Involvement of Thyrotroph Embryonic Factor in Calcium-mediated Regulation of Gene Expression*

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In the present study, we used an expression cloning strategy to identify transcription factors that bind specifically to a limited region of the inducible cAMP early repressor (ICER) promoter and regulate transcription. Murine thyrotroph embryonic factor (mTEF) was isolated and was shown to bind to a site located at nucleotides –117 to –108 from the transcriptional start site. Transient expression of reporter constructs containing either a consensus TEFRE or the icer/TEF binding site demonstrated that TEF-dependent transcription correlated with relative binding affinities, i.e. the consensus TEFRE bound TEF more tightly and was more responsive to TEF than the icer/TEFRE. Because the icer/TEFRE overlapped a cAMP response element, the responsiveness of these sequences to either cAMP or Ca²⁺ was tested. Although TEF expression had no effect on the cAMP-regulated transcriptional response of the ICER promoter, TEF did confer calcium responsiveness to these sequences. Calcium also modestly increased the TEF-mediated transcription from a consensus TEFRE. Additional studies using Ca²⁺-activated kinases indicate that Ca²⁺/TEF/TEFRE-regulated transcription may be mediated through Ca²⁺/calmodulin-dependent kinase (CaMK) IV. Moreover, studies with the icer/TEFRE in a CaMK IV-deficient cell line demonstrated that these cells were transcriptionally unresponsive to thapsigargin; however, responsiveness was restored by co-expression of the active CaMK IV. These studies are the first to demonstrate that TEF is a calcium-responsive transcription factor, and they suggest that there are two classes of TEF-regulated genes. One class, represented by a consensus TEFRE, is regulated by TEF in the resting cell; the second class, represented by icer/TEFRE, is regulated by TEF in the calcium-activated cell.

Transcriptional regulation is an important means through which cellular calcium fluxes exert their biological effects. Calcium-activated transcription is mediated through both cAMP response elements (CREs) and serum response elements located in the promoters of calcium-regulated genes. Elk-1 has recently emerged as a transcription factor that binds to the serum response element located in the c-fos promoter and is sensitive to calcium-dependent, mitogen-activated protein kinase activated signaling pathways (1, 2). The CRE-binding protein, CREB, is another transcription factor with a well established role in Ca²⁺-activated transcription. In this regard, the Ca²⁺/calmodulin-dependent kinase IV (CaMK IV) and mitogen-activated protein kinase pathways have been implicated in the Ca²⁺-specific phosphorylation and activation of CREB (3–6). CREB is also activated by cAMP/protein kinase A (PKA), and most CREs are responsive to both Ca²⁺ and cAMP when these pathways are functional in the same cell (reviewed in Ref. 7). The CREs located in the promoter of the inducible cAMP early repressor (ICER) gene stand as one exception to this general rule.

Expression of the ICER gene is up-regulated by cAMP/CREB in a number of different cell types. The ICER protein acts as a repressor of cAMP/CREB-mediated transcription and may also function as a tumor suppressor (8–10). The ICER promoter contains two pairs of CRE-like elements, CARE1/2 and CARE3/4, which are referred to as cAMP autoreponse elements (AREs) because they mediate down-regulation of ICER expression (8). Only the CARE3/4 pair is active in regulating cAMP-induced transcription in WEHI7.2 thymoma cells (9). CARE3/4 is of further interest because, unlike other similar CREs, CARE3/4 is unresponsive to Ca²⁺/CREB-mediated transcription (11). This unique regulation is presumably due to the promoter context in which it resides and to the binding of a putative repressor of calcium responsiveness (11). The ability for the ICER CAREs to discriminate between cAMP and Ca²⁺ signals suggests that unique transcriptional regulators distinct from CREB and ICER may also be involved in controlling ICER gene expression.

In a previous study, we identified sequences immediately surrounding two of the four ICER CAREs, CARE3 and CARE4, which limit Ca²⁺ responsiveness of the CAREs (11). As these sequences are likely to be required for binding of transcriptional regulatory proteins, we set out to identify factors that bind specifically to the ICER CARE3/4 promoter region and function as putative regulators of ICER gene expression. In the present study, we screened a mouse brain cDNA expression library for proteins that bind specifically to nucleotides –121 to –92 from the transcriptional start site of the ICER gene. These sequences surround and contain CARE3/4. We identified the murine homolog of thyrotroph embryonic factor (mTEF) as a CARE3/4-binding protein and a potential regulator of ICER protein kinase A; ICER, inducible cAMP early repressor; CARE, cAMP autoreponse element; EMISA, electrophoretic mobility shift analysis; TEF, thyrotroph embryonic factor; mTEF, murine TEF; TEFRE, TEF response element; cTEFRE, consensus TEFRE; CAT, chloramphenicol acetyl transferase; bp, base pairs; tk, thymidine kinase.

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\[1\] The abbreviations used are: CRE, cAMP response element; CREB, CRE-binding protein; CaMK, Ca²⁺/calmodulin-dependent kinase; PKA, protein kinase A; ICER, inducible cAMP early repressor; CARE, cAMP autoreponse element; EMISA, electrophoretic mobility shift analysis; TEF, thyrotroph embryonic factor; mTEF, murine TEF; TEFRE, TEF response element; cTEFRE, consensus TEFRE; CAT, chloramphenicol acetyl transferase; bp, base pairs; tk, thymidine kinase.

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gene expression. TEF is a member of the proline and acidic amino acid-rich subfamily of basic leucine zipper transcription factors, which also includes the albumin box-binding protein (12), hepatic leukemia factor (13), and the chicken TEF ortholog vitellogenin gene-binding protein (14). Like other proline and acidic amino acid-rich family members, TEF is known to constitutively activate transcription through binding to a TEF response element (TEFRE) (15, 16). Because the TEF-binding site in the ICER promoter overlapped CARE3, we investigated the ability of mTEF to activate transcription from this sequence and to modulate the response of these sequences to cAMP and to Ca\(^{2+}\). Here, we provide the first evidence that mTEF binds to a TEFRE in the ICER promoter and that it activates ICER gene transcription via a Ca\(^{2+}\)-dependent mechanism involving CaMK IV. Thus, in addition to being a constitutive transcriptional activator of TEFRE-mediated transcription, TEF may also be an important mediator of Ca\(^{2+}\)-dependent gene regulation through binding to TEFREs like that in the ICER promoter.

**EXPERIMENTAL PROCEDURES**

**Materials**—Thapsigargin was purchased from Alexis/LC Laboratories, dissolved in dimethyl sulfoxide, and stored at −80 °C. Forskolin was purchased from Sigma, dissolved in ethanol, and stored at −20 °C. Anti-Flag M2 monoclonal antibody was purchased from Sigma. Polyclonal antibody to TEF was generated by the Immunology Core Laboratory at Louisiana State University using an incompletely peptide corresponding to amino acids 150–163 of the murine sequence QPSETVSSTESSLKE. The resulting antiserum was verified for specificity to mTEF using Western blot analysis of bacterially expressed recombinant mTEF protein.

**Cell Culture**—Marine WEHI7.2 thymoma cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf bovine serum, 10 units/ml penicillin, and 0.1 mg/ml streptomycin. HepG2 human hepatocellular carcinoma cells (ATCC, Manassas, VA) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 10 units/ml penicillin, and 0.1 mg/ml streptomycin. All cells were cultured at 37 °C, 6% CO2, and 90% humidity.

**Screening of the Expression Library—**10\(^6\) clones from a mouse brain cDNA gt11 phage expression library (CLONTECH Laboratories) were plated and transferred to duplicate nitrocellulose membranes treated with isopropyl-1-thio-

**Screening for ICER CARE3/4 probed sequence:**

**Transfection Assay—**WEHI7.2 cells were transfected by lipofection as described (9). HepG2 cells were transfected using LipofectAMINE Plus (Life Technologies, Inc.) as directed by the manufacturer. All cells were transfected with 2–2.5 μg of DNA, of which 1 μg was reporter plasmid. Cells were treated with thapsigargin or forskolin, as indicated, 2.5 h after fresh medium was applied. After incubation for 15 h, all cells were harvested, and extracts were prepared and assayed for CAT activity (22).

**Western Blot Analysis—**WEHI7.2 and HepG2 nuclear extracts were prepared as described (9, 23). Crude extracts were prepared by solubilizing equal numbers of cells in SDS final sample buffer. Equal volumes of extract were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred overnight at 4 °C to Immobilon polyvinylidene fluoride membrane (Millipore Corp.). The blot was probed for 3 h at room temperature with monoclonal antibody to CaMK IV (Transduction Laboratoires) or with monoclonal antibody to CaMK IV (which was used for in-frame subcloning into pFlagMac and pCMV2Flag parent vectors (Sigma). pSG-TEF was generated by direct in-frame insertion of agt11 TEF cDNA into the eukaryotic pSG5 expression plasmid (Stratagene). Constitutively active kinase expression vectors for PKA (pRbSV-Cat-o) (18) and CaMK II (pRbSV-CaMKII) (19) have been described previously. pSG-CaMKIV was generated by subcloning the cDNA from pCMV-CaMKIV encoding for constitutively active CaMK IV (20) into pSG5. Constitutively active ERK1 and Raf1 expression plasmids were obtained from Dr. Joseph Baldassarre. The sequence of all new constructs used in these studies were verified using a Perkin-Elmer ABI Prism 377 automated DNA sequencer.

**Expression of Recombinant TEF—**DH5\(^{a}\) Escherichia coli were transformed with the pFlag-TEF expression plasmid and grown at 37 °C until A\(_{590}\) = 0.6–1.0. Isopropyl-1-thio-beta-D-galactosidase was added to a final concentration of 1 mM, and protein expression was induced for 3 h at 37 °C. Bacteria were harvested by centrifugation at 3000 × g; resuspended in 50 mM Tris-Cl, pH 8.0, 2 mM EDTA; and lysed by 15 min freezing and thawing. The lysate was cleared by centrifugation at 100,000 × g for 1 h, and the supernatant was then sonicated, and cellular debris was removed by centrifugation at 12,000 × g and verified for TEF expression by Western blot using M2 monoclonal anti-Flag antibody (Sigma) and the anti-TEF antibody.

**Electrophoretic Mobility Shift Assay (EMSA)—**EMSA analysis used procedures and conditions described previously (21). Briefly, protein extracts were preincubated for 15 min in binding buffer containing poly(dI-dC), antibodies, and competitor DNA where indicated. Radiolabeled CARE3/4 double-stranded DNA probe was then added, and binding reactions were continued for an additional 15 min. Protein-DNA complexes were then separated by electrophoresis using a 4% nondenaturing polyacrylamide gel and visualized by autoradiography. The CARE3/4 probe consists of the following sense strand sequence TEFRE-3′ to TEFRE-5′: 5′-GTCAGTGCTC-3′.

**ICER promoter activity—**Transient Transfection Assay—WEHI7.2 cells were transfected with the pCMV-Care-a expression plasmid (22) and a reporter plasmid containing ICER promoter sequences units of all new constructs used in these studies were verified using a Perkin-Elmer ABI Prism 377 automated DNA sequencer. The sequence of all new constructs used in these studies were verified using a Perkin-Elmer ABI Prism 377 automated DNA sequencer. The sequence of all new constructs used in these studies were verified using a Perkin-Elmer ABI Prism 377 automated DNA sequencer. The sequence of all new constructs used in these studies were verified using a Perkin-Elmer ABI Prism 377 automated DNA sequencer.
downstream from the fusion point and was within a strong consensus Kozak sequence, and it was assigned as the initiator codon. However, it is possible that additional 5' coding sequence may exist because no in-frame stop codons were present in this upstream sequence. A TAA stop codon was located at nucleotide 994 and was followed by 3' untranslated sequence. Database analysis indicated this cDNA encodes for a protein that shares 97 and 99% homology with human and rat TEF, respectively, and 80% homology with vitellogenin gene-binding protein (the chicken homolog of TEF) (Fig. 1B).

Therefore, this clone most likely represents mTEF.

DNA Binding Activity of Recombinant mTEF—Recombinant flag-tagged mTEF protein was generated in bacteria, and extracts were analyzed by EMSA for the ability to bind to a DNA probe containing the CARE3/4 sequence (Fig. 2A). No DNA binding activity was detected using increasing amounts of control bacterial extracts (Fig. 2B, lanes 2–6). However, a protein-DNA complex was readily detected in extracts from bacteria transformed with pFlag-mTEF (Fig. 2B, lanes 7–11). To confirm the presence of mTEF in this complex, the effect of a TEF-specific polyclonal antibody on the complex was tested (Fig. 2C). The anti-TEF antibody caused a supershift in this protein-DNA complex (Fig. 2C, compare lane 3 to lane 4), confirming that mTEF is binding to sequences within the CARE3/4 promoter region. As expected, no protein-DNA complex was observed with control bacteria extracts in the presence of antibody (Fig. 2C, lane 2). A nonspecific antibody had no effect on the TEF-DNA complex (data not shown).

Because the ICER promoter sequences used to isolate TEF do not contain a consensus TEFRE (cTEFRE) (GTTACGTAAT) (16, 24), we compared the ability of TEF to bind to CARE3/4 with its ability to bind to a cTEFRE (Fig. 3B). mTEF readily bound to radiolabeled CARE3/4, and approximately 50% of the binding activity was competed with 25-fold excess of unlabeled CARE3/4 (Fig. 3B, compare lanes 1 and 3). In comparison, a 5-fold excess of a cTEFRE resulted in complete competition of mTEF binding to CARE3/4 (Fig. 3B, compare lanes 1 and 5). These data indicate that mTEF binds to the CARE3/4 sequence, but with a relative binding affinity that is somewhat weaker than to a cTEFRE.

A polymerase chain reaction-based selective amplification and binding procedure was employed previously to define a consensus TEF binding site (16, 24). Comparison of these data with the sequences contained in CARE3/4 revealed that the DNA sequences within and surrounding each of the CARE3 and CARE4 elements may serve as TEF binding sites (Fig. 3C). To identify whether either of these two elements was responsible for TEF binding to CARE3/4, competition assays were performed (Fig. 3D). TEF binding to CARE3/4 was readily competed by CARE3 (Fig. 3D, lanes 4–7) but not by CARE4.
lanes 8–11), indicating that the minimal promoter fragment containing CARE3 functions as the principle binding site for TEF. The sequence of this TEF binding site, GTGACGTCAC, is 70% identical to a cTEFRE. The inability of TEF to bind to CARE4 may be due to a thymidine in the –1 position. Analysis by Haas et al. (24) suggests that a T in the –1 position is not permissive for TEF binding (Fig. 3C).

**Transcriptional Activation of the ICER Promoter by Ectopic Expression of mTEF in WEHI7.2 Thymocytes—** Previous reports demonstrated that co-expression of TEF with a cTEFRE-driven reporter construct results in the strong activation of the promoter (15, 16, 25). Therefore, we tested whether mTEF regulates transcription of a reporter gene linked either to a cTEFRE or to the TEF binding site located within the ICER promoter. WEHI7.2 thymoma cells were used for these studies because they expressed little or no TEF protein as assessed by Western blot analysis (data not shown), and the effect of TEF on transcription could be easily monitored. WEHI7.2 cells were transiently cotransfected with heterologous reporter constructs driven by CARE3/4 or two copies of CARE3 (each construct contains the icerTEF binding site), a cTEFRE, or a consensus CRE inserted upstream to a minimal thymidine kinase (tk) promoter and CAT reporter gene. mTEF overexpression led to a dramatic 15.3-fold induction in cTEFRE-driven reporter activity compared with cells transfected in the absence of mTEF (Fig. 4A). Paradoxically, mTEF overexpression resulted in relatively small increases in icerCARE3/4tkCAT and icerCARE3(2)tkCAT reporter activity (1.6- and 2.2-fold induction, respectively), even though these sequences contained the TEF binding site. However, this modest 2-fold increase was selective for the CARE3 element because mTEF expression had no effect on CRE-driven expression of the gphCRE(2)tkCAT reporter gene construct.

As mentioned previously, the ICER promoter is regulated by cAMP/CREB through CARE3/4, but it is unresponsive to regulation by Ca^{2+} (8, 9, 11). Because of this unique regulation and because the icerTEF binding site overlapped CARE3, we asked whether mTEF expression would affect the responsiveness of this region to either cAMP or Ca^{2+}. To test this, WEHI7.2 cells were transfected with the icerCARE3/4tkCAT reporter construct and treated with thapsigargin or forskolin to stimulate Ca^{2+} or cAMP-dependent transcription, respectively, and the effect of TEF expression was determined (Fig. 4B). Forskolin induced transcription of the CARE3/4 reporter...
nearly 5-fold, and this level of activation was not significantly affected by mTEF expression. In comparison, thapsigargin had minimal effects on transcriptional activation in the absence of mTEF (1.6-fold induction), but when mTEF was expressed in these cells, CARE3/4 was converted into a Ca²⁺-responsive element with greater than a 5-fold increase in CAT reporter activity. This effect was mediated through CARE3. As illustrated in Fig. 4C, when two copies of the CARE3/TEFRE and its flanking sequences were linked to tkCAT, the effect was even more dramatic. TEF expression led to a greater than 20-fold activation of this construct in thapsigargin-treated cells. In contrast to CARE3, the transcriptional response of CARE4 to calcium was unaffected by TEF expression (data not shown). To determine whether a cTEFRE was regulated by Ca²⁺ in a TEF-dependent manner, the TEFRE(2)tkCAT reporter plasmid was used. Thapsigargin treatment led to an additional 2-fold increase in CAT activity in cells expressing mTEF. Furthermore, the TEF/Ca²⁺-dependent transcription was specific for a TEFRE. Although the CREs within the gphCRE(2)tkCAT reporter construct responded to thapsigargin with a 22-fold increase in reporter expression, this calcium-regulated activity was unresponsive to mTEF expression in this system. Together, these data indicate that a TEF binding site is required for TEF/Ca²⁺-mediated transcription to be observed.

To verify that TEF-dependent, Ca²⁺-mediated transcriptional regulation of the icer/TEFRE occurs within the context of the native promoter, transient expression experiments were performed in WEHI7.2 cells using CAT reporter constructs driven by the native murine ICER promoter and 378 bp of 5′ untranslated sequence (ICER378-CAT) (Fig. 5). As illustrated in Fig. 5B, ICER378-CAT was only weakly responsive to calcium, in agreement with Krueger et al. (11). This promoter also demonstrated a 6.5-fold elevation of basal activity in the presence of ectopically expressed mTEF. However, when cells expressing mTEF were treated with thapsigargin, CAT reporter activity was dramatically increased more than 40-fold. Truncated promoter constructs (ICER171 and ICER123) were similarly regulated by thapsigargin and TEF (Fig. 5B). However, removal of the TEFRE/CARE3/4 region (ICER97-CAT) abolished the ability for TEF to confer Ca²⁺ responsiveness to the ICER promoter. When mTEF was expressed in these cells, there was no significant difference in activation of ICER97-CAT in the presence or absence of thapsigargin. Together, these data suggest that TEF, acting through the TEFRE in CARE3/4, is able to confer Ca²⁺ responsiveness to the native ICER promoter in WEHI7.2 cells.

**Fig. 3.** The TEF binding site overlaps the CARE3 element of the ICER promoter. A, sequences of the oligonucleotides used in the EMSA analyses. CARE3 and CARE4 are shown in boldface, the consensus TEFRE is boxed. B, 100 ng of mTEF bacterial extract was incubated with 32P-labeled CARE3/4 probe (lane 1) and with increasing amounts of unlabeled competitor CARE3/4 (lanes 2–4) or TEFRE (lanes 5–7) probes to compete for binding to radiolabeled CARE3/4. C, comparison of CARE3, CARE4, and CRE sequences (in boldface) with a consensus TEFRE. The matrix is adapted from Haas et al. (24) and Hunger et al. (16), denoted in the figure as Haas/Hunger. D, 32P-labeled CARE3/4 probe was incubated with 100 ng of control extract (lane 2), with 100 ng of mTEF bacterial extract (lane 3), or with 100 ng of mTEF bacterial extract with increasing amounts of unlabeled competitor CARE3 (lanes 4–7) or CARE4 (lanes 8–11) DNA to compete for binding to radiolabeled CARE3/4. Lane 1 contains the free probe. Shown are representative experiments.
by CaMK II, CaMK IV, and mitogen-activated protein kinase. To determine whether any of these may mediate the Ca\(^{2+}\)/TEF-associat
ed regulation of the ICER gene in WEHI7.2 thymocytes, mTEF was co-expressed with constitutively active mutants of CaMK II, CaMK IV, ERK1, and Raf1 or the catalytic subunit of PKA and assayed for transcriptional activation of ICER378-CAT (Fig. 6). PKA expression caused a greater than 20-fold induction of ICER378-CAT in the presence or absence of mTEF, supporting our previous observation that the ICER promoter is regulated by cAMP and that this regulation is unaffected by TEF expression. Constitutively active mutants of ERK1 or of Raf1 did not activate transcription of the ICER promoter in the presence or absence of TEF (Fig. 6). A constitutively active form of the Ca\(^{2+}\)-dependent CaMK II also had no effect on TEF-dependent ICER transcription. Only the constitutively active CaMK IV construct had an effect on ICER378-CAT reporter activity in these cells. Whereas CaMK IV had a modest 2.9-fold activation in the absence of TEF, TEF co-expression led to 44.2-fold increase in reporter activity compared with cells transfected with reporter and pSG5 ± SD (n ≥ 2).
data indicate that the Ca^{2+}-inducible, TEF-dependent regulation of ICER expression may be mediated specifically through CaMK IV in WEHI7.2 thymocytes.

Finally, the role of CaMK IV in activating TEF-dependent expression of the ICER promoter was examined in the HepG2 human hepatocellular carcinoma cell line. HepG2 cells do not express detectable levels of endogenous CaMK IV (Fig. 7A), and thus, the role of CaMK IV in this process could be tested directly. In HepG2 cells transfected with ICER378-CAT, mTEF overexpression led to a 2.7-fold activation that was not appreciably affected by thapsigargin treatment (4.7-fold induction) (Fig. 7B). Treatment of these cells with the calcium ionophore A23187 also had little effect on activation of ICER378-CAT in the presence or absence of TEF (data not shown). In contrast, constitutively active CaMK IV caused a 6.3-fold increase in ICER378-CAT reporter activity that was greatly enhanced when mTEF was overexpressed (21.9-fold induction). These data support the concept that Ca^{2+}/TEF-dependent activation of the ICER promoter is mediated through CaMK IV, and in the absence of the kinase, Ca^{2+} fluxes elicited by thapsigargin have little effect. Reporter assays using ICER378-CAT, ICER171-CAT, ICER123-CAT, and ICER97-CAT verified that CaMK IV/TEF-dependent activation of the ICER promoter in HepG2 cells was mediated specifically through the icer/TEFRE (Fig. 7C). The constructs containing the icer/TEFRE (ICER378-CAT, ICER171-CAT, and ICER123-CAT) each demonstrated a synergistic response to CaMK IV and TEF. This response was lost in the ICER97 construct from which the TEFRE was removed. Thus, in both WEHI7.2 and HepG2 cells, TEF-dependent activation by CaMK IV localizes to the region located between residues –123 and –97 of the ICER promoter, which contains the TEF binding site.

### DISCUSSION

Previously, we demonstrated that the CARE3/4 pair of CREs is uniquely responsive to cAMP but not calcium in WEHI7.2 cells (9, 11). The lack of a calcium effect may be due to the binding of a calcium-activated repressor of CREB/CRE-mediated transcription to sequences overlying CARE4. In the present study, we screened for factors that may contribute to the unique regulation of the ICER CRE regions. Using a 30-bp fragment of DNA containing CARE3/4 as a probe, the murine homolog of TEF was isolated from a brain cDNA expression library as a specific icer/CARE3/4-binding protein. We provide evidence that mTEF binds to a TEFRE located within the CARE3 region of the promoter. Although the ectopic expression of mTEF in WEHI7.2 cells had no effect on the levels of basal transcription of ICER reporter constructs containing this TEFRE, it was able to confer Ca^{2+} responsiveness to these constructs. Thus, TEF is distinct from the calcium-activated repressor described previously (11). Moreover, TEF is a Ca^{2+}-responsive transactivator that opposes the activity of the repressor by binding to an adjacent site on the ICER promoter and eliciting transcription.

In binding studies (Figs. 1 and 2), mTEF bound to sequences of the CARE3 in the CARE3/4 promoter region, although at a lower relative affinity than it bound to the cTEFRE GTTACGTAAAT. Examination of this region and comparison with established TEF binding sites (16, 24) reveals a putative TEFRE overlapping the CARE3. This 10-bp TEFRE is composed of the 8-bp CARE3 at its core, with 1 bp flanking on each side (GTGACGTCAC). Although the gphCRE contains an identical 8-bp core, the flanking bases are different (TTGAGCCTGCA). Moreover, on the 5’ side of the gphCRE is a thymidine residue, a residue that was identified as being nonpermissive for TEF binding (16, 24). Our findings are consistent with these earlier studies. First, TEF did not bind these sequences in our screening of the protein expression cDNA library (data not shown). Second, neither basal nor Ca^{2+}-activated expression of gphCRE/DoCAT was regulated by mTEF in WEHI7.2 cells (Fig. 3). Thus, it appears that TEFRE- and CRE-mediated transcriptional regulation can overlap when sequences surrounding the 8-bp CRE contain residues that form a functional 10-bp TEFRE.

The relative affinities of mTEF binding to a consensus TEFRE and the icer/TEFRE were mirrored by the basal transcriptional activation of these elements (Fig. 3). TEF overexpression in WEHI7.2 cells resulted in a dramatic increase in
transcription mediated through a cTEFRE, consistent with previous observations (15, 16). However, TEF overexpression had little effect on transcription mediated through the icer/TEFRE (Fig. 4). Only when cells were stimulated with thapsigargin was TEF able to dramatically activate transcription through the icer/TEFRE. Calcium activation of a cTEFRE was more modest, increasing reporter gene expression an additional 2-fold. These differences suggest that there are at least two classes of TEFREs. One class, represented by the cTEFRE, demonstrates high affinity binding and supports a relatively high level of transcription in the presence of TEF. This class shows only a modest response to calcium stimulation. The second class, of which the icer/TEFRE is a member, has a lower binding affinity, which contributes to its low level of TEF-mediated transcriptional activation. This class is dramatically affected by calcium fluxes in a TEF-dependent manner.

CaMK IV is a likely mediator of the Ca\textsuperscript{2+}/TEF-stimulated transcription. We analyzed cell lines that express or are deficient in CaMK IV, WEHI7.2 and HepG2 cells, respectively. In these systems, thapsigargin treatment of TEF-transfected cells readily activated CARE3/4 reporter gene expression in the WEHI7.2 cells but had no effect in the HepG2 cells. This clearly suggests that CaMK IV expression is required for Ca\textsuperscript{2+}/TEF-mediated effects. In support of this possibility, expression of a constitutively active CaMK IV in either the WEHI7.2 or the HepG2 cells resulted in the transcriptional activation of the icer/TEFRE. CaMK II, ERK1, and Raf1 were unable to elicit an effect (Fig. 6). There are multiple mechanisms possible for the effect of Ca\textsuperscript{2+} on TEF mediated transcription. One possibility is that a Ca\textsuperscript{2+}/CaMK IV-activated event leads to a increase in the binding affinity of TEF for the icer/TEFRE. Upon Ca\textsuperscript{2+}-induced binding to the element, TEF is constitutively active. Thus, the high affinity sites are only weakly affected because they may already be occupied by TEF. Evidence for a similar mechanism involving the affinity of CREB for binding to variant CREs has been reported (26). In this report, Nichols et al. (26) found that an asymmetrical CRE exhibited weak binding affinity for CREB in the unstimulated state, whereas a consensus CRE demonstrated significantly greater CREB binding affinity. In response to cAMP stimulation, CREB became phosphorylated and exhibited higher binding affinity for both classes of CREs; however, the increase in binding to the asymmetrical CRE was more dramatic. A second possible mechanism for Ca\textsuperscript{2+}/TEF-dependent transcription involves the Ca\textsuperscript{2+}-mediated activation of TEF. Although TEF is a constitutive transcriptional activator, a calcium-mediated modification such as phosphorylation may enhance its activity. This enhancement is most apparent on the icer/TEFRE due to its low basal level of activation. The AP-1 heterodimer provides a precedent for such a mechanism. Although AP-1 is constitutively active, phosphorylation by Jun-NH\textsubscript{2}-terminal kinase dramatically increases its activity (for review, see Refs. 27 and 28). Future studies will be directed at defining the mechanism whereby TEF induces transcription in a calcium-dependent manner.

These experiments provide the first evidence that TEF, a member of the proline and acidic amino acid-rich subfamily of basic leucine zipper transcription factors, may have an important role in Ca\textsuperscript{2+}-regulated gene expression. This regulation is dependent on both TEF and CaMK IV expression, suggesting an element of cellular specificity because all cells may not express both factors. In fact, the absence of endogenous TEF expression in the WEHI7.2 thymocytes may explain why ICER expression is not normally responsive to calcium in these cells. Other regulatory factors could be involved in the response, as well. For example, TEF can bind to DNA as a homo- or heterodimer, thus leading to another means of cell-specific transcriptional control. The lower basal level and higher relative stimulation of transcription through low affinity binding sites adds an additional level of complexity to the transcriptional activation. Elucidation of the mechanism by which CaMK IV and TEF are able to confer Ca\textsuperscript{2+} responsiveness to genes such as ICER could provide valuable insight as to the role TEF as a transcriptional regulator and contribute significant understanding of how Ca\textsuperscript{2+} signals modulate expression of target genes in response to specific extracellular stimuli.

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