Role of Transcriptional Repressor ICER in Cyclic AMP-mediated Attenuation of Cytokine Gene Expression in Human Thymocytes*

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Proliferating human medullary thymocytes can exhibit characteristic T helper cell type 1 cytokine responses exemplified by the immediate early expression of interleukin-2, interferon-γ, tumor necrosis factor-α, and lymphotoxin-β. Here we report that cyclic AMP-mediated attenuation of the transcription of T helper-1-specific cytokine genes in human medullary thymocytes correlates with the induction of the cyclic AMP-mediated transcriptional repressor ICER (inducible cyclic AMP early repressor). We show that ICER binds specifically to several NFAT/AP-1 (nuclear factor of activated T cells/activating protein-1) composite DNA sites essential for the activation of the interleukin (IL)-2 promoter as well as to a homologous DNA motif present in the proximal segment of the interferon-γ promoter. In the presence of the minimal NFAT DNA-binding domain, which is sufficient for both DNA binding and AP-1 complex formation, ICER and NFAT form NFAT/ICER ternary complexes on several NFAT/AP-1 DNA composite sites previously identified as essential for the expression of the immunoregulatory cytokines such as IL-2, IL-4, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor-α. In extracts prepared from human medullary thymocytes treated with forskolin and ionomycin, these composite sites bind endogenously expressed ICER either singly or in complexes. Moreover, in Jurkat cells, ectopically expressed ICER represses transcription from NFAT-mediated, phorbol ester/ionophore-activated IL-2, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor-α promoters. We present evidence that ICER interactions with NFAT/AP-1 composite DNA sites correlate with its ability to repress transcription. These findings provide further insight into the mechanisms involved in cyclic AMP-mediated transcriptional attenuation of cytokine expression.

It is well established that cyclic AMP signaling is inhibitory to T cell proliferation and effector functions. In particular, cyclic AMP inhibits the expression of T helper-1 cytokine genes (1–3). Earlier reported studies of fibroblasts showed that elevated levels of intracellular cyclic AMP inhibit upstream signal transduction pathways involved in cell growth and differentiation (4, 5). In contrast to fibroblasts, in which elevated levels of intracellular cyclic AMP inhibit extracellular signal-regulated kinases 1 and 2 and c-Jun NH2-terminal kinases involved in the signal transduction of mitogen-activated protein kinase pathways, T cell extracellular signal-regulated kinases 1 and 2 are insensitive to elevated levels of intracellular cyclic AMP (6). Moreover, the cyclic AMP-mediated inhibition of c-Jun NH2-terminal kinase in T cells shows delayed kinetics, an observation that correlates with the induction of the cyclic AMP-inducible early repressor ICER1 (7). In addition, overexpression of NFAT achieved by transfection of NFAT-encoding cDNAs to lymphoma cells abrogates the sensitivity of cyclic AMP-mediated inhibition of IL-2 gene expression (8, 9). Importantly, phosphorylation of amino-terminal serines of NFAT by protein kinase A does not prevent calcineurin-mediated translocation of NFAT to the nucleus, despite its ability to prevent IL-2 gene expression (10, 11). The notion that a newly synthesized transcriptional repressor rather than inhibition of upstream signal transduction pathways could be involved in the cyclic AMP-mediated transcriptional attenuation of T helper-1 cytokine expression was further strengthened by the reported alleviation of cyclic AMP-mediated inhibition of IL-2 expression in the presence of inhibitors of both RNA and protein synthesis (6).

ICER is a transcriptional repressor that appears to serve as a generalized negative regulator of the CREB and CREM families of transcription factors as well as other related bZIP family members (12–15). ICER isoforms represent a unique cyclic AMP-inducible CREM subfamily of transcription factors containing cyclic AMP-response elements within an internal P2 promoter. Because of autoregulation of the cyclic AMP-inducible P2 promoter, the expression of ICER can be intrinsically rhythmic. The rhythmic expression of ICER was first described in the pineal gland and in the hypothalamic-pituitary-gonadal axis (16, 17). However, the P2 promoter of ICER is also inducible in organs other than the pineal and hypothalamic-pituitary gonadal axis such as in specific subsets of T lymphocytes including human medullary thymocytes (7). Importantly, in the Jurkat T cell line ectopically expressed ICER can substitute for the inhibitory effects of cyclic AMP on the transcriptional attenuation of IL-2 promoter activity (7).

The NFAT (nuclear factor of activated T cells) and AP-1 (activating protein 1) represent two major transcription factor families implicated in the transcription of the IL-2 promoter in proliferating T lymphocytes (18–20). To address the possible mechanism by which ICER down-regulates IL-2 gene expres-

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1 The abbreviations used are: ICER, inducible cyclic AMP early repressor; AP-1, activating protein 1; bZIP, basic region/leucine zipper; CRE, cyclic AMP-response element; CREB, CRE-binding protein; CREM, CRE modulator protein; GM-CSF, granulocyte-monocyte colony-stimulating factor; IFNγ, interferon-γ; IL, interleukin; NFAT, nuclear factor of activated T cells; NFAT DBD; DNA binding domain of NFAT; PMA, 12-O-tetradecanoylphorbol 13-acetate; GST, glutathione S-transferase; TNF, tumor necrosis factor; CD28RE, CD28-responsive element; CAT, chloramphenicol acetyltransferase.
ision, we examined the binding of bacterially expressed ICER to all five NFAT motifs of the IL-2 promoter reported to be essential for the full induction of the IL-2 gene (21) either alone or in the presence of the minimal DNA-binding domain of NFAT (NFAT DBD). The highest affinity of ICER binding was found on a CD28-responsive element (CD28RE; −160 NFAT/AP-1 composite site) and −90 site, which is the motif in the IL-2 promoter that has striking sequence homology with the conserved proximal region (base pairs −73 to −48) of both the human and mouse promoters of the IFNγ genes (21, 22).

Moreover, certain NFAT/AP-1 composite sites that reside within the IL-4, GM-CSF, and TNF-α promoters resemble those located within the IL-2 promoter (23–27). It is believed that the mechanism underlying the actions of NFAT requires the binding of NFAT and/or NFAT/AP-1 to the NFAT/AP-1 composite binding motifs as ternary complexes (18). These complexes are believed to be essential for the transcriptional expression of immunoregulatory cytokines during T cell proliferation, such as IL-2, IL-4, GM-CSF, and TNF-α (19). Here we demonstrate that ICER binds to these NFAT/AP-1 composite DNA sites in vitro, either directly or indirectly via complex formation with the rel homology region of NFAT (NFAT DBD).

Furthermore, we detect the induction of ICER-immunoreactive complexes in extracts prepared from human medullary thymocytes treated with forskolin and ionomycin. Ectopically expressed ICER represses transcription from the IL-2, GM-CSF, and TNF-α promoters activated by ionomycin and phorbol ester, suggesting that the induction of ICER in response to cAMP may be responsible for the observed cAMP-mediated transcriptional attenuation of T helper-1 cytokine responses.

MATERIALS AND METHODS

Preparation of Human Medullary Thymocytes—Human thymus glands were obtained from children (ages 3 months to 4 years) undergoing corrective cardiac surgery. Thymocytes were fractionated over discontinuous Percoll gradients (Amersham Pharmacia Biotech) (28). Cells with densities of 1.060 < ρ < 1.070 were collected directly or indirectly via complex formation with the rel homology region of NFAT (NFAT DBD).

RNAse Protection Analysis—RNA extraction was performed as described (Qiagen). RNA probes hCK1 and hCK3 were purchased from Pharmingen and labeled with [α-32P]CTP using reagents from an RNA probe kit (Ambion). These probes were used for RNAse protection studies according to the protocol provided by Ambion (RPAlI ribonuclease protection assay kit).

Western Blot Analysis—Separation of whole cell proteins (50 μg) was carried out by SDS-polyacrylamide gel electrophoresis (10%) for 2 h at 40 mA in Tris/glycine buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS) at room temperature. The proteins were electrophoretically transferred (0.4 A overnight at 4 °C in 10 mA Tris/glycine buffer with 12% methanol on Immobilon P membrane (Millipore Corp.). The membrane was blocked in a Tris-buffered saline solution containing 0.05% Tween 20 (Sigma) and 5% nonfat dry milk (Bio-Rad) for 1 h with gentle agitation at room temperature. For immunological detection, the same solution without dry milk but containing the ICER or CREM-specific antisera (CS4) diluted 1:10,000 was agitated for 1 h, followed by three washes, with subsequent incubation with horseradish peroxidase conjugated to a secondary antibody (Amer sham Pharmacia Biotech) diluted 1:5000 for 1 h, followed by nine washes, and finally developed using an ECL kit (Amer sham Pharmacia Biotech).

Expression and Purification of Recombinant Proteins—Human ICER II cDNA was subcloned into the pEXKg vector (Amer sham Pharmacia Biotech) based on bacteriophage T7 (GST) fusion protein. The pGSTagCREB construct was described previously (29). Purifications of both ICER and CREB were carried out with minor modifications according to the protocol previously established for CREB (29). NFATpXS(1–187), encompassing the minimal DNA-binding domain of NFATp (gift from Dr. A. Rao), was expressed in bacteria as a hexahistidine-tagged protein and purified as reported previously (30). Recombinant c-Fos (Fos(139–243)) and c-Jun (Jun(187–334)) (gift from Dr. T. Kerppola) were purified from Escherichia coli overexpression strains by nickel chelate affinity chromatography (31, 32).

Nuclear Extracts and Gel Mobility Shift Assay—Whole cell extracts were prepared by high salt extraction using 50 mM Hepes, 250 mM NaCl, 5 mM EDTA, 0.5 mM dithiothreitol, and protease inhibitors (20 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mg/ml phenylmethylsulfonyl fluoride) as described previously (30). Binding reactions were performed in a 15-μl reaction volume containing 20 mM HEPS, 1 mM MgCl2, 50 mM KCl, 12% glycerol, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.2 μg of poly(dI-dC) as an unspecific competitor and recombinant proteins or bacterially expressed proteins as indicated. Where indicated, unlabelled competitor oligonucleotides in excess were added and incubated for 10 min at room temperature. Samples were run on a 4% polyacrylamide gel in 0.5× TBE at 200 V for 2 h following a 2-h prerun at 4 °C. The dried gels were exposed for autoradiography overnight. The oligonucleotides encompassing NFAT composite sites of the following human promoters were used: IL-2 (−45), 5′-ctagCCATTG-TGACACCCCTAATATTCTTCCAGAATTA-3′; IL-2 (−90), 5′-ctagCATCTTGGAAAAATGTATGTAATTGACAT-3′; IL-2 (−151), 5′-ctagATGAGAAGAAAAATGGAATTGTTT-3′; IL-2 (−160), 5′-ctagAAAGATTTCCAGAGAACCAGTTCTACAAGA-3′; GM-CSF (−90), 5′-gagateCCCTTCGGTTTCCTGCGGCACT-3′; GM-CSF (−420), 5′-gagateCTCTTCTCTCTCAAGAATGCACTGAGGA-3′; GM-CSF (−550), 5′-gagateGAGAGGAAGAAGCAGTACCATAAAAGAN-3′; IL-4 (−80), 5′-gagateTACTCTGCACTTGGAATGATTAATAATTCTTAAGTTCTATC-3′; TNF-α (−95), 5′-gagateTCCAGTCTCCAGGCTTTCTCCAGGAGA-3′; and cre (mouse c-fos DNA sites). The highest affinity of ICER binding was found at the presence of the minimal DNA-binding domain of NFAT (NFAT DBD). The highest affinity of ICER binding was found on a CD28-responsive element (CD28RE; −160 NFAT/AP-1 composite site) and −90 site, which is the motif in the IL-2 promoter that has striking sequence homology with the conserved proximal region (base pairs −73 to −48) of both the human and mouse promoters of the IFNγ genes (21, 22).

RESULTS

cAMP-mediated Transcriptional Attenuation of T Helper-1 responsive Cytokine Genes in Human Medullary Thymocytes—

Under the experimental conditions used, human medullary thymocytes exhibited the characteristics of naive T helper-0 and T helper-1 cells with predominant IL-2 and IFNγ expression documented by RNase protection used for the evaluation of mRNA levels of multiple cytokines (IL-2, IL-4, IL-5, IL-9, IL-10, IL-13, IL-14, IL-15, interleukin-β, IFNγ, human migration inhibitory factor, TN-α, TNF-β, LIF, and transforming growth factors β1, β2, and β3) (Fig. 1). Stimulation of human medullary thymocytes with a combination of phorbol ester and ionomycin significantly induced the synthesis of mRNAs encoding IL-2, IFNγ and to a lesser extent also TNF-α and LIF (Fig. 1A, lane 4; Fig. 1B, lane 4). At the same time, cotreatment with forskolin or 8-bromo-cAMP reduced the cellular mRNA levels of IL-2,
IFNγ, TNF-α, and Ltβ (Fig. 1A, lanes 5 and 6; Fig. 1B, lanes 5 and 6). We propose that at least part of this transcriptional attenuation is based on cAMP-mediated expression of the transcriptional repressor ICER and a subsequent blockade of NFAT/AP-1 composite DNA sites essential for T helper-1 cytokine expression. The inhibition by ICER may occur either directly through binding to the DNA element or indirectly via protein-protein interactions such as to the rel homology domain of NFAT (NFAT DBD).

Cyclic AMP-mediated Attenuation of T Helper-1 Cytokine Transcription in Human Medullary Thymocytes Correlates with cAMP-Mediated Induction of the Transcriptional Repressor ICER—To test the proposed mechanism involved, we first sought evidence for the presence of ICER protein in human medullary thymocytes after forskolin treatment to explore whether ICER may interact with important NFAT/AP-1 enhancer motifs of the IL-2 gene (21) (Fig. 2A). Four out of five NFAT sites in positions –90, –135, –160, and –280 were previously characterized as NFAT/AP-1 composite sites due to their inherent ability to bind to the NFAT/AP-1 complex in a cooperative fashion (34). The fifth NFAT site in the most proximal position, NFAT –45, does not bind to the NFAT/AP-1 complex and was determined to be exclusively an NFAT binding site (34). Bacterially expressed ICER, as well as ICER expressed in COS cells (data not shown), binds to all five NFAT sites, although to different extents (Fig. 2B). The strongest binding was observed to the NFAT/AP-1 composite sites in positions –90 and –160, an intermediate binding to the –135 NFAT/AP-1 composite site, and weak binding to the most proximal and distal NFAT and NFAT/AP-1 sites in positions –45 and –280, respectively. The binding specificity of recombinant ICER was evaluated using a CREM-specific antisera (CS4) that “supershifts” ICER bound to specific oligonucleotides containing individually the five DNA motifs, leaving nonspecific binding unaffected (Fig. 2B, lanes 1–10) as well as by using control oligonucleotide encompassing the first 21-base pair repeat (H21) of the human T-cell lymphotrophic virus type I long terminal repeat promoter containing the CRE-like motif (35) (Fig. 2B, lanes 11 and 12).

ICER Binding to NFAT/AP-1 Composite Sites in the Presence of NFAT DBD—To better understand the role of the interactions of ICER with NFAT/AP-1 composite DNA motifs, we examined the effectiveness of ICER binding to the DNA motifs in the presence of NFAT DBD. This domain of NFAT, which has the highest degree of conservation among NFAT family members (36), is both necessary and sufficient for DNA binding as well as to associate AP-1 with NFAT (30). All NFAT motifs on the IL-2 promoter examined, with the exception of the NFAT/AP-1 composite site in position –90, bound NFAT effi-

FIG. 1. Cyclic AMP-mediated attenuation of T helper-1 cytokine transcription in human (h) medullary thymocytes correlates with cAMP-mediated induction of the transcriptional repressor ICER. RNA from untreated (U) medullary thymocytes and forskolin (F)- or 8-bromo-cAMP (Br)-treated human medullary thymocytes in the absence (lanes 2 and 3) or presence of phorbol ester and ionomycin (FMA + Iono; lanes 5 and 6) were scored for cytokine expression using RIBOQUANT probes hCK1 (A) and hCK3 (B) in the RNase protection assay. Also shown are the corresponding RNase-protected probes following hybridization with yeast tRNA in the presence (+) (lane 7) or absence (−) (lane 8) of RNase. Templates for the analysis of hL32 and hGAPDH housekeeping genes were included to allow assessments of total RNA levels (Pharmingen). Note that each probe (lane 8) migrates more slowly than its protected band; this is due to flanking sequences in the probe that are not protected by mRNA. C, Western immunoblotting using ICER-specific antiserum generated against a peptide encompassing the ICER-specific exon (7) shows induction of ICER in medullary thymocytes after 3 h but not after 12 h of forskolin treatment. There is no ICER protein detectable before treatment or in forskolin-treated human cortical thymocytes, which corresponds to a lack of detectable ICER mRNA reported previously in cortical thymocytes (7).
both in the presence of CS4 (lanes 3) (160 motif of the IL-2 promoter (o nt h e TATA box of the IL-2 promoter. B, bacterially expressed ICER binds in boiled total bacterial lysate specifically to −90 (lanes 3 and 4) and −160 CD28RE motifs (lanes 7 and 8) and to a limited extent also to the rest of the motifs. The binding of ICER to these motifs is specific, since it is recognized by CS4 CREM-specific antiserum (CS4), causing a specific supershift (sICER), while the nonspecific complex (NS) remains unperturbed both in the presence of CS4 (+) and normal rabbit antiser (−). The control CRE consists of an oligonucleotide encompassing the 21-base pair repeat of human T-cell lymphotrophic virus type I long terminal repeat (H21 CRE) (35). C, in vitro binding of purified recombinant ICER and NFAT DBD (NFAT) proteins yields NFAT/ICER ternary complex (NF/IC) on CD28RE motif −160 (lane 12) and to a lower extent also on the NFAT −45 motif (lane 3). D, ICER and truncated Fos and Jun proteins (AP) form similar complexes (NF/IC versus NF/AP) in the presence of NFAT DBD (NFAT) on the −160 motif of the IL-2 promoter (lanes 3 and 9), which are recognized by the CS4 anti-CREM (C) (lane 4) and DX anti-Fos (D) (lane 10) or K25 anti-Jun (K) (lane 11) and to a limited extent also by R59 NFAT (R) antiser (lane 12), respectively. Both unlabeled oligonucleotides NFAT (ap) (lanes 6 and 13) and AP (top) (lanes 7 and 14) efficiently compete for complex.

**FIG. 2.** ICER binding and formation of an NFAT/ICER complex with NFAT/AP-1 composite DNA-binding sites of the IL-2 promoter. A, list of NFAT/AP-1 composite sites of IL-2 promoter delineated previously to be essential for IL-2 expression (34) used in electromobility shift assay analysis. NFAT and AP-1 (top) denote domains of homology between NFAT/AP-1 composite sites and consensus sequences for NFAT and AP-1 in human IL-2 promoter, respectively. Numbers on the left correspond to the relative distance of the depicted DNA-binding motifs from the TATA box of the IL-2 promoter. B, bacterially expressed ICER binds in boiled total bacterial lysate specifically to −90 (lanes 3 and 4) and −160 CD28RE motifs (lanes 7 and 8) and to a limited extent also to the rest of the motifs. The binding of ICER to these motifs is specific, since it is recognized by CS4 CREM-specific antiserum (CS4), causing a specific supershift (sICER), while the nonspecific complex (NS) remains unperturbed both in the presence of CS4 (+) and normal rabbit antiser (−). The control CRE consists of an oligonucleotide encompassing the 21-base pair repeat of human T-cell lymphotrophic virus type I long terminal repeat (H21 CRE) (35). C, in vitro binding of purified recombinant ICER and NFAT DBD (NFAT) proteins yields NFAT/ICER ternary complex (NF/IC) on CD28RE motif −160 (lane 12) and to a lower extent also on the NFAT −45 motif (lane 3). D, ICER and truncated Fos and Jun proteins (AP) form similar complexes (NF/IC versus NF/AP) in the presence of NFAT DBD (NFAT) on the −160 motif of the IL-2 promoter (lanes 3 and 9), which are recognized by the CS4 anti-CREM (C) (lane 4) and DX anti-Fos (D) (lane 10) or K25 anti-Jun (K) (lane 11) and to a limited extent also by R59 NFAT (R) antisera (lane 12), respectively. Both unlabeled oligonucleotides NFAT (ap) (lanes 6 and 13) and AP (top) (lanes 7 and 14) efficiently compete for complex.

**FIG. 3.** ICER protein can interact directly with NFAT DBD. Decreasing amounts of NFAT DBD protein retained on Sepharose matrix with equal amounts of GST-linked ICER (GST-ICER, lanes 1, 3, and 6) beside a negative control represented by equivalent amounts of GST matrix alone (GST, lanes 4, 2, and 7) interact specifically in a GST pull-down assay. Lane 5 represents the input of NFAT DBD, which is equivalent to protein added to GST-ICER beads retained in GST pull-down in lane 3. In contrast, GST-CREB (lane 10) does not interact with comparable amounts of NFAT DBD protein (input, lane 8). Lane 9 represents GST-CREB beads alone. NFAT DBD retained on GST-ICER Sepharose beads was separated by SDS-polyacrylamide gel electrophoresis and visualized by Western blotting using the NFAT-specific antibody R59.
ICER Binds Directly to the Conserved Proximal Motif of IFNγ Promoter—Unlike the CD28RE (−160 motif), which shows equally high affinity for both ICER and NFAT, the NFAT/AP-1 motif of the IL-2 promoter in position −90, which has striking homology to the conserved proximal element of the IFNγ promoter, does not interact with NFAT DBD (Fig. 4).

Studies performed on the conserved proximal motifs of both human and mouse IFNγ promoters demonstrated high affinity ICER binding and a lack of NFAT binding or NFAT/ICER complex formation in the presence of NFAT/DBD (Fig. 4), a situation similar to that observed on the homologous −90 motif of the IL-2 promoter (Fig. 2).

NFAT/AP-1 Composite Sites in the Context of the GM-CSF, IL-4, and TNF-α Promoters Bind ICER either Alone or in Complexes—NFAT/AP-1 binding sites have been shown previously to be essential for the efficient activation of the GM-CSF, IL-4, and TNF-α promoters. Therefore, we examined the binding to these sites of ICER and NFAT, both as purified recombinant proteins and in extracts prepared from human medullary thymocytes treated with forskolin and ionomycin (Fig. 5). These studies demonstrated that ICER can bind either by itself or in complexes with NFAT DBD to these composite sites in the promoters of the GM-CSF, IL-4, and TNF-α promoters, similar to the experiments using the binding site motifs of the IL-2 and IFNγ promoters.

The GM-420 DNA motif strongly bound the purified NFAT/ICER complex (Fig. 5B, lane 6), whereas the GM-330 and GM-550 motifs bound the complex much more weakly (Fig. 5B, lanes 3 and 9). It is noteworthy that the GM-420 motif has been shown to constitute the essential enhancer core of the GM-CSF promoter (25). Likewise, NFAT/ICER readily formed a complex with the −80 element of the IL-4 and −95 element of the TNF-α promoter (Fig. 5B, lanes 12 and 15, respectively). Interestingly, the k3 motif of the TNF-α promoter, which contains an “inverted CRE” motif adjacent to the NFAT composite site (27), created a complex with an electrophoretic mobility different from those observed on the motifs of the IL-2, IL-4, and GM-CSF promoters (Fig. 2C and Fig. 5B). Furthermore, NFAT DBD alone formed a much slower mobility complex (lane 14), suggesting that NFAT may bind to the TNF-α motif as oligomers.

The treatment of isolated human medullary thymocytes with forskolin and ionomycin readily induced the expression of ICER (Fig. 5, D and E) not seen in uninduced thymocytes (Fig. 5C). The binding of ICER to the oligonucleotides containing the NFAT/AP-1 composite sites is inhibited by competition of the binding with a CRE-containing oligonucleotide (Fig. 5D, lanes 4, 7, and 10) or interference of the binding with antisera to ICER (C-Ab) (Fig. 5E, lanes 4, 7, and 10).

Ectopically Expressed ICER Represses NFAT-mediated Activation of IL-2, GM-CSF, and TNF-α Promoters—To determine whether ICER expression could supplant the effect of forskolin in transcriptional attenuation of various cytokine promoters observed in medullary thymocytes, ICER (isoform II) was expressed in Jurkat T cells in transient transfection assays. Expression of ICER down-regulated the human IL-2, GM-CSF, and TNF-α promoters activated by the combined treatment of the cells with PMA and ionomycin, whereas ectopic expression of neither isoform of ICER did not prove to have any significant effect on VP16-mediated transactivation of (3× GAL4)-CRE-CAT under the same conditions (Fig. 6). Thus, ICER can be induced by, and substituted for, forskolin in the transcriptional down-regulation of the calcineurin-dependent, NFAT/AP-1-mediated transactivation of IL-2, GM-CSF, and TNF-α promoters when induced by PMA and ionomycin.

DISCUSSION

The mechanism of cAMP-mediated inhibition of cytokine expression in proliferating T lymphocytes has been attributed to cAMP-mediated inactivation of upstream signal transduction pathways directing the proliferation of T lymphocytes. Although this hypothesis was supported by studies of fibroblasts (5), it was not confirmed by studies of T cells (6). Surprisingly, several protein kinases required for T cell prolifera-
tion were found to be insensitive or to exhibit a delayed response to high levels of intracellular cAMP (6). Here we provide evidence that in human medullary thymocytes expression of the transcriptional repressor ICER correlates with a delayed cAMP-mediated transcriptional attenuation of T helper-1 cytokine responses.

Footprinting and electrophoretic mobility shift analysis of the IL-2 promoter revealed (34) that the originally defined AP-1 site at position −2150 of the IL-2 promoter (37, 38), a major CD28RE that contains an upstream NFAT binding site (39, 40), represents a novel NFAT/AP-1 composite site at position −2160 (34). A reexamination of the original observations in which NF-kB was identified as a major component of the complex (41, 42) determined that NFAT is a prevalent component of the complex that binds the CD28RE in vivo (34, 43). Our findings indicate that the CD28RE (−160 composite site of IL-2 promoter) effectively binds ICER either alone or in an NFAT/ICER complex. These findings may be important for obtaining a better understanding of a direct cAMP-mediated transcriptional attenuation of IL-2 expression. In addition, a potential indirect role of ICER has been demonstrated in transgenic mice overexpressing the dominant negative CREB mutant (a functional homologue of ICER), which impairs the expression of IL-2 and IFN-γ expression is dependent on the activity conferred by each of the individual DNA motifs (22, 34), a demonstration of a direct binding of ICER and/or the formation of an inhibitory NFAT/ICER complex on any of these NFAT/AP-1 composite sites could provide an explanation for the mechanism involved in the transcriptional attenuation of IL-2 and IFN-γ expression mediated by cAMP. These findings correlate with observations in which the conserved proximal motif of the IFN-γ promoter was reported to be inhibited by forskolin in proliferating thymocytes of mice made transgenic with an IFN-γ promoter-luciferase reporter gene (45). These findings further suggest that both NFAT/AP-1 motifs, either those that directly bind ICER or those that form NFAT/ICER complexes, could convey ICER-mediated transcriptional attenuation.

It appears that numerous NFAT/AP-1 composite sites previously identified in the context of GM-CSF, IL-4, and TNF-α
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promoters as essential determinants of their expression (23, 25, 27) can associate with ICER. This property does not seem to be a universal feature shared by all NFAT/AP-1 composite sites tested because we find that relatively minor differences in DNA sequences have a profound effect on both the binding of ICER and the formation of NFAT/ICER complexes. An example of this circumstance is represented by the GM-CSF promoter in which the GM –420 element shows binding for ICER and NFAT/ICER complexes, whereas both neighboring motifs GM –330 and GM –550 show only a modest binding of ICER and/or formation of NFAT/ICER complexes. The strong binding of ICER to the GM –420 element, previously defined by deletion analysis as the essential core of the GM-CSF enhancer (25), suggests that ICER may play an important role in transcriptional attenuation of GM-CSF expression. Similar binding studies performed with several NFAT/AP-1 composite sites important in the context of IL-4 and TNF-α promoters show that these sites, previously shown to be essential for efficient expression (23, 26, 27), bind ICER either alone or in complexes similarly to the motifs of the IL-2 and IFNγ promoters. It remains to be determined whether the induction of ICER can selectively modulate T helper-1 versus T helper-2 cytokine expression in peripheral blood T lymphocytes.

We have reported previously that human medullary but not cortical thymocytes synthesize ICER mRNA after 3 h of forskolin treatment (7). Western immunoblot analysis using an ICER-specific antiserum confirmed that in these conditions the ICER mRNA is translated efficiently into ICER protein. Moreover, endogenously expressed ICER protein was detected in extracts prepared from human medullary thymocytes treated with forskolin and ionomycin using oligonucleotide probes containing NFAT/AP-1 DNA motifs that are able to form NFAT/ICER complexes in vitro. In contrast to bacterially expressed ICER, endogenously expressed ICER in medullary thymocytes shows an altered mobility in gel shift assays, suggesting that posttranslational modification(s) may be involved in the regulation of the binding properties of ICER and/or degradative pathways involved in its proteolysis in vivo. The ICER-containing complexes that are immunoreactive to ICER-supershifting antiserum are efficiently competed by oligonucleotides containing CRE or NFAT motifs. NFAT antisera that are unable to recognize directly bound ICER still affect the mobility of ICER-containing complexes, suggesting the possibility of the formation of NFAT/ICER complexes in vivo. The ambiguity of the DNA-protein complexes in extracts of thymocytes observed on gel shift assays may be due to posttranslational modifications of the proteins involved (data not shown) and/or their potential consequences for DNA binding. At this point, the possibility cannot be excluded that proteins other than ICER and NFAT that contain homologous bZIP or rel homology regions may also participate in the formation of ICER-containing complexes. Finally, ectopic expression of ICER in Jurkat cells demonstrates that ICER, in agreement with its binding capabilities, can also effectively inhibit NFAT-mediated, phorbol ester/ionophore-induced expression of IL-2, GM-CSF, and TNF-α promoters.

In conclusion, inducible ICER expression in developing human medullary thymocytes as well as in certain subset(s) of human peripheral blood lymphocytes (7) and monocytes (work in progress) could significantly influence their respective effector function(s). The proposed inhibitory effects on effector function of the immune system mediated by ICER may be related to its ability to bind (mask) a wide range of CRE and AP-1 motifs and/or its ability to inactivate certain transcription complexes via protein-protein interactions.

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FIG. 6. ICER isoform II represses transcription from NFAT/AP-1-activated cytokine promoters stimulated by PMA and ionomycin (P + I treatment). A, promoter-reporter, IL-2-CAT (human interleukin-2 (32)); CD28RE (–160 AP-Luc; gift from A. Rao); GM-CSF-CAT (35) (human granulocyte-macrophage colony stimulating factor), TNF-α-Luc (contains the sequence from –614 to –20 of the human tumor necrosis factor-α plg2; gift from S. L. McKnight). A control (32): GAL4)-CR-CAT (with three GAL-4 binding sites substitut-

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