classes of corepressors including Cabin1 and HDAC4 in resting T cells.

To assess whether the putative MEF2-binding site in the IL-2 promoter is responsible for the binding of MEF2, we employed the CHIP assay using transiently transfected plasmid encoding either wile type IL-2 promoter or a mutant in which the putative MEF2-binding site has been mutated. As shown in Fig. 2B, MEF2 was bound to the transiently transfected IL-2 promoter either in the absence or the presence of stimulation with PMA and ionomycin (Lanes 2 and 3), consistent with the outcome of endogenous CHIP assay (Figure 2A). Mutation of the putative MEF2-binding sequence in the IL-2 promoter (pIL2-Luc/Mut) completely abolished the binding of MEF2 to the transiently transfected IL-2 promoter (Figure 2B, Lanes 4 and 5). Note that fewer PCR cycles were used for the amplification of the Nur77 promoter DNA such that the endogenous DNA is hardly detectable under these conditions (Figure 2B, Lane 6). Together, these results indicate that MEF2 is bound to the IL-2 promoter through the putative MEF2-binding site.

Overexpression of MEF2 enhances the activation of the IL-2 promoter in a MEF2 corepressor sensitive manner--The binding of MEF2 to the IL-2 promoter suggests that MEF2 may be involved in the transcriptional regulation of IL-2. To assess this possibility, we overexpressed MEF2 together with an IL-2 luciferase reporter gene and determined whether upregulation of MEF2 had any effect on the IL-2 reporter gene expression. It was anticipated that overexpression of MEF2 will alter the ration of MEF2 to endogenous MEF2 corepressors, enhancing the transcriptional

activity of MEF2 as previously observed (16). Indeed, overexpression of MEF2 enhanced the IL-2 reporter gene activation in response to stimulation by PMA and ionomycin (Figure 3). As MEF2 is known to be sensitive to expression of its corepressors Cabin1 or HDAC4, we also determined the effect of overexpressing Cabin1 and HDAC4 on the activation of the IL-2 reporter by PMA and ionomycin. Overexpression of HDAC4 or Cabin1 significantly blocked the activation of the IL-2 reporter gene in response to PMA and ionomycin (Figure 3). In contrast, overexpression of HDAC1, which does not directly bind to MEF2 (even though it is part of the Cabin1-mSin3 repression complex), had a negligible effect on IL-2 promoter activation. In addition to the MEF2-binding domain, Cabin1 also contains a calcineurin-binding domain (CNBD), which may account for the inhibition of IL-2 reporter gene expression by blocking calcineurin activity. We thus employed a truncation mutant of Cabin1 lacking the calcineurin-binding domain (Cabin1ΔCNBD) (14). When Cabin1ΔCNBD was overexpressed in Jurkat T cells, it remained capable of blocking IL-2 reporter gene activation, albeit with lower potency than the full-length Cabin1. These results further support the proposition that MEF2 is intimately involved in the activation of the IL-2 promoter.

Removal of the MEF2-binding site in the IL-2 promoter or blocking MEF2 activity inhibits activation of the IL-2 reporter gene--To assess the role of MEF2 in IL-2 transcription in response to calcium signaling, we mutated the putative MEF2-binding site in the IL-2 luciferase reporter gene. This mutation was shown to be defective in MEF2 binding in vivo by the CHIP assay (Figure 2B). The activation of the mutant reporter gene (pIL2-Luc/Mut) suffered from a significant decrease in response to stimulation by PMA and ionomycin (Figure 4). Unlike the wild type reporter, the MEF2 mutant reporter gene could not be further activated by overexpression of MEF2, nor could it be further inhibited by HDAC4, which requires MEF2 to associate with the IL-2 promoter. Thus, the MEF2-binding site in the IL-2 promoter plays a critical role in its activation.

If MEF2 is required for IL-2 promoter activation, it is predicted that downregulation of MEF2 activity or its protein level should cause inhibition of IL-2 reporter gene expression. As a preliminary assessment of the importance of MEF2 in IL-2 activation, we expressed a dominant negative form of MEF2D (dnMEF2) missing the C-terminal transactivation domain (33,34), and determined its effect on the IL-2 reporter gene activation stimulated by PMA and ionomycin. Overexpression of the dnMEF2 mutant significantly inhibited the activation of the IL-2 reporter gene (Figure 3), suggesting that MEF2 is required for IL-2 promoter activation.

Downregulation of MEF2 by RNA interference blocks transcription of endogenous IL-2 gene in response to stimulation by TCR agonists--To further assess the role of MEF2 in TCR-mediated IL-2 transcriptional activation in vivo, we applied RNA interference to downregulate the expression of MEF2 in primary human T cells and determined the effect on endogenous IL-2 mRNA synthesis and protein secretion. There are four isoforms of MEF2, MEF2A to MEF2D. The most abundant isoform of MEF2 expressed in T cells is MEF2D. After screening several regions of MEF2D cDNA for an effective siRNA, we identified an siRNA construct which reduced MEF2D expression in transfected Jurkat T cells by approximately 10 fold (data not shown). To deliver the siRNA efficiently to primary human T cells, we used lentiviral vectors capable of transducing both activated and unstimulated T cells (28,29). We generated a hairpin RNA expression construct, pFUP-siMEF2D, and the corresponding empty vector pFUP (with the U6 promoter), based on the lentiviral vector FUGW co-expressing GFP (26), and produced recombinant lentiviruses encoding the MEF2D siRNA.

Purified CD4+ T cells from human peripheral blood were cultured with IL-7 and transduced by either vector. The gene transduction efficiency by both vectors was estimated to be around 80%, based on GFP expression in flow cytometric analysis (not shown). As determined by Western blot analysis, the MEF2 siRNA construct significantly decreased the expression of MEF2D (Figure 5A, upper panel). The effect of the siRNA construct appeared to be specific for

MEF2D, as it had no effect on the protein level of endogenous tubulin (Figure 5A, lower panel). When the virally transduced human primary T cells (including untransduced T cells) were stimulated with a mixture of anti-CD3 and anti-CD28 antibodies, IL-2 transcription was seen in cells transduced with the empty vector pFUP (Figure 5B). A significant decrease in IL-2 mRNA synthesis was observed in the T cell population transduced with the MEF2D siRNA viruses. In agreement with the decrease in RNA level, the level of IL-2 protein secreted into the media was also dramatically reduced in human T cells transduced with MEF2D siRNA viruses (Figure 5C). In contrast, the expression of CD69, a marker associated with T cell activation that is independent of NFAT, is not affected (data not shown), indicating that the downregulation of IL-2 transcription is not due to an indirect effect of MEF2D siRNA on NFAT. The reduction of MEF2D and IL-2 expression in transduced human T cells was likely underestimated by these analyses, as 20% of the human T cells were untransduced, and expressed normal levels of MEF2 and IL-2. These observations strongly suggest that MEF2 is necessary for the transcriptional activation of IL-2 during peripheral T cell activation.

As a unique calcium regulated transcription factor, MEF2 has been shown to play a key role in transmitting calcium signals from cytosol into the nucleus to regulate expression of genes involved in thymocyte apoptosis and muscle cell differentiation. We have shown that the same calcium signaling module is involved in regulating transcription of IL-2 gene during T cell activation. Thus, two distinct calcium signaling modules, one centered around MEF2 and the other consisting of calcineurin and NFAT, are simultaneously involved in conferring calcium responsiveness to the IL-2 promoter (Supplemental Figure 1). Our identification and confirmation of the MEF2-binding site in the IL-2 promoter suggests that more MEF2-binding sites are likely to be found in the promoters of other genes previous unknown to be regulated by MEF2. It is worth noting that a number of cytokines other than IL-2 are upregulated in the CabinΔC mice (24). We have identified non-perfect MEF2-binding sites in the promoter regions of those cytokines similar to the MEF2-binding site in the IL-2 promoter reported here (Figure 1). It is likely that MEF2 and

its associated corepressors including Cabin1, HDAC4 and other factors play a similar role in regulating the transcription of additional cytokine genes. That knockdown of MEF2D expression in primary human T cells caused a significant decrease in the synthesis of IL-2 mRNA and secretion of IL-2 in primary human T cells in response to stimulation by TCR agonists indicate that MEF2 is indispensable for activation of the IL-2 promoter. These results also suggest that MEF2 may serve as a potential target for developing novel immunosuppressants that block IL-2 production and T cell activation.

FOOTNOTE

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FIGURE LEGENDS

Fig. 1. Binding of MEF2 to the putative MEF2-binding site in the IL-2 promoter *in vitro*. (A) The putative MEF2-binding sequence in the context of the IL-2 promoter with the putative MEF2-binding sequence and the TATA box underlined. (B) Electrophoretic gel mobility shift assay to detect the binding of MEF2 to the putative MEF2-binding sites in the IL-2 promoter. Lane 1, probe with no cell extract; 2, probe plus cell extract; 3, probe plus extract in the presence 50-fold excess of cold probe; 4, probe plus cell extract in the presence of non-immunized rabbit sera; 5, probe plus cell extract in the presence of anti-MEF2 antibodies; 6, probe plus cell extract in the presence of 50-fold mutant cold probe.

Fig. 2. MEF2 is bound to the IL-2 promoter via the putative MEF2-binding site *in vivo* and is capable of recruiting Cabin1 and HDAC4 to the IL-2 promoter. (**a**) CHIP assay to determine the binding of MEF2 (Lanes 2 and 3), Cabin1 (Lanes 4 and 5) and HDAC4 (Lanes 6 and 7) to the endogenous IL-2 promoter. Each set of CHIP samples were prepared from Jurkat T cells either stimulated with PMA and ionomycin (P/I) or without stimulation. (**b**) CHIP assay using transiently transfected wild type IL-2 reporter plasmid (pIL2-Luc/WT) or a mutant in which the putative MEF2-binding site has been mutated (pIL2-Luc/Mut).

Fig. 3. MEF2 enhances, and dominant negative (dn) MEF2 or MEF2-specific repressors inhibit, IL-2 reporter gene expression. Jurkat T cells were transfected with IL-2 luciferase reporter plasmid along with plasmids expressing various proteins as indicated. Cells were treated with PMA plus ionomycin for 8 h before they were lysed for measurement of luciferase and β-galactosidase activities.

Fig. 4. The MEF2-binding site on the IL-2 promoter is required for IL-2 promoter activation. Wild type IL-2 luciferase reporter plasmid (pIL2-Luc/WT) and a plasmid with mutations on the

putative MEF2-binding site (pIL2-Luc/Mut) were transfected into Jurkat T cells along with other expression plasmids as shown. Cells were treated with PMA plus ionomycin for 8 h before they were lysed for measurement of luciferase and β-galactosidase activities.

Fig. 5. RNA interference of MEF2 expression leads to inhibition of IL-2 promoter activation. (**a**) Specific inhibition of MEF2 expression by MEF2D siRNA. Lysates prepared from primary human T cells transduced with viruses harboring either empty pFUP vector or pFUP-siMEF2D were subjected to Western blot analysis using anti-MEF2D (top panel) or anti-tubulin (bottom panel) antibodies. (**b**) RNA interference with MEF2 expression inhibited IL-2 mRNA synthesis in response to stimulation by anti-CD3 and anti-CD28. The two populations of primary human T cells were stimulated with a combination of anti-CD3 and anti-CD28 antibodies. Total RNA was prepared from each sample and subjected to RT-PCR analysis. (**c**) RNA interference with MEF2 expression inhibited IL-2 secretion in response to stimulation by anti-CD3 plus anti-CD28 antibodies. IL-2 protein secreted into the culture medium was determined by ELISA.

A

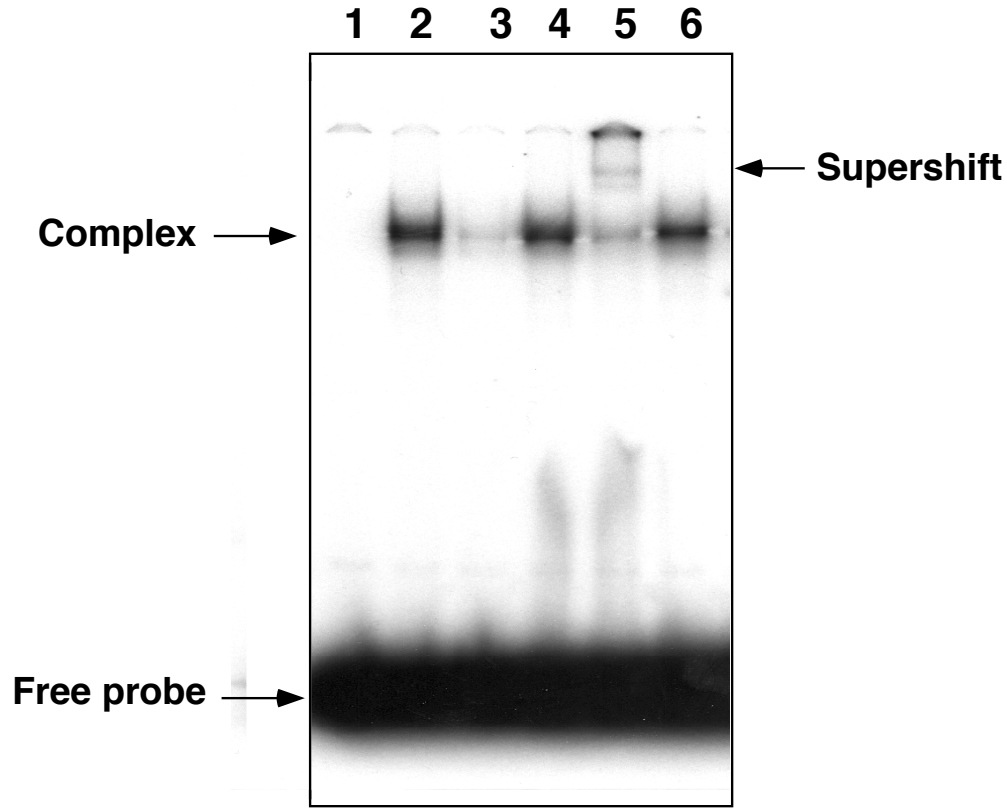
5'-CCCCATATTATTTTTCCAGCATTAACAGTATAAA-3' Mouse

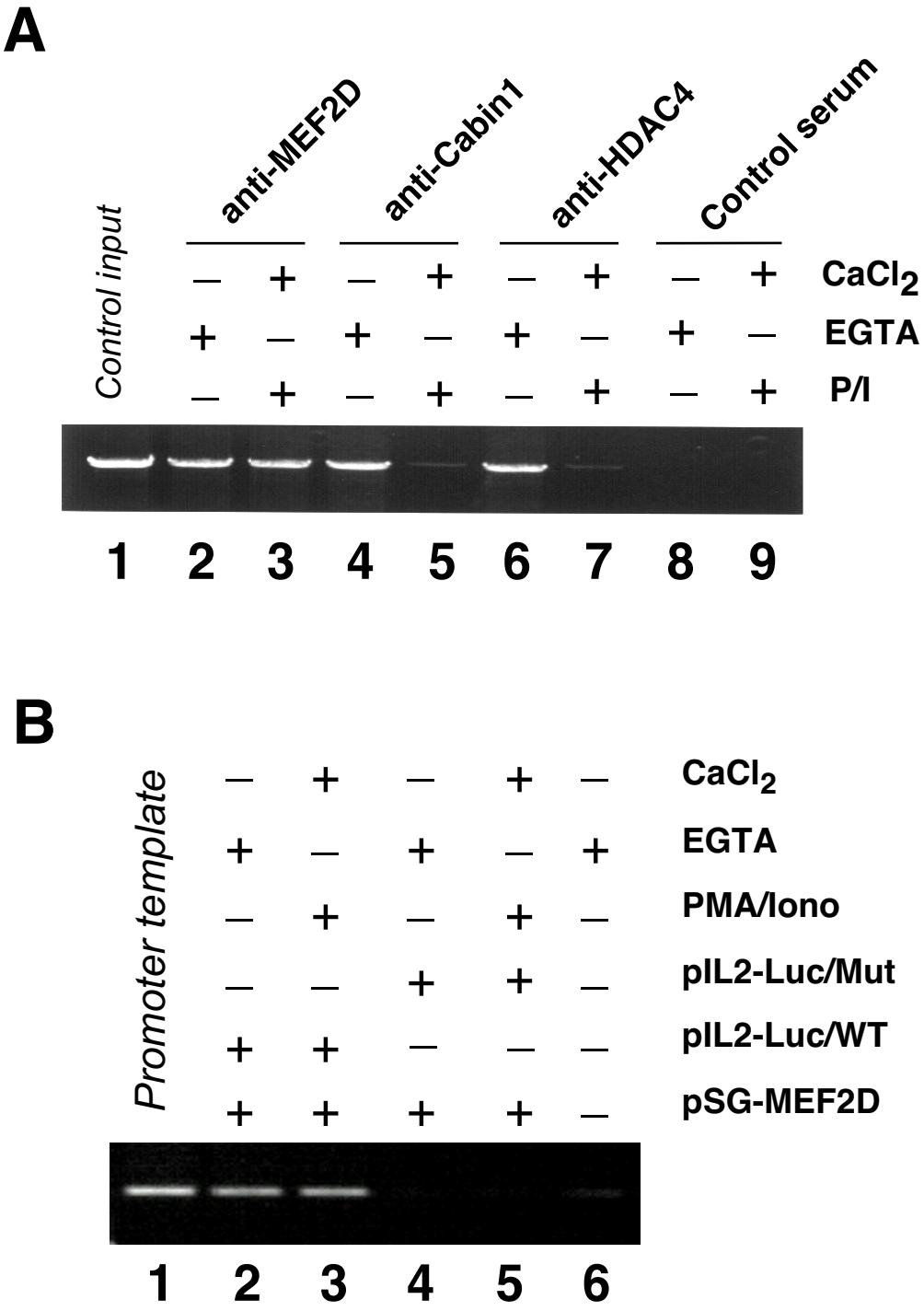
5'-CCCCATAATATTTTTCCAGAATTAACAGTATAAA-3' Human

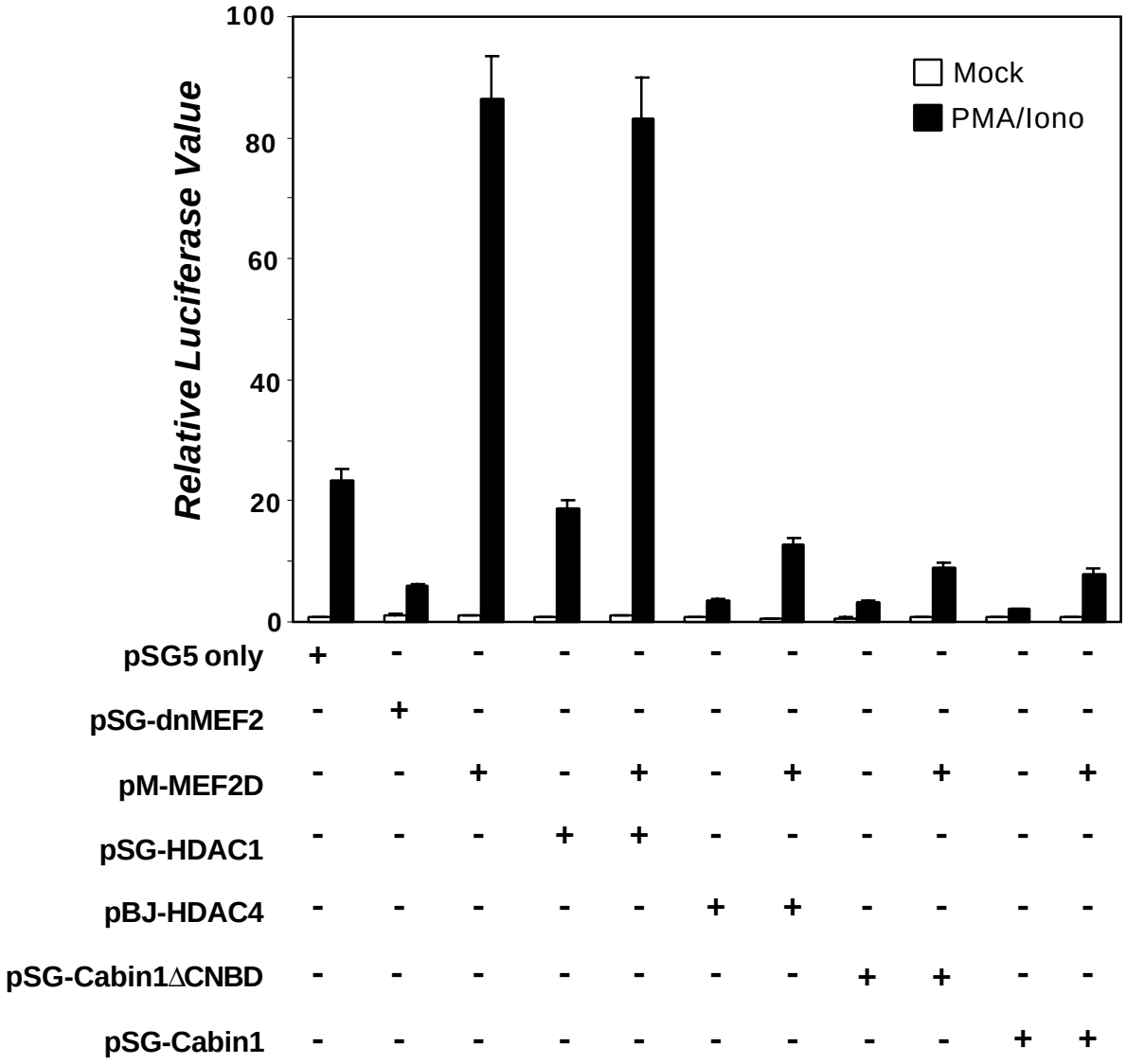
MEF2-Binding Site

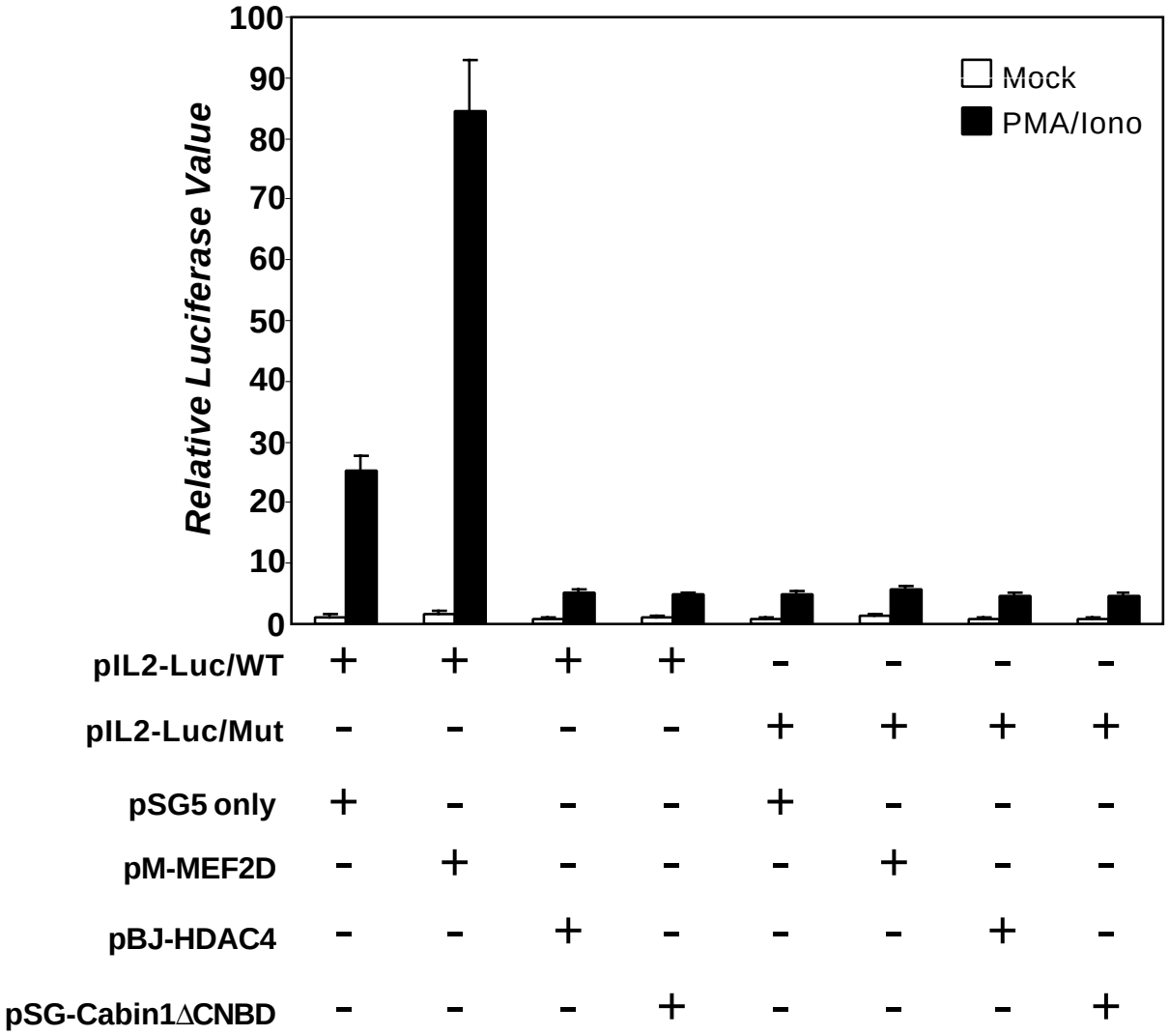
TATA Box

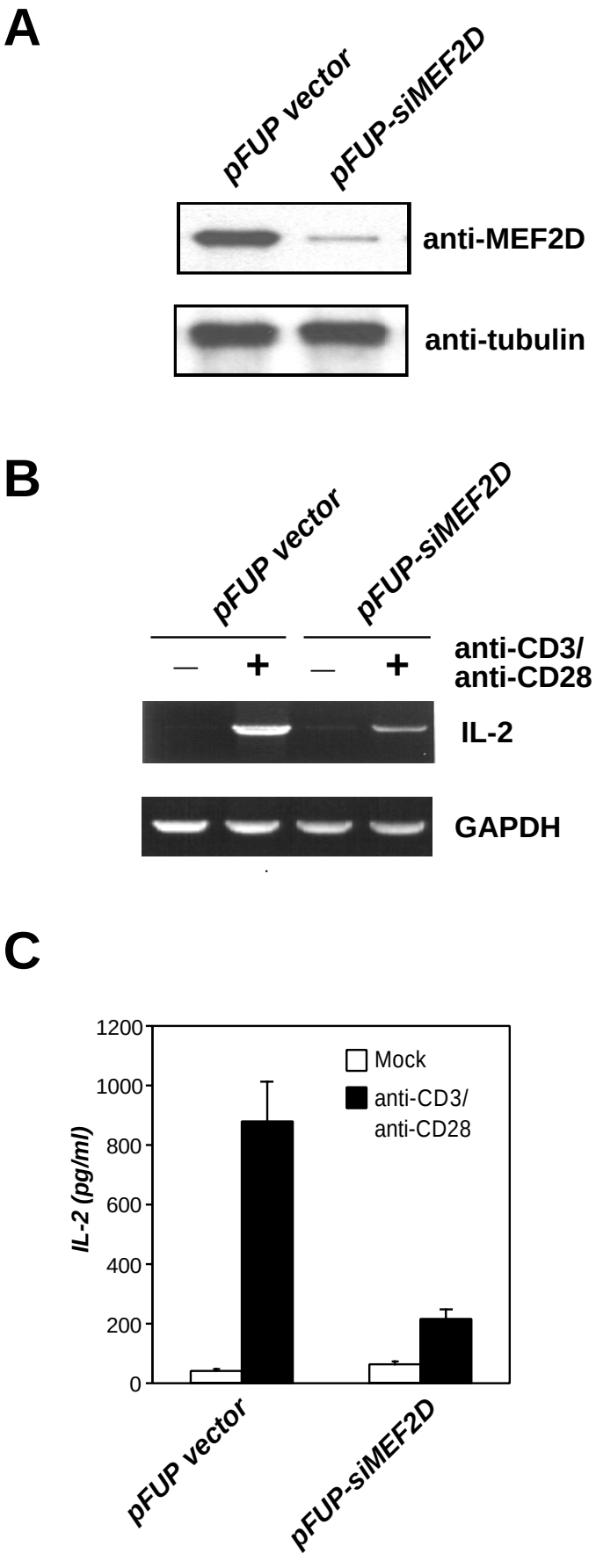
B




A
anti-MEF2D
anti-Cabin1
anti-HDAC4
Control serum
Control input
– + – + – + – + CaCl2
+ – + – + – + – EGTA
– + – + – + – + P/I
1 2 3 4 5 6 7 8 9
B
Promoter template
– + – + – CaCl2
+ – + – + EGTA
– + – + – PMA/Iono
– – + + – pIL2-Luc/Mut
+ + – – – pIL2-Luc/WT
+ + + + – pSG-MEF2D
1 2 3 4 5 6


Relative Luciferase Value
100
80
60
40
20
0
Mock
PMA/Iono
pSG5 only + - - - - - - - - - -
pSG-dnMEF2 - + - - - - - - - - -
pM-MEF2D - - + - + - + - + - +
pSG-HDAC1 - - - + + - - - - - -
pBJ-HDAC4 - - - - - + + - - - -
pSG-Cabin1ΔCNBD - - - - - - - + + - -
pSG-Cabin1 - - - - - - - - - + +


Relative Luciferase Value
100
90
80
70
60
50
40
30
20
10
0
Mock
PMA/Iono
pIL2-Luc/WT + + + + - - - -
pIL2-Luc/Mut - - - - + + + +
pSG5 only + - - - + - - -
pM-MEF2D - + - - - + - -
pBJ-HDAC4 - - + - - - + -
pSG-Cabin1ΔCNBD - - - + - - - +


A
pFUP vector
pFUP-siMEF2D
anti-MEF2D
anti-tubulin
B
pFUP vector
pFUP-siMEF2D
—
+
—
+
anti-CD3/
anti-CD28
IL-2
GAPDH
C
1200
1000
800
600
400
200
0
IL-2 (pg/ml)
Mock
anti-CD3/
anti-CD28
pFUP vector
pFUP-siMEF2D

Suppl. Figure 1 **Pan et al.**