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Transcriptional regulation of nuclear orphan receptor, *NOR-1*, by Ca^{2+}/calmodulin-dependent protein kinase cascade

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Abstract We found that transcription of *NOR-1* (neuron-derived orphan receptor-1) is induced by the Ca^{2+}/calmodulin-dependent protein kinase IV (CaM-KIV) cascade by differential hybridization screening of a cDNA array using probes prepared from SH-SY5Y cells infected with recombinant adenoviruses expressing Ca^{2+}/CaM-independent mutants of CaM-KIV and CaM-K kinase (CaM-KK). Induction of *NOR-1* mRNA expression by the CaM-KIV cascade was confirmed by RT-PCR. Promoter analyses indicate that CaM-KIV cascade response element is located between −162 bp and −42 bp in the 1.7 kb *NOR-1* promoter containing triple cAMP response elements. Disruption of each element significantly reduced the promoter activity, indicating the direct transcriptional regulation of *NOR-1* by CaM-KIV cascade.

Key words: Ca^{2+}; Ca^{2+}/calmodulin-dependent protein kinase IV cascade; Neuron-derived orphan receptor-1; Ca^{2+}/calmodulin-dependent protein kinase kinase; Differential screening

1. Introduction

Ca^{2+}/calmodulin-dependent protein kinases (CaM-Ks) constitute a diverse group of enzymes that are involved in many cellular responses and are activated by increasing intracellular calcium concentrations. Previous studies have demonstrated that two multifunctional CaM-Ks, CaM-KI and IV, are activated by phosphorylation of the Thr in their activation loop by an upstream CaM-K kinase (CaM-KK). This strongly upregulates their catalytic activities (reviewed in [1,2]). This CaM-K cascade has been demonstrated to be involved in the activation of CaM-KIV in response to Ca^{2+} mobilization in transfected COS-7 cells [3], Jurkat cells [4], and cultured hippocampal neurons [5]. It has also been shown that the CaM-KIV cascade is important in regulating Ca^{2+}-dependent gene expression by phosphorylating transcription factors such as cAMP response element (CRE) binding protein (CREB) [6–8]. Furthermore, a recent study indicates that the phosphorylation of CREB is significantly attenuated in transgenic mice carrying dominant negative CaM-KIV and that this disrupts late phase long term potentiation [9]. The consolidation/retention phase of hippocampus-dependent memory is also impaired in these mice. In addition, mice deficient in CaM-KIV show that the CaM-KIV-mediated pathway plays an important role in the function and development of the cerebellum and is critical for male and female fertility [10–12]. Thus, the CaM-KIV pathway plays important roles in various physiological events. Identifying genes that are induced by the CaM-KIV cascade will further aid our understanding of the physiological significance of this signaling pathway. Previous studies have shown that the expression of several genes, including *c-fos* [6], *BDNF* [13], and *insulin* [14], are induced by the Ca^{2+}-dependent CaM-KIV-mediated pathway. Here we performed differential hybridization screening of a cDNA array to identify CaM-KIV cascade-inducible genes. This approach revealed that *NOR-1* (neuron-derived orphan receptor-1), a member of the nuclear receptor family, is a target gene of the CaM-KIV cascade.

2. Materials and methods

2.1. Plasmids and cDNAs

cDNAs of Ca^{2+}/CaM-independent mutant of CaM-KIV (CaM-KIVc, 305HMDT to DEDD), CaM-KIV kinase-negative mutant (CaM-KIVd, 305HMDT to DEDD, K71E), CaM-KK constitutively active mutant (CaM-KKc, residues 1–434) and CaM-KK kinase-negative mutant (CaM-KKd, residues 1–434, K157A) in the pME18s expression plasmids (DNAX Res. Inst. Inc.) were previously described [3,15] (Fig. 1A). *NOR-1* promoter and deletion mutants subcloned in pGV-B plasmid (Wako) were constructed as previously described [16]. Mutagenesis of *NOR-1* promoter was performed by Gene Editor site-directed mutagenesis system (Promega) using mutagenic oligonucleotides as follows: mtCRE1, 5′-GGGAGGAGGAGGGACTGGTAG-CGTCCCATG-3′; mtCRE2, 5′-AGCGTCCCATGGCCAGTCATT-GACGTCTCG-3′; mtCRE3, 5′-CATGGCGTCACATACTGGTCT-CGCATTCCA-3′. Mutations were confirmed by nucleotide sequencing using an ABI 377 automated sequencer.

2.2. Cell culture, transfection and luciferase assay

COS-7 cells and SH-SY5Y cells were maintained as previously described [17]. For transfection assay, COS-7 cells were plated at six-well dishes on the day prior to transfection. COS-7 cells were transfected with a luciferase reporter plasmid fused with the 1.7 kb *NOR-1* promoter together with either 0.8 μg of empty vector (Mock) or 0.4 μg of CaM-KIV and CaM-KK expression plasmids as indicated using LipofectAmine PLUS reagent (Gibco-BRL) according to the manufacturer's instructions. Cells were treated 16 h after transfection with or without 1 μM ionomycin for another 8 h. After transfection, COS-7 cells were incubated in opti-MEM medium for 24 h, then harvested with 150 μl of lysis buffer (25 mM glycyl glycine, pH 7.8, 8 mM $MgSO_4$, 1 mM EDTA, 1% Triton X-100, 5% glycerol, 1 mM dithiothreitol (DTT)), centrifuged at 15 000 rpm at 4°C. For luciferase as-

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Abbreviations: NOR-1, neuron-derived orphan receptor-1; CaM-K, Ca^{2+}/calmodulin-dependent protein kinase; CRE, cAMP response element

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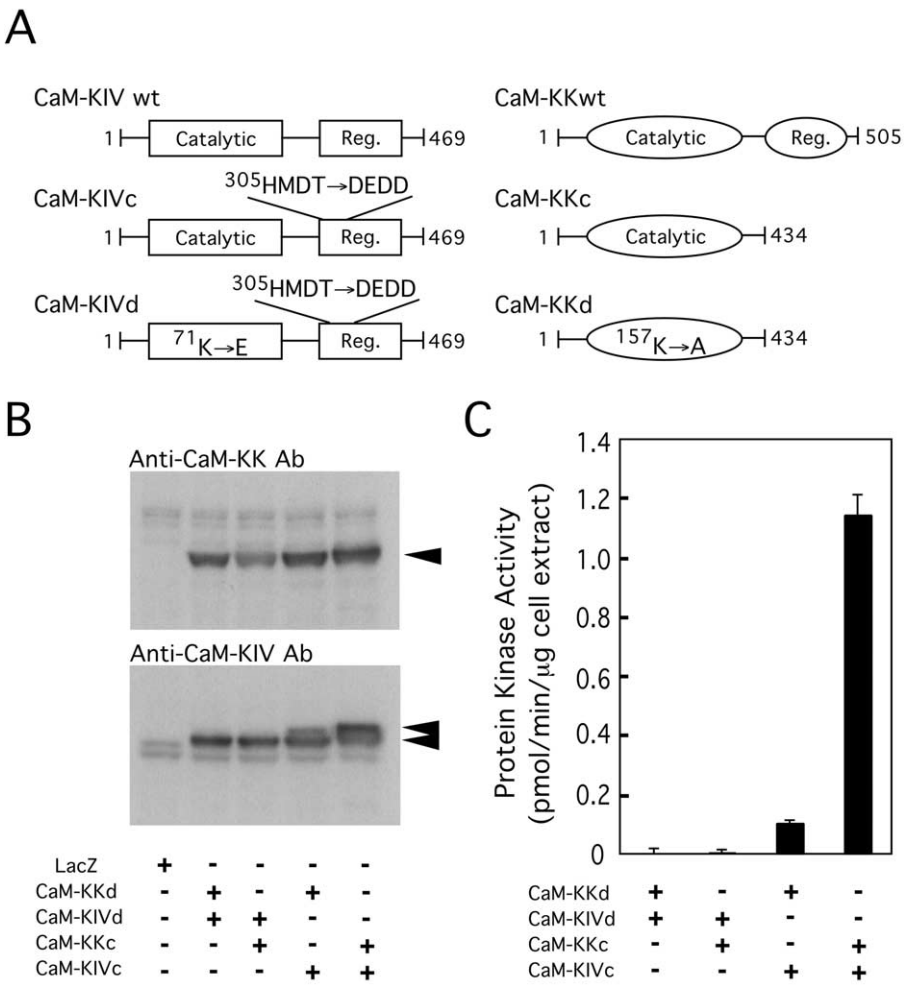

Fig. 1. Expression of the CaM-KIV cascade in SH-SY5Y cells infected with recombinant adenoviruses. A: Schematic representation of Ca^{2+}/CaM-independent (CaM-KIVc and CaM-KKc) and kinase-negative (CaM-KIVd and CaM-KKd) mutants of CaM-KIV and CaM-KK. wt, wild-type. B: Western blot analyses of the expression of CaM-KK and CaM-KIV mutants in adenovirus-infected SH-SY5Y cells, including SH-SY5Y cells infected with an adenovirus containing the β-galactosidase gene (LacZ), by using either anti-CaM-KK (upper panel) or anti-CaM-KIV (lower panel) antibodies. Arrowheads indicate exogenous CaM-KK (upper panel) and CaM-KIV (lower panel) proteins. C: Activation of CaM-KIVc by CaM-KKc in SH-SY5Y cells. Protein kinase activity of CaM-KIVc in adenovirus-infected cell extracts was measured in the presence of EGTA and STO-609. Results represent the mean ± S.D. of three experiments.

say, 50 μl of supernatant was used. Luciferase activity was measured using PicaGene LT7.5 Luminescence kit (Toyo Inki) and presented by light units normalized with the total amount of protein.

2.3. Expression of CaM-KK or CaM-KIV mutants in SH-SY5Y neuroblastoma cells using recombinant adenovirus infection

Recombinant adenoviruses carrying cDNAs of CaM-KK or CaM-KIV were produced by Adeno-X Expression System (Clontech) as previously described [17]. For virus infection, confluent SH-SY5Y cells in six-well culture plates were infected with viruses at a multiplicity of infection of 20 plaque-forming units/cell at 37°C for 1 h. After infection, viruses were aspirated and cells were further cultured in RPMI medium containing 10% fetal bovine serum. The cells were washed with phosphate-buffered saline and lysed with 150 μl of lysis buffer (150 mM NaCl, 20 mM Tris–Cl pH 7.5, 10% glycerol, 2 mM EDTA, 1% NP-40, 0.2 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, 1 μM microcystin-LR) on ice and then subjected to 7.5% SDS–PAGE followed by Western blotting using either anti-CaM-KIV antibody (1:4000, Transduction Lab.) or anti-CaM-KK (1:2000, Transduction Lab.).

2.4. Protein kinase assay

Total cell lysates (8 μg of protein) prepared as described above were incubated at 30°C for 5 min in 25 μl of CaM-KIV kinase buffer (50 mM HEPES, pH 7.5, 10 mM $Mg(Ac)_2$, 1 mM dithiothreitol, 200 μM [γ-^{32}P]ATP (1000–2000 cpm/pmol), 2 mM EGTA and 40 μM syntide-2 in the presence of 10 μg/ml STO-609, a CaM-KK inhibitor [17]. Protein kinase activity was measured as previously described [18].

2.5. Differential hybridization of cDNA microarray

Atlas cDNA expression neurobiology array (Clontech) was probed with [α-^{32}P]dCTP-labeled cDNA synthesized from total cellular RNA that was isolated from the adenovirus-infected SH-SY5Y cells using Trizol reagent (Gibco-BRL). The synthesis of radiolabeled probes, hybridization and washing of the array filters were carried out according to the manufacturer's protocol. Hybridization membranes were then exposed to BioMAX MS film (Kodak) at −80°C for 3 days.

2.6. RT-PCR analysis

For RT-PCR, 1 μg of total RNA prepared from the adenovirus-infected SH-SY5Y cells was reverse-transcribed to generate the first strand cDNA using AMV reverse transcriptase (Life Science). Synthesized cDNA was used as a template in PCR using Titanium Taq polymerase (Clontech) with pairs of primers for *c-fos* (forward primer: 5′-GCCTCTCTTACTACCACTCAC-3′, reverse primer: 5′-TAGTTGGTCTGTCTCCGCTTG-3′), *G3PDH* (forward primer: 5′-TGAAGGTCGGAGTCAACGGATTTGG-3′, reverse primer: 5′-CATG-

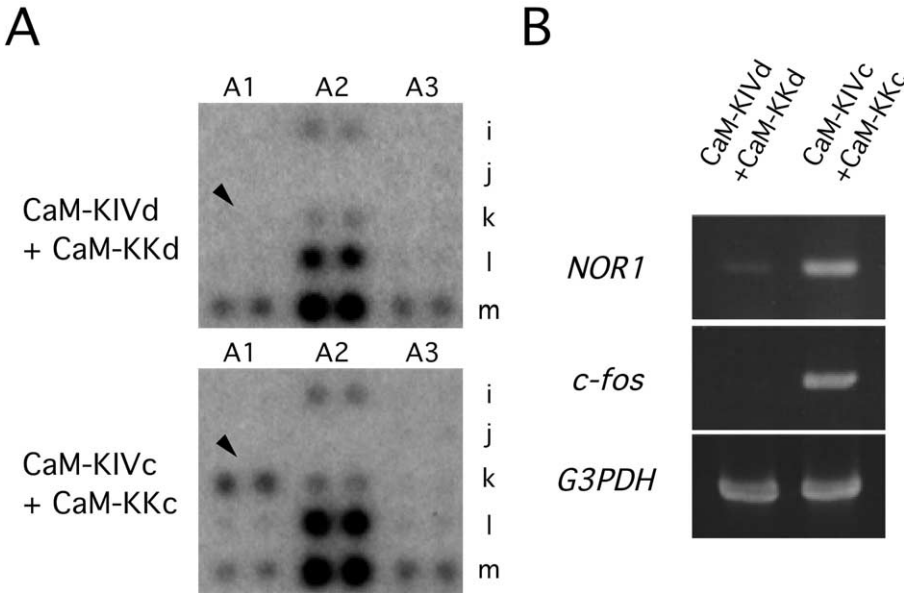

Fig. 2. The CaM-KK/CaM-KIV cascade induces *NOR-1* gene expression. A: Induction of *NOR-1* gene expression by CaM-KKc and CaM-KIVc in SH-SY5Y was detected by differential hybridization using a neurobiology cDNA expression array. ^{32}P-labeled cDNA probes synthesized from total cellular RNA prepared from SH-SY5Y cells expressing either CaM-KKc/CaM-KIVc (lower panel) or CaM-KKd/CaM-KIVd (upper panel) as described in Fig. 1A were hybridized to two identical gene array membranes (Atlas Human Neurobiology Array, Clontech). Each gene is represented by duplicate spots. Arrows indicate duplicate spots of the *NOR-1* gene. B: RT-PCR analysis to detect *NOR-1* transcripts induced by CaM-KKc and CaM-KIVc. RT-PCR was performed using cDNA templates from total cellular RNA prepared from SH-SY5Y cells expressing either CaM-KKc/CaM-KIVc (right lane) or CaM-KKd/CaM-KIVd (left lane). *G3PDH* and *c-fos* were also amplified as a negative and a positive control, respectively.

TGGGCCATGAGGTCCACCAC-3′) and *NOR-1* (forward primer: 5′-TAATCAGGAGCAGTGGAGCAGTGAG-3′, reverse primer: 5′-TAAGACACATCCTACCCTGGCGAAC-3′). PCR products were then separated on a 2% agarose gel and stained with ethidium bromide.

3. Results and discussion

To identify the gene(s) whose expression is induced by the CaM-KK/CaM-KIV cascade, we generated recombinant adenoviruses carrying the Ca^{2+}/CaM-independent form of CaM-KIV (CaM-KIVc, 305HMDT-DEDD) [15], constitutively active CaM-KK (CaM-KKc, 1–434) [3] and kinase-negative versions of equivalent mutants. After SH-SY5Y neuroblastoma cells were infected with various combinations of recombinant adenoviruses carrying the CaM-KIV and CaM-KK mutants, the ability of the cell extracts to phosphorylate the syntide-2, a peptide substrate that can be phosphorylated by CaM-KIV, was measured in the absence of Ca^{2+}/CaM (Fig. 1C). This protein kinase assay measuring CaM-KIV activity was performed in the presence of STO-609, a specific inhibitor of CaM-KK [17], to avoid activation of CaM-KIV during the kinase assay by contaminating CaM-KKc proteins in the cell extract. As expected, no protein kinase activity was observed in the extract of cells expressing the kinase-deficient CaM-KIV mutant (CaM-KIVd) in combination with either the constitutively active CaM-KK or the kinase-deficient CaM-KK mutant (CaM-KKd). In contrast, the extract of cells expressing the Ca^{2+}/CaM-independent form of CaM-KIVc exhibited a weak syntide-2 phosphorylation activity that was further upregulated (8–10-fold) by co-expression of the constitutively active CaM-KKc. This is consistent with the in vitro activa-

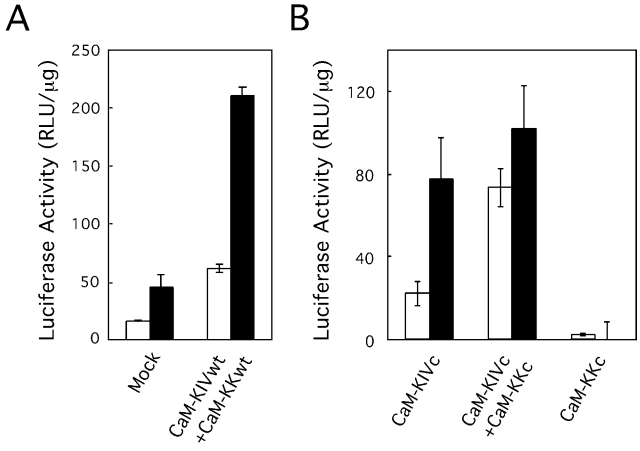

Fig. 3. Induction of *NOR-1* promoter activity by the CaM-KK/CaM-KIV cascade. A: Transcriptional activity of the 1.7 kb *NOR-1* promoter [16]. COS-7 cells were transfected with a luciferase reporter plasmid fused with the 1.7 kb *NOR-1* promoter together with either 0.8 μg of empty vector (Mock) or 0.4 μg of CaM-KIV wild-type and CaM-KK wild-type expression plasmids as indicated. Cells were treated 16 h after transfection with vehicle (DMSO, open bar) or 1 μM ionomycin (closed bar) for another 8 h followed by measurement of luciferase activity. B: CaM-KKc and CaM-KIVc synergistically induce *NOR-1* promoter activation. COS-7 cells were transfected with 1.7 kb *NOR-1* reporter plasmid and 0.4 μg of expression plasmid encoding CaM-KIVc and/or CaM-KKc as indicated. Cells were treated 16 h after transfection with either vehicle (DMSO, open bar) or 1 μM ionomycin (closed bar) for another 8 h followed by measurement of luciferase activity. Results represent the mean ± S.D. of three independent transfections.

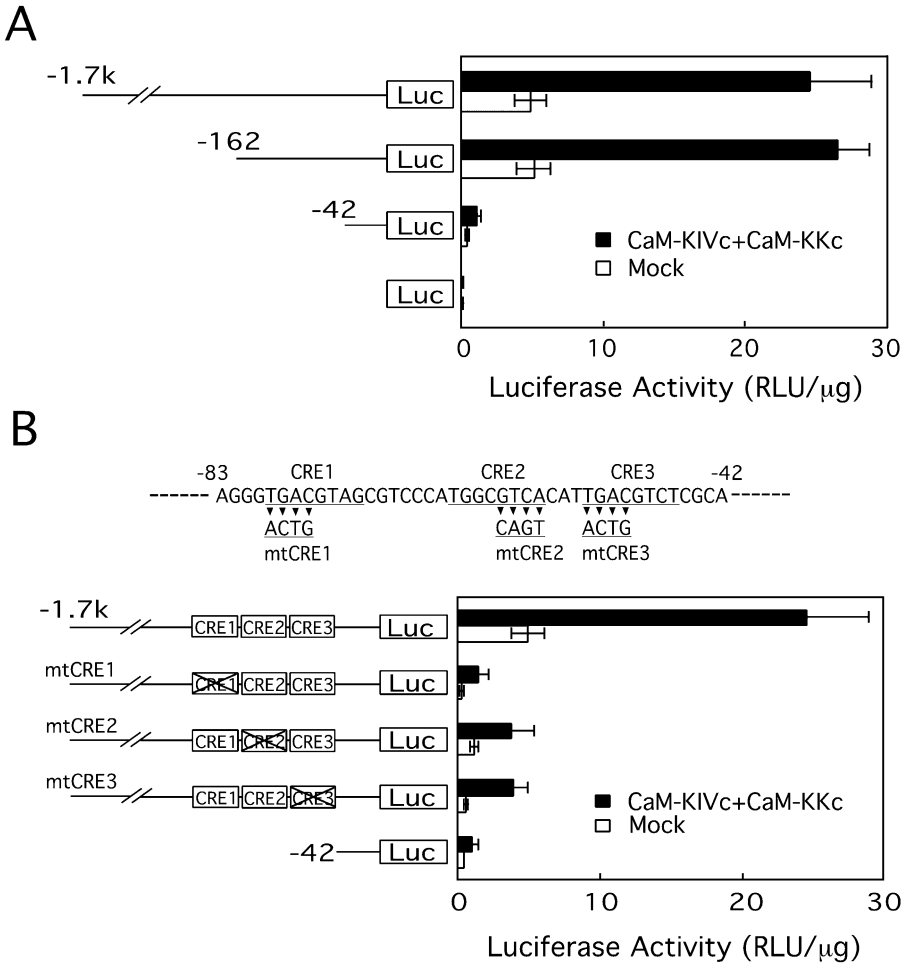

Fig. 4. CREs in the *NOR-1* promoter are the CaM-KIV cascade response elements. A: Mapping of CaM-KIV cascade response elements in the *NOR-1* promoter. Promoter activities of the various indicated deletion mutants of the *NOR-1* promoter were analyzed as described in Fig. 3 in either transfected COS-7 cells with an empty vector (Mock, open bar) or COS-7 cells co-transfected with the CaM-KIVc and CaM-KKc expression plasmids (closed bar). B: Mutagenesis analysis of CREs in the *NOR-1* promoter. Promoter activities of 1.7 kb *NOR-1* reporter genes containing mutants of each putative CRE (mtCRE1, mtCRE2 and mtCRE3) were analyzed as described in Fig. 3 in either transfected COS-7 cells with an empty vector (Mock, open bar) or COS-7 cells co-transfected with the CaM-KIVc and CaM-KKc expression plasmids (closed bar). Nucleotide sequence indicates the position of the putative CREs in the *NOR-1* promoter [16]. The mutagenized nucleotides in each CRE are also indicated. Results represent the mean ± S.D. of three independent transfections.

tion of CaM-KIVc by CaM-KKc that has been demonstrated previously [19]. The cell extracts were also subjected to Western blot analysis using the anti-CaM-KK and anti-CaM-KIV antibodies (Fig. 1B). The CaM-KIVc band was supershifted on the SDS–PAGE gel when the CaM-KIVc-expressing cells had been co-infected with CaM-KKc. No supershift of the kinase-deficient CaM-KIV mutant (CaM-KIVd) co-infected with CaM-KKc was observed while Thr196 is intact in this mutant, which is a phosphorylation site for CaM-KK, indicating that the hyper-autophosphorylation of CaM-KIV enhanced by CaM-KK results in the supershift. These results indicate that CaM-KKc phosphorylates Thr196 of CaM-KIVc in SH-SY5Y cells resulting in induction of CaM-KIVc activity.

We prepared a ^{32}P-labeled cDNA probe from total cellular RNA isolated from adenovirus-infected SH-SY5Y cells expressing either CaM-KIVc/CaM-KKc or the kinase-deficient CaM-KIVd/CaM-KKd mutants as a control. These probes were used with identical neurobiology cDNA expression arrays containing 588 genes, which did not include CaM-KIV target genes such as *c-fos* and *BDNF*. We compared the panels hybridized with the two different probes (Fig. 2A) and found that expression of the *NOR-1* gene was significantly upregulated (arrowhead) by the expression of CaM-KKc/CaM-KIVc. *NOR-1* has previously been identified as a member of the nuclear orphan receptor [20,21]. It is noteworthy that we could not identify any other genes on this cDNA array whose expressions were significantly induced by CaM-KKc/CaM-KIVc.

To confirm the induction of *NOR-1* by the CaM-KIV cascade, we performed RT-PCR analysis using total cellular RNA prepared from adenovirus-infected SH-SY5Y cells. As shown in Fig. 2B, *NOR-1* mRNA is significantly induced by the expression of CaM-KKc/CaM-KIVc while *G3PDH* is not. *c-fos*, a target gene of CaM-KIV, was also induced by CaM-KKc/CaM-KIVc expression [6]. Notably, we found by RT-PCR analysis that CaM-KKc/CaM-KIVc expression also strongly induces the expression of *Nur77* (*NGFI-B*), another nuclear orphan receptor (data not shown).

To examine whether *NOR-1* promoter activity is directly

regulated by the CaM-KK/CaM-KIV cascade, we used a reporter gene fused with the 1.7 kb promoter of *NOR-1* that has previously been isolated [16]. COS-7 cells were co-transfected with wild-type CaM-KIV and CaM-KK. As shown in Fig. 3A, stimulation of these cells with ionomycin to mobilize Ca^{2+} strongly induced *NOR-1* promoter activity. When the cells were transfected with CaM-KIVc only, ionomycin stimulation stimulated *NOR-1* promoter activity four-fold, indicating the presence of endogenous CaM-KK in COS-7 cells (Fig. 3B). In the absence of Ca^{2+} mobilization, CaM-KIVc-mediated transcriptional activation of the *NOR-1* promoter was enhanced (~four-fold) by co-transfection with CaM-KKc. These observations correlate well with the activation of CaM-KIVc by CaM-KKc in unstimulated SH-SY5Y cells (Fig. 1C). Thus, the *NOR-1* promoter is directly activated by the CaM-KK/CaM-KIV cascade in intact cells.

We used reporter genes fused with various truncated *NOR-1* promoters to identify the CaM-KIV cascade response *cis*-element in the promoter (Fig. 4A). COS-7 cells were transfected with these reporters as well as with CaM-KIVc and CaM-KKc. The CaM-KIV cascade-induced *NOR-1* promoter activity of the reporter gene truncated at −162 bp was indistinguishable from that of the 1.7 kb promoter. However, further truncation of 120 bp significantly reduced the enhancement of the promoter activity by the CaM-KIV cascade. This indicates that the region between −162 bp and −42 bp in the *NOR-1* promoter contains the CaM-KIV cascade response element. Recently, three copies of putative CRE (CRE1, CRE2 and CRE3) have shown to be located in this region [16]. We tested whether these CREs are involved in the CaM-KIV cascade by using 1.7 kb *NOR-1* promoters containing mutations in one of the CRE elements (Fig. 4B). Mutations of any of the CREs (mtCRE1, mtCRE2 and mtCRE3) significantly impaired the promoter activity indicating that all the CREs in the *NOR-1* promoter are required for the full induction of *NOR-1* gene transcription by the CaM-KIV cascade.

In summary, we identified *NOR-1* as a CaM-KK/CaM-KIV cascade-inducible gene by using differential hybridization screening with probes derived from adenovirus-infected cells expressing Ca^{2+}/CaM-independent and inactive forms of CaM-KK and CaM-KIV. This approach will be useful in identifying other gene(s) whose transcription is induced by the CaM-KK/CaM-KIV cascade as well as by other signal transduction pathways. *NOR-1* has been identified as a member of the family of nuclear orphan receptor type transcription factors [20,21]. A recent study has revealed that *Nur77* (*NGFI-B*), another nuclear orphan receptor, is also induced by the CaM-KIV-mediated pathway in T-lymphocytes [22]. We confirmed such CaM-KIV-mediated induction of *Nur77* transcription by RT-PCR analysis in this study. The transcription of *Nur77* has been shown to be upregulated by CaM-KIV in a MEF2-dependent manner [22]. We show here, however, that *NOR-1* transcription induced by CaM-KK/CaM-KIV cascade is dependent on three CREs in the *NOR-1* promoter. This correlates well with previous studies indicating that CREB is a target of CaM-KIV [6–8]. It has been suggested that the physiological function of the nuclear orphan receptors is to promote apoptosis [23]. In thymocytes, the induction of *Nur77* is involved in negative selection, an event in thymic development characterized by apoptosis of T-cells whose T-cell receptor recognizes host tissue too strongly [24,25]. Further study will be necessary to examine the involvement of the CaM-KIV cascade in inducing the apoptosis of various cell types by regulating the transcription of nuclear orphan receptor family proteins.

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