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Calcium-Dependent Activation of TNF Family Gene Expression by Ca\(^{2+}\)/Calmodulin Kinase Type IV/Gr and Calcineurin

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CD40 ligand (L), TNF-\(\alpha\), and FasL, members of the TNF family of cytokines, all are expressed by T lymphocytes shortly after activation. Transcription of these genes can be induced by stimulation of T cells by calcium ionophore alone and requires the calcineurin-dependent transcription factor NF of activated T cells (NF-AT). We have examined a second calcium-dependent signaling pathway, mediated by calcium/calmodulin-dependent kinase IV (CaMKIV) in transcriptional activation of TNF family genes. In reporter gene assays using constructs driven by the promoters of human CD40L, FasL, or TNF-\(\alpha\) along with vectors expressing constitutively active CaMKIV and calcineurin, we have demonstrated that each promoter is activated by calcineurin and CaMKIV in a synergistic fashion. Furthermore, specific inhibition of CaMKIV by chemical means and by a dominant negative mutant of CaMKIV impairs the ionomycin-induced activity of all three promoters as well as protein expression of CD40L and TNF-\(\alpha\). Our results indicate that activation of gene expression by calcineurin and CaMKIV is common to members of the TNF cytokine family.


CD40 ligand (L), TNF-\(\alpha\), and FasL, members of the TNF family of cytokines, exhibit a significant degree of structural similarity but mediate distinct effector functions. All are synthesized as type II membrane proteins with conservation of ~20–25% of the extracellular domain sequence (1) and are expressed as membrane-bound trimers that can be proteolytically cleaved to become soluble proteins. These molecules mediate critical aspects of the inflammatory response, including B cell growth and differentiation (2), migration and activation of inflammatory cells (3), and apoptosis-mediated cytotoxicity (1, 3).

Lymphocyte activation results in increased expression of these molecules by distinct but overlapping signaling mechanisms. The inhibition of expression of CD40L (4), FasL (5), and TNF-\(\alpha\) (6) by cyclosporin A (CsA) demonstrates their requirement of the calcium-dependent phosphatase calcineurin, which dephosphorylates the NF of activated T cells (NF-AT), enabling NF-AT to translocate into the nucleus (7) in which it associates with activation protein 1 (AP-1), composed of a complex of fos and jun proteins, to form a fully active transcription factor (8). Both NF-AT and AP-1 have been shown to be necessary for activation of the CD40L promoter (9, 10). Similarly, NF-AT is required for FasL promoter activation along with Ras-dependent signals (5), implying activation of the mitogen-activated protein (MAP) kinase cascade and ultimate induction of AP-1 elements. TNF-\(\alpha\) demonstrates a tissue-specific requirement for either NF-kB or NF-AT/AP-1 signaling pathways for promoter activation (11). In activated T cells, TNF-\(\alpha\) expression requires binding of ATF-2/Jun proteins to a cyclic AMP-responsive element (CRE) in conjunction with NF-AT (12).

Elevated intracellular levels of calcium also activate calcium/calmodulin-dependent protein kinases (CaMK), including CaMKII and CaMKIV. This family of kinases is independent of calcineurin but, like calcineurin, requires Ca\(^{2+}\)/calmodulin binding for activation. Previous work by us and others has demonstrated the capacity of CaM kinases to modulate transcription factor activity. CaMKIV, which is expressed selectively in neurons and in T lymphocytes, phosphorylates and activates CRE-binding protein (CREB) and subsequent CREB-dependent transcription including transcription of the immediate early gene c-fos (13, 14). CaMKIV also activates AP-1, likely by induction of c-fos, and a constitutively active mutant of CaMKIV can activate AP-1-dependent reporter gene transcription (15, 16). Activity of CaMKIV is rapidly and transiently induced by elevated [Ca\(^{2+}\)]\(_{i}\), peaking at 1 min after TCR engagement and declining to baseline levels within 15 min (15). This result suggests that wholly calcium-dependent signal transduction pathways, consisting of calcineurin and CaM kinases, may be able to mediate transcription requiring NF-AT along with AP-1 or CREB. Indeed, ionomycin alone is sufficient to induce TNF-\(\alpha\) mRNA (6) and CD40L protein expression (17), indicating that calcium-dependent signaling is essential and sufficient for gene expression.

In this study, we have examined the role of CaMKIV in calcium-dependent activation of the TNF family of cytokines. We have found that a constitutively active mutant of CaMKIV participates...
synergistically with constitutively active calcineurin in promoter activation of TNF-α, CD40L, and FasL. CaMKIV appears to mediate actual calcium-dependent transcription of these genes, as specific inhibition of CaMKIV by chemical means or by a dominant negative mutant of CaMKIV impairs both the ionomycin-induced promoter activity of all three molecules and protein expression of CD40L and TNF-α. Identification of CaMKIV as a novel regulatory molecule may provide new therapeutic strategies targeting the potent cytokines of the TNF family.

Materials and Methods

Cells

All transfections were conducted in Jurkat thymoma cells (American Type Culture Collection, Manassas, VA) grown in RPMI 1640 supplemented with 10% FCS (complete medium). Cell surface staining was performed on primary human CD4+ T cells. PBMC were isolated from whole blood by Ficoll density gradient centrifugation and enriched for CD4+ cells as determined by flow cytometry.

Reporter plasmid construction

Reporter plasmids incorporating the CD40L, FasL, and TNF-α promoters were prepared as follows: CD40L promoter/luciferase (CD40L-luc), a 563-bp fragment spanning −495 to +67 relative to the start of transcription site of the CD40L gene (9), was derived by PCR from human genomic DNA using sense and antisense primers fitted with HindIII sites (underlined): 5′ (sense) primer, 5′-AAGCTTCCTGAGCAGGTTCATTGATT-3′; and 3′ (antisense) primer, 5′-AAGCTTGTGTTGATGTTAAATTGTGAAAG-3′. The fragment was subcloned into the HindIII site upstream of a luciferase reporter gene in the pGL3 (Promega, Madison, WI). CD40L-luc includes both previously identified NF-AT binding motifs, located at −259 to −265 and at −62 to −69 relative to the transcription start site (9).

FasL promoter/luciferase (FasL-luc), a 1073-bp fragment spanning −1035 to +38 relative to the start of the transcription site of the FasL gene (5), was isolated using sense and antisense primers fitted with HindIII sites (underlined): 5′ (sense) primer, 5′-GCAAATAGCAGAATCCGACCTG 3′; and 3′ (antisense) primer, 5′-AAGCTTTCGGAGCCCAACTGTTGATAC-3′. A 995-bp fragment, spanning −957 to +38 relative to the start of transcription site was excised at a native HindIII site and the engineered HindIII sites and subcloned into the Smal/HindIII sites in the pGL3 basic vector (Promega).

The pGL3/TNF-α-luc vector, a 316-bp fragment spanning −221 to +95 relative to the start of transcription site of the TNF-α gene (18), was derived by PCR using sense and antisense primers fitted with BglII and HindIII sites, respectively (underlined): 5′ (sense) primer, 5′-AGATCTGGAGTGTGAGGGGTATCCTTGATG-3′; and 3′ (antisense) primer, 5′-AAGCTTGTGTTGATGTTAAATTGTGAAAG-3′. The fragment was subcloned into the BglII/HindIII sites upstream of a luciferase reporter gene in the pGL2 Luc basic vector (Promega).

Expression vectors

Vectors expressing wild-type (wt), constitutively active (c), and dominant negative (dn) forms of CaMKIV as well as a constitutively active mutant of CaMKII were created in the pSG5 vector (Stratagene, La Jolla, CA) as previously described (16, 19).

The plasmid pBJ5-CNMMUT2B.19, here termed CNM, expresses a constitutively active mutant of murine calcineurin α subunit (amino acids 1–394) using the expression vector pBJ5 as described (20). NF-ATc2 was created as described by subcloning cDNA encoding the cytoplasmic component of NF-ATc2 into pBJ5 (16). CNM and NF-ATc2 were kind gifts from Gerald Crabtree (Stanford University, Palo Alto, CA).

CMV-luciferase (CMV-luc) contains a luciferase gene under control of the immediate early promoter region of CMV (16) and was a kind gift from Ken Murphy (Washington University, St. Louis, MO).

Transient transfection and luciferase reporter gene assays

Jurkat T cells were suspended at 2.0 × 10^6/ml in complete medium and transfected by electroporation at 250 V and 950 μF. After electroporation, cells were cultured in complete medium for 1–2 h. As indicated, CaS (100 ng/ml, Calbiochem, San Diego, CA) or KN62 (10 μM, Calbiochem) were added to the cultures for 60 min before stimulation. Transfected cells were left unstimulated or were stimulated for 24 h with ionomycin (Calbiochem) at 2.5 μM, or as otherwise indicated. Cells then were lysed with detergent buffer (Promega), and luciferase activity was measured in 25 μl of lysate after the addition of 50 μl luciferin substrate (Promega) in a 96-well plate luminometer (Labsystems Luminoskan, Needham Heights, MA). Statistical analysis was performed by means of the paired Student’s t test using StatView software (Abacus Concepts, Berkeley, CA).

Cell surface staining

CD4+ T cells were stimulated for 6 h with ionomycin at 2.5 μM, or as otherwise indicated, and assayed for cell surface expression of CD40L by flow cytometry on a FACSscan (Becton Dickinson Immunocytometry Systems, San Jose, CA) as described previously (21) using a FITC-conjugated CD40L mAb or a FITC-conjugated isotype control mAb (Ancell, Bayport, MN). As indicated, cells were incubated for 60 min before stimulation in CsA (100 ng/ml) or KN62 (10 μM), and cell viability was determined by trypan blue exclusion. Three independent experiments were performed, and a representative experiment is shown.

Detection of recombinant CaM kinase protein expression

Jurkat T cells cotransfected with CaMKIV(dn) and each TNF promoter/luciferase reporter gene construct were resuspended at 10 × 10^6 cells/ml and lysed by pipetting on ice in a buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 42 mM DTT, and 0.01% bromophenol blue, followed by shearing of DNA by repeated vigorous passage of the lysate through a 25-g needle. The lysates were boiled 3 min and cleared of insoluble material by centrifugation at 16,000 × g for 10 min at 4°C. A total of 20 μl of lysate (equivalent of 0.2 × 10^6 cells) were resolved by SDS-PAGE (10% acrylamide) and electoblotted onto polyvinylidene fluoride membranes (Immobilon-P; Millipore, Bedford, MA). The membranes were incubated for 1 h in blocking buffer (20 mM Tris (pH 7.6), 150 mM NaCl, 0.1% Tween 20 detergent, and 5% nonfat dry milk), washed, and incubated overnight at 4°C with rabbit polyclonal IgG anti-FLAG epitope Ab (Santa Cruz Biotechnology, Santa Cruz, CA) at 0.5 μg/ml in blocking buffer. The membranes were washed and incubated for 1 h at room temperature with HRP-linked anti-rabbit IgG (New England Biolabs, Beverly, MA) diluted 1:1,000 in blocking buffer. After washing, the blots were developed by the Phototope-HRP Western Blot Detection system (New England Biolabs). Images were detected using the GS 525 densitometer (Bio-Rad Laboratories, Hercules, CA) and analyzed using Molecular Analyst software (Bio-Rad Laboratories).

Results

Ionomycin alone is capable of inducing promoter activity and cell surface expression of TNF family molecules

Calcium-dependent signal transduction in T cells has been shown to be sufficient for expression of TNF-α, CD40L, and FasL. To verify that calcium-dependent signals regulate TNF family expression at the level of promoter activation, luciferase reporter vectors driven by TNF family member promoters were transfected into Jurkat T cells. The transfected cells were stimulated with increasing concentrations of ionomycin and assayed 24 h later for luciferase activity. Ionomycin activated the TNF-α, CD40L, and FasL promoters in a dose-related manner from 0.5 to 5.0 μM (Fig. 1). Ionomycin induced no luciferase activity in cells transfected with the empty parent vector pGL3B. To evaluate the ability of calcium-dependent signaling to lead to protein expression, we examined ionomycin-stimulated CD4+ T cells for CD40L surface expression and for intracellular TNF-α accumulation by flow cytometry. Stimulation with ionomycin resulted in a dose-dependent increase both in CD40L cell surface expression and in intracellular staining for TNF-α (data not shown). These findings indicate that calcium-dependent signaling pathways provide the transcription factors required for promoter activation and are sufficient for protein expression of TNF family members.

Inhibition of CaM kinases suppresses TNF family promoter activity and cell surface expression

The crucial role of calcineurin in TNF-α (6), CD40L (4), and FasL gene expression (5) is well known. To evaluate the relative regulatory roles of CaM kinases and calcineurin, we tested the effect of

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KN62 and CsA on ionomycin-induced promoter activity. KN62 is a cell-permeable chemical inhibitor of CaMKII and IV that has no inhibitory effect on protein kinase A or C activity (13, 22, 23). CsA is a specific inhibitor of calcineurin that consequently prevents activation of NF-AT. Jurkat T cells were transfected with TNF-α-luc, CD40L-luc, FasL-luc, or pGL3B (15 μg), rested for 1 h, and then stimulated with ionomycin at the indicated concentrations. After 24 h, luciferase activity was determined for triplicate samples and normalized relative to the activity from unstimulated cells. Each bar represents the mean ± SEM relative luciferase activity from three independent experiments.

We further questioned whether CaM kinases mediate ionomycin-induced expression of CD40L on the surface of CD4+ T cells. Purified human CD4+ T cells were preincubated in KN62 or CsA for 60 min before stimulation with ionomycin. KN62 expression was nearly abolished by CsA (mean, 83% reduction). There was a small disparity in the degree of suppression of promoter activity by KN62. CD40L promoter activity was more sensitive to the effects of CaM kinases because it contains only 500 bp of the CD40L promoter and lacks the potential intronic and enhancer elements possessed by the endogenous CD40L gene. Alternatively, the explanation may lie in the differences between Jurkat T cells, used in the reporter gene assays, and primary CD4+ T lymphocytes. The difference in stimulation times for reporter gene assays and cell surface expression (6 and 24 h, respectively) resulted from our determinations of the peaks of luciferase activity and of CD40L surface expression. It appeared that this was not an explanation for the disparity in CsA sensitivity, as promoter activity measured at 6 h was also totally abolished by CsA (data not shown). The impairment of CD40L promoter activity and CD40L cell surface expression by KN62, an inhibitor of CaM kinases that does not inhibit calcineurin, strongly suggests that CaM kinases are involved in the calcium-induced activation of the endogenous gene. Similarly, we found that KN62 led to a 40% inhibition of ionomycin-induced expression of TNF-α by intracellular cytokine staining (data not shown). KN62 had no effect on lymphocyte viability and did not affect the expression of CD69 on activated CD4+ lymphocytes (data not shown). Specific inhibition of TNF family protein expression by pharmacologic inhibition of CaM kinases supports the role of CaM kinases in regulating calcium-induced expression of these genes.

Constitutively active and dominant negative forms of CaMK IV regulate TNF family promoter activity

Our finding that pharmacologic inhibition of CaM kinases could impair TNF family promoter activity and cell surface expression prompted us to examine the effect of constitutively active form of

FIGURE 1. Ionomycin alone is capable of inducing TNF family promoter activity. Jurkat T cells were transfected with TNF-α-luc, CD40L-luc, FasL-luc, or pGL3B (15 μg), rested for 1 h, and then stimulated with ionomycin at the indicated concentrations. After 24 h, luciferase activity was determined for triplicate samples and normalized relative to the activity from unstimulated cells. Each bar represents the mean ± SEM relative luciferase activity from three independent experiments.

FIGURE 2. Inhibitors of calcineurin and CaMKIV suppress TNF family promoter activity and cell surface expression. A, Reporter gene assays. Jurkat T cells were transfected with TNF-α-luc, CD40L-luc, FasL-luc, or pGL3B (15 μg) and incubated in KN62 or CsA for 60 min before stimulation with ionomycin. KN62 significantly (p < 0.05) reduced promoter activity of all three constructs by >70% (Fig. 2A). As expected, inactivation of calcineurin by CsA impaired promoter activity by >99%. Activity of a vector containing the luciferase gene driven by the CMV promoter, CMV-luc, was unaffected by either inhibitor. The degree of suppression of promoter activity by KN62 was similar among TNF-α, CD40L, and FasL, suggesting that regulation of expression by CaM kinases is shared by these members of the TNF family of genes.

B, Flow cytometric analysis of cell surface expression. Purified human CD4+ T cells were cultured in complete medium for 1 h in the presence of no inhibitor, KN62, or CsA before stimulation with ionomycin for 24 h. Cells were then stained with a FITC-conjugated CD40L mAb (shaded area) or a FITC-conjugated isotype control mAb (open area) and analyzed by flow cytometry. Percent cells expressing CD40L are indicated in parentheses. These results are representative of three independent experiments.
CaMK IV in TNF family gene expression. We investigated the influence on promoter activity of vectors expressing constitutively active forms of CaMKIV(c) and calcineurin (CNM) cotransfected into Jurkat T cells along with TNF family promoter/luciferase reporter gene constructs. To assess the specificity of the effect of CaMKIV(c), a construct expressing a constitutively active mutant of CaMKII(c) was cotransfected along with CNM. Then, promoter activity was determined in the absence of any chemical or receptor-derived stimulation of the transfected cells. TNF-α-luc, CD40L-luc, or FasL-luc was cotransfected into Jurkat T cells with CNM and CaMKIV(c) or CaMKII(c). CNM alone induced a 2-fold increase in TNF-α promoter activity, which was enhanced to a 12-fold induction by coexpression of CaMKIV(c) (Fig. 3). CNM alone was also sufficient to induce a 15-fold increase in CD40L promoter activity, which increased to nearly 40-fold by coexpression of CaMKIV(c). CNM alone similarly led to a nearly 10-fold increase in FasL promoter activity, but this was augmented to a 50-fold induction by the coexpression of CaMKIV(c). No luciferase activity was detectable in cells transfected with the parent vector pGL3B. Coexpression of CaMKIV(c) alone induced no significant promoter activity in any tested construct. In contrast, coexpression of CaMKII(c) partially impaired the CNM-induced activity of both the TNF-α and CD40L promoter but had no effect on the CNM-induced activity of the FasL promoter. Cotransfection of CaMKIV(c) alone had neither a positive nor a negative effect on the activity of the TNF family promoter constructs (data not shown). CaMK II has been found to inhibit IL-2 gene transcription, possibly through inhibition of AP-1 (24) and inactivate CREB by dual phosphorylation (25), which may explain the observed inhibition of CNM-induced promoter activation in our experiments. The heterogeneity in relative activity between the promoters may reflect differences in the promoter length contained in each construct and in native responsiveness to calcium-dependent signaling. These results demonstrate that CaMKIV activates gene expression of TNF family members in synergy with calcineurin. This activity is specific for CaMKIV, as demonstrated by the contrasting inhibitory effect of CaMKII.

FIGURE 3. TNF family promoters are activated by constitutively active CaMKIV and calcineurin. Jurkat T cells were transfected with TNF-α-luc, CD40L-luc, FasL-luc, or pGL3B (15 μg) along with CNM (2 μg), CaMKII(c) (10 μg) or CaMKIV(c) (10 μg), or with the empty parent vectors pBJ5 (2 μg) and pSG5 (10 μg). After 24 h, luciferase activity was determined for triplicate samples and normalized relative to the activity from cells transfected with empty parent vectors. Each bar represents the mean ± SEM relative luciferase activity from three independent experiments.

Although KN62 appears to be a specific inhibitor of CaM kinases, we sought further evidence for the role of CaMKIV by inhibiting its activity in an alternate manner. Therefore, we tested the effect of cotransfecting vectors expressing wt CaMKIV and a dominant negative mutant of CaMKIV on ionomycin-induced promoter activity. Jurkat T cells were transfected with TNF-α-luc, CD40L-luc, FasL-luc, or pGL3B (15 μg) along with CaMKIV(c), CaMKIV(wt), CaMKIV(dn), or with the empty parent vector pSG5 followed by stimulation with 2.5 μM ionomycin. Luciferase activity was determined after 24 h. Each bar represents the mean ± SEM relative luciferase activity from two independent experiments. B. Lysates of 0.2 × 10⁶ Jurkat T cells transfected with CaMKIV(dn) along with each TNF family promoter reporter construct were resolved by SDS-PAGE and probed for expression of CaMKIV(dn) by anti-FLAG epitope Ab as described in Materials and Methods.

FIGURE 4. A dominant negative mutant of CaMKIV impairs ionomycin-induced TNF family promoter activity. A, Jurkat T cells were transfected with TNF-α-luc, CD40L-luc, FasL-luc, or pGL3B (15 μg) along with CaMKIV(c), CaMKIV(wt), CaMKIV(dn), or with the empty parent vector pSG5, cotransfected with CNM along with CaMKIV(c), CaMKIV(c), or with the empty parent vector pSG5 (15 μg), or with the empty parent vector pSG5 (15 μg). After 24 h, luciferase activity was determined for triplicate samples and normalized relative to the activity from cells transfected with empty parent vectors. Each bar represents the mean ± SEM relative luciferase activity from three independent experiments. B. Lysates of 0.2 × 10⁶ Jurkat T cells transfected with CaMKIV(dn) along with each TNF family promoter reporter construct were resolved by SDS-PAGE and probed for expression of CaMKIV(dn) by anti-FLAG epitope Ab as described in Materials and Methods.
was not likely to have resulted from variations in transfection efficiency, as this was addressed by cotransfection of a fixed quantity of a β-galactosidase reporter gene construct in each experiment (as described in Materials and Methods). We performed immunoblotting analysis of cells transfected with each reporter gene construct along with the vector expressing the dominant negative mutant of CaMKIV to evaluate the relative expression of CaMKIV(dn) (Fig. 4B). Expression of CaMKIV(dn) protein was expressed at approximately equivalent levels regardless of the cotransfected reporter gene construct, indicating that the difference in reporter gene constructs had no influence on the expression vectors. It appeared that CaMKIV(dn) was not as effective at inhibiting reporter gene constructs had no influence on the expression vectors. It appeared that CaMKIV(dn) was not as effective at inhibiting reporter gene constructs had no influence on the expression vectors. It appeared that CaMKIV(dn) was not as effective at inhibiting reporter gene constructs had no influence on the expression vectors. It appeared that CaMKIV(dn) was not as effective at inhibiting reporter gene constructs had no influence on the expression vectors. It appeared that CaMKIV(dn) was not as effective at inhibiting reporter gene constructs had no influence on the expression vectors. It appeared that CaMKIV(dn) was not as effective at inhibiting reporter gene constructs had no influence on the expression vectors. It appeared that CaMKIV(dn) was not as effective at inhibiting reporter gene constructs had no influence on the expression vectors. It appeared that CaMKIV(dn) was not as effective at inhibiting reporter gene constructs had no influence on the expression vectors.

Expression of CaMKIV to evaluate the relative expression of CaMKIV(dn) (Fig. 5). The degree of activity induced by CaMKIV alone reflected constitutively active AP-1 or calcineurin alone can induce a modest degree of TNF family promoter activity as well as expression of CD40L and of TNF-α. This finding implies that calcium-dependent signal transduction pathways are able to provide the transcription factors required not only for promoter activation but also for induction of transcription and translation of endogenous TNF family genes. Our findings indicate that CaMK IV provides a signal that acts with the calcineurin/NF-AT pathway to activate the promoters of CD40L, FasL, and TNF-α. Furthermore, the inhibition of CD40L and of TNF-α protein expression by an inhibitor of CaMKIV indicates that this pathway is involved in the calcium-induced activation of the endogenous gene.

Although CD40L, FasL, and TNF-α differ markedly in their effector functions, they share the characteristic of early expression by activated T cells after stimulation by Ag. Stimulation via interaction of the TCR/CD3 complex with Ag/MHC or cross linking of TCR/CD3 by anti-CD3 Abs initiates parallel kinase cascades that lead to activation of phospholipase C followed by metabolism of inositol lipids to inositol triphosphate, triggering both release of calcium from intracellular stores and formation of diacylglycerol (30–33). These events set in motion two of the major signal transduction pathways leading to cell activation and cytokine production: the calcium/calcmodulin-dependent enzymes (e.g., calcineurin and the CaM kinases) and protein kinase C (PKC), which is activated by diacylglycerol. In addition, the ras/raf/MAP kinase cascade is activated in parallel with PKC by a series of GTP binding proteins, although PKC itself also activates this series of enzymes. Stimulation by TCR/CD3 can be imitated by the combination of calcium ionophore, which activates calcium dependent signaling, and phorbol ester, which directly activates PKC.

Constitutively active forms of CaMK IV and NF-AT cooperate to induce TNF family promoter activity

NF-AT requires cooperative binding with fos and jun proteins for stable DNA binding and transcription factor activity (26). Therefore, our finding that constitutively active calcineurin alone can induce a modest degree of TNF family promoter activity in transfected Jurkat T cells raised the possibility that calcineurin-dependent pathways may be able to provide both NF-AT and AP-1 or CREB signals. This was supported by a recent report indicating that CsA and FK506 can inhibit CREB activity, providing indirect evidence of a role for calcineurin in the activation of CREB (27). It was also possible that the CNM-induced activity represented activation of pathways distinct from NF-AT that have been shown to be activated or induced by calcineurin, including NFIL2A (OAP/Oct-1) (28) and c-rel (29). Alternatively, we hypothesized that the activity induced by calcineurin alone reflected constitutively active AP-1 or CREB proteins that may be present in a transformed cell line such as Jurkat. To test this hypothesis, we cotransfected constitutively active forms of NF-AT and CaMKIV(c) with TNF family promoter/luciferase reporter constructs. Like CNM, NF-ATC2 alone induced modest activity in each promoter that was enhanced greatly by cotransfection of CaMKIV(c) (Fig. 5). The degree of activity induced by NF-AT alone and in combination with CaMKIV was of a magnitude comparable to the activity induced by CNM. These data indicate that in Jurkat T cells NF-AT can activate TNF family promoters and suggest that the effect of CNM on our promoter constructs in Jurkat T cells is mediated largely if not wholly by the activation of NF-AT.

Discussion

In this study, we have identified a novel role for the CaM kinases in regulating the expression of members of the TNF family of cytokines. We have demonstrated that stimulation by ionomycin alone in a wide range of doses is sufficient to induce TNF family promoter activity as well as expression of CD40L and of TNF-α. This finding implies that calcium-dependent signal transduction pathways are able to provide the transcription factors required not only for promoter activation but also for induction of transcription and translation of endogenous TNF family genes. Our findings indicate that CaMK IV provides a signal that acts with the calcineurin/NF-AT pathway to activate the promoters of CD40L, FasL, and TNF-α. Furthermore, the inhibition of CD40L and of TNF-α protein expression by an inhibitor of CaMKIV indicates that this pathway is involved in the calcium-induced activation of the endogenous gene.
have demonstrated previously that CaMKIV activates AP-1 and CREB-dependent transcription (14, 16). Therefore, CaMKIV and calcineurin can provide a wholly calcium-dependent means of transcriptional activation of genes requiring binding of both CREB/AP-1 and NF-AT. Our data demonstrate a critical regulatory role for CaMKIV in calcium-dependent TNF family gene expression and provide a model that explains the ability of elevated intracellular calcium to provide both the calcineurin-dependent NF-AT and the CaMKIV-dependent CREB/AP-1 signals described above. Determination of which CREB/AP-1 family proteins are the exact transcription factors that are activated by CaMKIV and that cooperate with NF-AT in each TNF family promoter will be the subject of further study.

In support of this model, CaMKIV(c) has been demonstrated to activate transcription of the EBV protein BZLF1 by inducing binding of a CREB/AP-1 site in the BZLF1 promoter (14). This activity requires binding of a calcium-dependent CsA-sensitive factor at a neighboring element and consequently is augmented greatly by coexpression of constitutively active calcineurin. Activation of expression of the TNF family of genes by CaMKIV does not appear to be a generalized effect on transcription, as cotransfection of CaMKIV(c) with CNM does not up-regulate activity of a construct containing the HIV-1 long terminal repeat driving the luciferase reporter gene (R.L.F., unpublished observations).

The significance of CaMKIV-mediated transcriptional activation is supported by a recent report demonstrating a constitutive association in Jurkat T cells between CaMKIV and protein phosphatase 2A (PP2A), a serine-threonine phosphatase that can impair the ability of CaMKIV to phosphorylate CREB (35). Inactivation of CaMKIV by PP2A provides an explanation for the rapid loss of CaMKIV activity despite the persistence of elevated [Ca\(^{2+}\)] after T cell stimulation. This finding suggests that CaMKIV activity requires close regulation, providing further indirect evidence of an important role for CaMKIV in transcriptional activation in lymphocytes. Although PP2A and protein phosphatase 2B (calcineurin) share certain characteristics of substrate specificity and inhibitor resistance, calcineurin uniquely requires Ca\(^{2+}\)/calmodulin for activation (36). The ability of overexpressed calcineurin to act in synergy with CaMKIV in our experiments indicates that, unlike PP2A, calcineurin does not have an inhibitory effect on CaMKIV. Furthermore, pretreatment with CsA has neither a positive nor a negative effect on TCR/CD3-mediated CaMKIV activation in T lymphocytes, indicating that CaMKIV is not influenced directly by calcineurin (T.A.C., unpublished observations).

The demonstration that common pathways regulate expression of TNF family members with such different functions raises the question of how these molecules are expressed differentially by activated T lymphocytes. One mechanism for determining differential expression of TNF family members by cytokines and costimulatory molecules. Anti-CD3-induced expression of CD40L on murine T cells is inhibited by IFN-γ on Th1, Th2, and splenic T cells, whereas TGF-β inhibits CD40L expression on Th2 cells (37). We also have found that IFN-γ impairs CD40L expression induced by PMA and ionomycin on primary human T lymphocytes by ~50% (F.M.L., unpublished observations). In addition, engagement of the accessory molecule CD28 has been shown to stimulate or enhance CD40L surface expression (38–40). TNF-α expression is inhibited by IL-10 (41) and enhanced by cell adhesion molecules (42). FasL is expressed largely on Th1 cells, and therefore its expression may be inhibited by Th2 cytokines (43). In addition, a recent report provides evidence for speculation that differential expression in Th1 vs Th2 cells may be determined by the intensity of the intracellular calcium signal itself, which is selectively lost in mature Th2 cells but retained in Th1 cells (44). Definition of the pathways that provide specific expression of each of these molecules will be the subject of further investigations.

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