Calcium-dependent Activation of Nuclear Factor Regulated by Interleukin 3/Adenovirus E4 Promoter-binding Protein Gene Expression by Calcineurin/Nuclear Factor of Activated T Cells and Calcium/Calmodulin-dependent Protein Kinase Signaling*

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An increase in the intracellular Ca^{2+} concentration controls a diverse range of cell functions, including gene expression, apoptosis, adhesion, motility, and proliferation. We have investigated Ca^{2+} regulation of gene expression in rat aortic smooth muscle cells. We found that the expression of nuclear factor regulated by interleukin 3 (NFIL3)/adenovirus E4 promoter-binding protein (E4BP4)/basic region/leucine zipper (bZIP) type of a transcription factor that has a very important function in cell survival, was activated by thapsigargin (TG). This activation was inhibited by chelation of extra- or intracellular Ca^{2+}, suggesting that the induction by TG was dependent on the elevation of [Ca^{2+}]. Specific inhibition of calcineurin or calcium/calmodulin-dependent protein kinase (CaM kinase) by chemical means impaired the TG-induced NFIL3/E4BP4 expression. Expression of dominant negative forms of calcineurin or nuclear factor of activated T cells (NFAT) inhibited the induction of NFIL3/E4BP4 mRNA by TG. These results suggest that intracellular Ca^{2+} plays a critical role in regulating gene expression of NFIL3/E4BP4 by calcineurin/NFAT and CaM kinase signaling in vascular smooth muscle cells.

Calcium signals regulate numerous cell functions, including gene expression, some forms of programmed cell death, motility, secretion, proliferation, and cell survival (1). ([Ca^{2+}]), can be raised experimentally by a number of agents, including the micromolar calcium ATPase inhibitor TG (1, 2). We investigated the regulation of gene expression in rat aortic smooth muscle cells (RASMC) exposed to TG by utilizing the fluorescent differential display technique (3). We compared the complementary DNA fragments from differentially expressed RASMC mRNAs following 3 h treatment with either vehicle or 100 nM TG. This analysis resulted in the identification of the rat homologue of NFIL3/E4BP4 as a TG-inducible gene.

NFIL3/E4BP4 was originally isolated as a novel member of the bZIP family of DNA-binding proteins that displays an unusual DNA binding specificity which overlaps that of the activating transcription factor family and cAMP response element-binding protein (4). NFIL3/E4BP4 repressed promoter activity and this repression was mediated through the cAMP response element/activating transcription factor-like site (5). NFIL3/E4BP4 might play a role in the glucocorticoid repression of several genes, because NFIL3/E4BP4 was induced by the synthetic glucocorticoid dexamethasone (6). Moreover, NFIL3/E4BP4 repressed the stimulating activity of box α, which is an essential element that positively regulates the transcription of human hepatitis B virus genes (7). The potent suppression of box α activity by NFIL3/E4BP4 may contribute to the silencing of hepatitis B virus gene expression (7).

NFIL3/E4BP4 also plays an important role in the expression of IL-3 in T cells (8). In mouse pro-B cell lines, NFIL3/E4BP4 was also regulated as a delayed-early IL-3 responsive gene, requiring de novo protein synthesis (9). In the absence of IL-3, enforced expression of human NFIL3/E4BP4 promoted the survival but not the growth of IL-3-dependent pro-B cells, indicating that induction of NFIL3/E4BP4 is one of the mechanisms through which IL-3 suppresses apoptosis (9).

Recently, it has been found that the expression of NFIL3/E4BP4 was regulated by oncogenic Ras mutant proteins through both the Raf-mitogen-activated protein kinase and the phosphatidylinositol 3-kinase pathways in murine Pro-B lymphocytes (10). Ras-NFIL3/E4BP4 pathways may be common targets for a variety of oncogenes (10).

In this paper, we describe the cloning and calcium regulation of expression of rat NFIL3/E4BP4 in RASMC. We show that TG induced rat NFIL3/E4BP4 gene expression by activating the calcineurin/NFAT and by CaM kinase signaling. Thus, calcium

ER, endoplasmic reticulum; BAPTA-AM, 1,2-bis(2-aminophenox)-ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl; CsA, cyclosporin A; dnNFAT, dominant-negative nuclear factor of activated T cells; ERR, extracellular regulated kinase; NFIL-3, nuclear factor regulated by interleukin 3; E4BP4, adenovirus E4 binding protein; CaM, calmodulin; CaM kinase, calcium/calmodulin-dependent protein kinase.
signaling may be critical for the regulation of NFIL3/E4BP4 through calcineurin/NFAT and CaM kinase signaling in vascular smooth muscle cells.

EXPERIMENTAL PROCEDURES

Reagents—Thapsigargin, calcium ionophore A23187, phorbol 12-myristate 13-acetate (PMA), actinomycin D, and cycloheximide were purchased from Sigma. Cyclosporin A, FK506, KN-93, and PD98059 were purchased from CalBiochem. KN-92 was purchased from SeikagakuKogyo Co. Tokyo, Japan.

Cloning of Rat NFIL3/E4BP4 cDNA—For cloning the 3'-half of the rat NFIL3/E4BP4 cDNA, we performed a rapid amplification of cDNA ends (RACE) (11). The 3'-half of the full-length cDNA was obtained using the primers 5'-TTACGCCAAGTCCGGATATGAAG-3' and 5'-CCACTCTAAAATGACGTCTAGGCGG-3' for the first RACE and 5'-GGAGATGGCCTAGCAACCTGGACAGCAT-3' and 5'-ACTCTCATAT-AGGACGCTGAGCCGC-3' for the second RACE. A rat heart Marathon cDNA amplification kit (CLONTECH) was used for the template. Unfortunately, we could not obtain the 5'-half of the full-length cDNA by RACE, and so we performed polymerase chain reactions (PCR) using a degenerate primer. The 5'-half of the full-length cDNA was obtained by using the primers 5'-AAGGKMCTGCRGACRTTAYCC-3' and 5'-ATGTTCTGCACCTGCGAGAACGAG-3'. The PCR products were sequenced using automated DNA sequencing (Applied Biosystems).

Northern Blotting Analysis—Northern blotting analysis was carried out using Rat Multiple Tissue Northern blots (CLONTECH). 32P-Random prime-labeled fragment of the rat NFIL3/E4BP4 cDNA (nucleotides 1093–1701) probe was synthesized with Strip-EZ DNA (Ambion). The heat-denatured DNA probe was added to 2 x 106 cpm/ml to ExpressHyb hybridization solution (CLONTECH). The probe was hybridized to the blots for 1 h at 65 °C. The membrane was washed according to the manufacturer's protocol and exposed to x-ray film at –80 °C. The filter was re-probed with 32P-random prime-labeled fragment of the rat β-actin cDNA to ensure approximately equal loading of RNA samples.

Plasmid Constructs—The dominant negative human calcineurin (12) and the calciumbinding region (aa 101–122) (18), and a novel transcrip- tion factor are a region of basic amino acids that could bind DNA (aa 79–93) (17), a region containing leucine residues repeated ev- ery seven amino acids (aa 101–122) (18), and a novel transcrip- tion factor are a region of basic amino acids that could bind DNA (aa 79–93) (17), a region containing leucine residues repeated ev- ery seven amino acids (aa 101–122) (18), and a novel transcrip- tion factor are a region of basic amino acids that could bind DNA (aa 79–93) (17), a region containing leucine residues repeated ev- every seven amino acids (aa 101–122) (18), and a novel transcrip- tion factor are a region of basic amino acids that could bind DNA (aa 79–93) (17), a region containing leucine residues repeated ev- ery seven amino acids (aa 101–122) (18), and a novel transcrip-
hybridization was performed to analyze the tissue distribution of rat NFIL3/E4BP4 (Fig. 3). A 2.5-kilobase transcript could be detected in all tissues except the pancreas. Modest expression was seen in heart, lung, and skeletal muscle. Strongest expression was seen in liver, although human E4BP4 is expressed very little in the liver (7). The blot was subsequently reprobed with a radiolabeled rat β-actin cDNA to ensure equal loading.

**Real-time RT-PCR analysis was then performed to confirm the differential expression of NFIL3/E4BP4.** As shown in Fig. 4B, pretreatment of A7r5 cells with the extracellular Ca\(^{2+}\) chelating agent EGTA resulted in significant inhibition of TG-induced expression of NFIL3/E4BP4. Almost complete inhibition was observed when A7r5 cells were treated with 10 μM BAPTA-AM, a cell permeable Ca\(^{2+}\) chelating agent (Fig. 4B). These results suggest that increased cytosolic Ca\(^{2+}\) concentration is necessary to induce NFIL3/E4BP4 gene expression by TG.

**Effects of inhibitors of Ca\(^{2+}\)/calmodulin-dependent Protein Phosphatase and Protein Kinases on the Induction of NFIL3/E4BP4 mRNA by TG**—Because increased cytosolic Ca\(^{2+}\) concentration is necessary to induce NFIL3/E4BP4 gene expression by TG, we investigated the effects of Ca\(^{2+}\)/calmodulin-dependent protein phosphatase and protein kinases on NFIL3/E4BP4 expression. Cyclosporin A (CsA) and FK506, in complex with calcineurin and CaM kinase. These two inhibitors were investigated along with KN-93, an inactive analog of KN-93, displayed much less inhibition than KN-93. These results suggest that the calcium dependent activation of NFIL3/E4BP4 is mediated by calcineurin and CaM kinase.

**Fig. 1. Nucleotide and predicted amino acid sequences of rat NFIL3/E4BP4 cDNA (accession number AY004663).** The amino acid sequence of rat NFIL3/E4BP4 is shown in the single-letter code below the nucleotide sequence. The canonical polyadenylation signal is underlined.

**Induction of NFIL3/E4BP4 mRNA by TG Treatment**—Real-time RT-PCR analysis was then performed to confirm the differential expression results indicating that rat NFIL3/E4BP4 was a TG-inducible gene. As shown in Fig. 4A, treatment with TG resulted in a marked increase in rat NFIL3/E4BP4 mRNA levels. An elevation in NFIL3/E4BP4 mRNA level was apparent within 3 h, peaked at 6 h of treatment (2-fold) and expression returned to almost basal levels by 12 h.

**Dose dependence of NFIL3/E4BP4 mRNA induction by TG** was also examined. The induction was increased from 1 to 30 nM with almost no further increases at higher concentrations (Fig. 4B).

**TG-induced Expression of NFIL3/E4BP4 mRNA Is Dependent on Elevation of Intracellular Ca\(^{2+}\)—**TG induces both elevations of intracellular Ca\(^{2+}\) concentration (2) and an endoplasmic reticulum (ER) stress response (20). We therefore tested whether a range of different stress-inducing conditions could prevent expression of the NFIL3/E4BP4 mRNA.

As shown in Fig. 5B, pretreatment of A7r5 cells with the extracellular Ca\(^{2+}\) chelating agent EGTA resulted in significant inhibition of TG-induced expression of NFIL3/E4BP4. Almost complete inhibition was observed when A7r5 cells were treated with 10 μM BAPTA-AM, a cell permeable Ca\(^{2+}\) chelating agent (Fig. 5B). These results suggest that increased cytosolic Ca\(^{2+}\) concentration is necessary to induce NFIL3/E4BP4 gene expression by TG.
We next used the protein synthesis inhibitor cycloheximide to determine whether TG-induced expression of NFIL3/E4BP4 mRNA was dependent on de novo protein synthesis. As shown in Fig. 7, A7r5 cells were either left untreated or treated with TG for 3 h with or without pretreatment of cycloheximide. The pretreatment of cycloheximide to TG-stimulated cells did not significantly prevent NFIL3/E4BP4 gene induction (Fig. 7). These results indicate that the induction of NFIL3/E4BP4 mRNA by TG does not require de novo protein synthesis for transcriptional activation.

**TG Regulates NFIL3/E4BP4 Gene Expression by Mechanisms Involving Calcineurin**—The immunosuppressive drugs CsA and FK506 target the phosphatase activity of calcineurin. But it has also been reported that CsA and FK506 could target other proteins (e.g., JNK (24) and p38 (25)). To further verify that calcineurin is involved in TG-induced expression of NFIL3/E4BP4 mRNA, we transfected dominant negative calcineurin (12) into A7r5 cells. Calcineurin dephosphorylates NFAT proteins and induces their translocation from the cytoplasm into the nucleus. The dominant negative calcineurin is exclusively cytoplasmic and interferes with NFAT translocation (12). As shown in Fig. 8, the dominant negative calcineurin (H101Q/H290Q) caused inhibition of TG-induced expression of NFIL3/E4BP4 mRNA compared with the control. These data indicate that calcineurin activity is required for the induction of NFIL3/E4BP4 mRNA expression by TG.

**DISCUSSION**

This study has demonstrated that the expression of NFIL3/E4BP4 mRNA is induced by calcium signaling pathways. The endoplasmic reticulum calcium-ATPase inhibitor TG depletes Ca$^{2+}$ from internal stores, leading to Ca$^{2+}$ influx and thus, to a sustained increase in [Ca$^{2+}$]$_i$ (1). TG also induces an ER stress response (20). We demonstrated that chelation of extracellular and intracellular Ca$^{2+}$ by EGTA and BAPTA-AM, respectively, suppressed the induction of NFIL3/E4BP4 mRNA.
by TG. We also demonstrated that the induction of NFIL3/E4BP4 mRNA by tunicamycin, which induces an ER stress response (20) without disturbing Ca\textsuperscript{2+} homeostasis, was less than with TG or A23187. These results suggest that an increase in [Ca\textsuperscript{2+}] is critically involved in NFIL3/E4BP4 mRNA induction by TG.

Fluxes in the amount of intracellular Ca\textsuperscript{2+} are important determinants of gene expression (1). Of the many types of Ca\textsuperscript{2+}-binding proteins, calmodulin (CaM) is of major importance in vascular smooth muscle cell function. Ca\textsuperscript{2+}/CaM regulates functional proteins such as calcineurin (28) and CaM kinase (29) that play important roles in gene expression in vascular smooth muscle cells. To evaluate the relative regulatory roles of calcineurin and CaM kinase, we tested the effect of inhibitors of calcineurin and CaM kinase on TG-induced NFIL3/E4BP4 mRNA expression. We have demonstrated that both CsA and FK506 inhibited the induction of NFIL3/E4BP4 mRNA. The induction of NFIL3/E4BP4 mRNA by TG was also inhibited by KN-93. These results indicate that TG induces the NFIL3/E4BP4 gene expression by calcineurin and CaM kinase-dependent mechanisms.

To further verify that TG involves calcineurin in the induction of NFIL3/E4BP4 mRNA, we transfected dominant negative calcineurin into A7r5 cells. Expression of dominant negative calcineurin strongly suppressed the induction of NFIL3/E4BP4 mRNA by TG. All of these results strongly suggest that calcineurin plays a critical role in the selective induction of NFIL3/E4BP4 mRNA by TG. Calcineurin regulates immune response genes through dephosphorylation of NFAT (28).

Following dephosphorylation, NFAT is translocated into the nucleus where it binds either directly to DNA or in a complex with members of the bZIP transcription factors family such as AP-1 (30) and subsequently activates gene transcription. NFAT proteins are present in diverse cell types including vascular smooth muscle cells (31).

We tested the involvement of NFAT in NFIL3/E4BP4 mRNA expression by using the dnNFAT molecule. Inhibition of NFAT-mediated transcription by the dnNFAT resulted in significant suppression of the induction of NFIL3/E4BP4 mRNA by TG. These data indicate that NFAT is required for the expression of NFIL3/E4BP4 gene by TG. The NFAT family has a rel-type DNA-binding domain which lacks sequences in NF\textsubscript{B} p50 that interact with DNA (28). In addition, a critical arginine involved in DNA binding by NF\textsubscript{B} is substituted with a histidine in NFAT family members (28). NFAT family members have evolved to interact weakly with DNA and to require a partner for high-affinity DNA binding at most sites (28). Complexes induced through other signaling pathways such as AP-1 (30, 32) or cell type-specific proteins such as MEF2 (33–35), GATA2 (36), GATA4 (37), or others (38, 39) can provide the partner. Although the human NFIL3/E4BP4 gene is mapped to 9q22 (40), the complete genome sequences of human, mouse, and rat NFIL3/E4BP4 are currently unknown. Homo sapiens chromosome 9 clone RP11-440G5 (GenBank\textsuperscript{TM} accession number AL353764) appears to contain the human NFIL3/E4BP4 gene. The structure of the NFIL3/E4BP4 gene and the exact mechanisms by which NFAT activates the expression of NFIL3/E4BP4 are still to be determined.

KN-93 prevents the activation of CaM kinase II by interacting with the calmodulin-binding domain of the kinase (23). As shown in Fig. 6, 1 \textmu M KN-93 markedly reduced the TG-induced NFIL3/E4BP4 expression, whereas 1 \textmu M KN-92, an inactive analog of KN-93, displayed much less inhibitory effects than KN-93. These results suggest that CaM kinase signaling is involved in the TG-dependent NFIL3/E4BP4 induction. Although KN-93 show a high degree of selectivity for CaM kinase, other nonspecific effects on cellular function cannot be ruled out. For example, KN-93 suppressed voltage-dependent K\textsuperscript{+} channels in vascular myocytes (41). It is reported that inhibition of voltage-dependent K\textsuperscript{+} channels in T lymphocyte resulted in suppression of calcium signaling and NFAT-driven gene expression (42). To obtain more direct evidence for the

**Fig. 3. Tissue distribution of rat NFIL3/E4BP4.** Two \textmu g/lane of poly(A)\textsuperscript{+} RNA, prepared from the indicated rat tissues, were analyzed by Northern blotting using probes derived from cDNA for rat NFIL3/E4BP4 (upper panel) or rat \beta-actin (lower panel).
involvement of CaM kinase signaling, we transfected a CaM kinase II isoform to A7r5 cells. Expression of the CaM kinase II isoform in A7r5 cells resulted in enhanced elevation of TG-induced NFIL3/E4BP4 expression, relative to empty vector control expression (data not shown). These data suggest that CaM kinase signaling is involved in the TG-dependent NFIL3/E4BP4 induction. Additional studies are required to define that the CaM kinase responsible for TG-induced NFIL3/E4BP4 expression is CaM kinase II or other CaM kinases.

CaM kinase II has been reported to mediate the activation of c-Fos (1), which can form heterodimeric complexes with c-Jun (e.g. AP-1). It is possible that calcineurin and CaM kinase II activate NFAT and AP-1 expression, respectively, and that NFAT and AP-1 stimulate the NFIL3/E4BP4 mRNA expression in concert with each other. Another possibility is that calcineurin and CaM kinase signaling pathways may act in parallel to preferentially target NFAT and MEF2, respectively, because CaM kinase I (35) and CaM kinase IV (33–35) can activate the MEF2 transcription factors. The CaM kinases can also activate cAMP response element-binding protein and serum response element-binding protein (1). Therefore, it is possible that these transcription factors may mediate some of the effects of TG on the induction of NFIL3/E4BP4 independently of NFAT. CaM kinase II can also mediate the activation of the ERK pathway following stimulation of calcium influx by ionomycin in vascular smooth muscle cells (43). Thus, CaM kinase II may serve to regulate NFIL3/E4BP4 expression via ERK1/ERK2. In fact, we found that TG-dependent NFIL3/E4BP4 induction was suppressed by pretreatment with 10 μM PD98059, a specific inhibitor of MEK1/2 (data not shown).

In the mouse pro-B cell lines Baf-3 and FL5.12, expression of NFIL3/E4BP4 was regulated by oncogenic Ras mutant proteins through both the Raf-MAP kinase and phosphatidylinositol 3-kinase pathways (10). The effect of the MAP kinase pathway in TG-induced NFIL3/E4BP4 mRNA expression in RASMC remains to be studied.

In the human T cell lines MLA144 and HUT78, NFIL3/E4BP4 mRNA levels of NFAT. CaM kinase II can also mediate the activation of the ERK pathway following stimulation of calcium influx by ionomycin in vascular smooth muscle cells (43). Thus, CaM kinase II may serve to regulate NFIL3/E4BP4 expression via ERK1/ERK2. In fact, we found that TG-dependent NFIL3/E4BP4 induction was suppressed by pretreatment with 10 μM PD98059, a specific inhibitor of MEK1/2 (data not shown). In Baf-3 cells, the expression of NFIL3/E4BP4 was regulated by oncogenic Ras mutant proteins through both the Raf-MAP kinase and phosphatidylinositol 3-kinase pathways (10). The effect of the MAP kinase pathway in TG-induced NFIL3/E4BP4 mRNA expression in RASMC remains to be studied.

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increased after PMA stimulation, although the amount of NFIL3/E4BP4 mRNA did not change following PMA stimulation in S-LB-1 T cells (8). We found that PMA did not induce the expression of NFIL3/E4BP4 mRNA in RASMC (data not shown). These results suggest that there are some differences in the cellular proteins reacting after PMA stimulation between these cell lines.

It is very important to know the physiological significance of NFIL3/E4BP4 induction. It is known that NFIL3/E4BP4 functions as an anti-apoptotic transcription factor in murine IL-3-dependent Pro-B lymphocytes including Baf-3 and FL5.12 cells (9, 10). In the absence of IL-3, enforced expression of the human NFIL3/E4BP4 promoted the survival but not the growth of IL-3-dependent pro-B cells, indicating that induction of NFIL3/E4BP4 is one of the mechanisms through which IL-3 suppresses apoptosis (9). The downstream factors through which NFIL3/E4BP4 delays apoptosis in IL-3-deprived Baf-3 cells have as yet not been identified. One possibility is that NFIL3/E4BP4 induces the expression of cytokine in Baf-3 cells and...
blocks apoptosis through an autocrine mechanism, because this transcription factor has been reported to transactivate the IL-3 promoter in T cells. We found that rat IL-3 mRNA (44) was induced by TG in RASMC, although NFAT mRNA (44) was not induced by TG in RASMC (data not shown). Although NFAT blocks apoptosis through an autocrine mechanism, because this induction of NFIL3/E4BP4 expression in RASMC. The induction of NFIL3/E4BP4 by calcium signaling might have some critical functions in apoptosis in vascular smooth muscle cells.

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