Identification of a Novel Cyclosporin-sensitive Element in the Human Tumor Necrosis Factor α Gene Promoter

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Summary

Tumor necrosis factor α (TNF-α), a cytokine with pleiotropic biological effects, is produced by a variety of cell types in response to induction by diverse stimuli. In this paper, TNF-α mRNA is shown to be highly induced in a murine T cell clone by stimulation with T cell receptor (TCR) ligands or by calcium ionophores alone. Induction is rapid, does not require de novo protein synthesis, and is completely blocked by the immunosuppressant cyclosporin A (CsA). We have identified a human TNF-α promoter element, κ3, which plays a key role in the calcium-mediated inducibility and CsA sensitivity of the gene. In electrophoretic mobility shift assays, an oligonucleotide containing κ3 forms two DNA protein complexes with proteins that are present in extracts from unstimulated T cells. These complexes appear in nuclear extracts only after T cell stimulation. Induction of the inducible nuclear complexes is rapid, independent of protein synthesis, and blocked by CsA, and thus, exactly parallels the induction of TNF-α mRNA by TCR ligands or by calcium ionophore. Our studies indicate that the κ3 binding factor resembles the preexisting component of nuclear factor of activated T cells. Thus, the TNF-α gene is an immediate early gene in activated T cells and provides a new model system in which to study CsA-sensitive gene induction in activated T cells.

The human TNF-α gene encodes a cytotoxic protein with diverse biological activities including the ability to promote T and B cell growth (1-8 and for reviews see references 9-11). TNF-α is produced by multiple cell types including lymphocytes after viral infection or stimulation through cell-surface receptors (12-19). In primary T cells for example, TNF-α mRNA is induced upon stimulation with the phorbol ester (PMA) plus activating antibodies to CD3 polypeptides (12), which are closely associated with the TCR. TNF-α mRNA is also induced in T cells by stimulation with PMA alone (12, 18), and by PMA plus calcium ionophore (12, 15), which mimics stimulation through the TCR-CD3 complex (for reviews see references 20 and 21).

The promoter sequences required for virus, LPS (16), PMA (18, 22-25), and TNF-α induction (26) of the human TNF-α gene have been identified. These studies have yielded a complex picture of TNF-α gene regulation in that the promoter sequences required for inducibility have varied with the cell type and stimulus studied. Although the 5' flanking region of the human TNF-α gene contains three sequences, κ1, κ2, and κ3, that match the consensus sequence for a binding site for the transcription factor NF-κB, the induction of the human TNF-α gene by virus, LPS, or PMA in the cell lines studied does not appear to be primarily mediated by NF-κB (16, 18). Thus, in contrast to the NF-κB-mediated inducible transcription of other promoters containing NF-κB binding sites (for a review see reference 27), and in contrast to studies with the murine TNF-α gene in which NF-κB binding sites have been implicated in LPS induction in macrophages (28, 29), studies of the human TNF-α gene promoter argue against a major role for NF-κB in the virus, LPS, or PMA induction of the gene (16, 18).

The immunosuppressive agents cyclosporin A (CsA) and FK506, inhibit the transcription of many cytokine genes in activated T cells (15, 30-32). CsA and FK506, when complexed to their intracellular receptors (cyclophilin and FKBP, respectively), bind to and inhibit the activity of the calcium- and calmodulin-dependent phosphatase calcineurin (33; for reviews see references 34 and 35). Recent studies (36-39) have suggested that calcineurin regulates the activity of at least two elements in the IL-2 gene promoter, nuclear factor of activated T cells (NFAT) and NFIL2A. The nuclear factors binding to these two elements appear to be distinct: the NFAT

1 Abbreviations used in this paper: CAT, chloramphenicol acetyl transferase; CsA, cyclosporin A; n, nucleotide; NFAT, nuclear factor of activated T cells; OAP-40, octamer-associated protein.
site binds to a lymphoid-specific factor, NFAT (40), which consists of a preexisting DNA-binding subunit (NFATp) in association with fos and jun proteins (41-43), whereas the NFIL2A site binds oct-1 and oct-2 proteins in association with an inducible octamer-associated protein, OAP-40 (44). Calcineurin may directly dephosphorylate NFATp (45), however its effect on the activity of the Oct-OAP complex may be more involved.

Using an untransformed murine T cell clone that responds to stimulation with antigen and TCR ligands (46, 47), the TNF-α gene is shown to be one of the earliest genes transcribed after T cell activation. Induction of TNF-α mRNA does not require de novo protein synthesis but is sensitive to CsA. Thus, the TNF-α gene represents a new model system in which to study CsA-sensitive gene induction. We have identified a TNF-α promoter element, k3, as a CsA-sensitive regulatory element required for TNF-α gene transcription in activated T cells. The requirements for induction of the k3 binding factor parallel closely the requirements for induction of endogenous murine TNF-α mRNA levels: both TNF-α mRNA and the k3 binding factor are rapidly induced by TCR ligands or by calcium ionophore alone. Their induction does not require de novo protein synthesis and is blocked by CsA. Our studies indicate that the nuclear k3 binding factor is present in unstimulated T cells and that it resembles the preexisting component of NFAT. Activation of the k3 binding factor appears to require posttranslational modification and/or translocation to the nucleus via a calcium-dependent, CsA-sensitive pathway.

Materials and Methods

Cell Culture, Activation, and Transfections. The murine IL2-dependent T cell clone Ar-5 (48) was grown and activated with mAbs to murine CD3e (145-2C11; oLCD3) or to a framework determinant of the murine TCR-α/β (H57-597; TCR-cr for the times indicated) before the addition of antibodies or ionomycin. The ability of cydoheximide and 40 μM anisomycin (both from Sigma Chemical Co., St. Louis, MO) for 30 min or with 1 μM CsA (Sandoz, Vienna, Austria) before the addition of antibodies or ionomycin. The ability of cydoheximide and anisomycin to block protein synthesis under these conditions was verified by testing the effect of these reagents on the reagents on the EBV lytic switch gene, BZLF1, whose transcription is dependent on de novo protein synthesis (50). Transfections were performed as described (47) and cells were activated the next day with αCD3 or ionomycin and harvested ~18 h later. Chloramphenicol acetyl transferase (CAT) assays were performed as previously described (18).

RNA Analysis. RNA was prepared from Ar-5 cells as previously described (51). 32P-labeled RNA probes were prepared from SP6 γ-actin (52) and a murine TNF-α probe as previously described (16, 51). RNA fragments protected from RNase cleavage were separated by electrophoresis on a 6% denaturing polyacrylamide gel.

Preparation of Cellular Extracts and EMSAs. Nuclear extracts were prepared as previously described (49). To prepare cytosolic extracts, unstimulated Ar-5 cells were harvested by centrifugation, washed with PBS and resuspended and lysed at a concentration of 2.5 × 10^7 cells/ml in a buffer containing 50 mM NaCl, 20 mM Tris, pH 7.5, 10 mM iodoacetamide, 2 mM PMSE, 0.1 mM EDTA, 25 μM leupeptin, 100 μg aprotinin, and 0.05% NP-40. The cells were first centrifuged at 200 g to remove nuclei, and then centrifuged further at 100,000 g for 60 min. The 100,000 g supernatant was then adjusted to a concentration of 1.5 M ammonium sulfate, and the precipitated proteins were collected by centrifugation at 10,000 g. The protein pellets were resuspended in a buffer containing 100 mM NaCl, 20 mM Hepes, pH 7.4, 10 mM iodoacetamide, 2 mM EDTA, 2 mM PMSE, 25 μM leupeptin, 100 μg/ml aprotinin, and 10% glycerol, and were extensively dialyzed against the same buffer without iodoacetamide and with 0.5 mM DTT.

EMSA using nuclear proteins were performed using ~5 μg nuclear protein in a total volume of 15 μl containing 4 mM Hepes, pH 7.4, 90 mM NaCl, 20 mM KCl, 0.08 mM EDTA, 9% glycerol, 133 μg/ml poly (dIdC), and 0.125 ng 32P-endabeled oligonucleotide. For competition assays, a 200-fold excess of unlabeled oligonucleotide was added to the reaction. Binding reactions were incubated on ice for 15 min. Unbound oligonucleotide and protein-oligonucleotide complexes were separated by electrophoresis at 4°C on Tris/borate/EDTA/4% acrylamide gels as described earlier (49).

Cytosolic extracts (0.5–1 μg protein) were assayed for DNA binding in a reaction mix containing 4 mM Hepes, pH 7.4, 100 mM NaCl, 9% glycerol, 0.7 mg/ml BSA, 17 μg/ml poly (dIdC), and 0.125 ng 32P-endabeled oligonucleotide, followed by electrophoresis under the same conditions described for nuclear extracts.

Methylation interference assays were performed as described (49). The sequences of the synthetic oligonucleotides used in the gel shift and methylation interference assays were as follows: k2, (5'-GATCCGGAGCTCATGGTTTCTCCACA-3'); k1, (5'-GATCCGGAGCCCA-3'); k2, (5'-GATCCGGTTTCTCCACA-3'); NF-κB (WT), (5'-CAGAGGAGCCTTCAGGA-3'); NF-κB (M5'), (5'-CAGAGGAGCCTTCAGGA-3'); NF-κB (M3'), (5'-CAGAGGAGCCTTCAGGA-3'); NF-κB (M3'), (5'-CAGAGGAGCCTTCAGGA-3'); and APL, (5'-CTGATCAACACAATTTCCCTTGAGC-3'); and APL, (5'-CTGATCAACACAATTTCCCTTGAGC-3').

Plasmids. The TNF-α promoter/CAT gene constructs and the -61 TNF-α CAT and (k3)'-61 TNF-α CAT constructs have been previously described (16). The k3 mutant constructs were constructed by subcloning a TNF-α promoter fragment into M13mp18 and performing in vitro mutagenesis with a Mutagenex M13 in vitro Mutagenesis Kit (Biorad Laboratories, Richmond, CA). Mutants were confirmed by dideoxy sequencing. Promoter sequences from -199 to +87 nucleotides relative to the TNF-α transcription start site were amplified by PCR and were subcloned into the POClAT vector (53) and reconfirmed by sequencing. The wild-type -199 to +87 TNF-α sequences were also subcloned in this manner, ensuring that all of the constructs were isogenic.

Results

Regulation of the Endogenous Murine TNF-α Gene in the Murine T Cell Clone, Ar-5

TCR Stimuli. To characterize the transcriptional induction of the TNF-α gene in activated T cells, the untransformed murine T cell clone, Ar-5, was stimulated with antibodies directed towards the CD3 complex (αCD3) or the TCR-α/β (TCR-α). TNF-α mRNA levels were increased strikingly within 30 min of stimulation by either αCD3 or
Figure 1. Anti-CD3 and anti-TCR-α/β induction of TNF-α gene expression in murine Ar-5 T cells. Cells were induced with anti-CD3 (αCD3) or anti-TCR-α/β (αTCR) for 30 min or 4 h as indicated, in the presence or absence of cyclosporin A (CsA) or cycloheximide (CHX) and anisomycin (ANI) as described in Materials and Methods. RNase mapping of TNF-α and γ-actin mRNA was carried out with 5 μg of total cellular RNA. A 32P-labeled mouse TNF-α probe was used to map murine TNF-α mRNA levels and an RNA probe complementary to the γ-actin gene was used as an internal control for mRNA levels as previously described (15). The γ-actin probe was made to have a sp act one fifth of the mouse TNF-α probe. (Left) Positions of the TNF-α and γ-actin protected fragments.

Figure 2. Ionomycin induction of TNF-α gene expression in murine Ar-5 T cells. (A) Cells were mock (-) induced, induced with 100 nM or 1 μM ionomycin for 30 or 60 min in the presence or absence of 1 μM CsA as indicated. CsA was added 10 min before induction with ionomycin. (B) Cells were mock (-) induced or induced for 30 min with 1 μM ionomycin alone or in the presence of CsA or CHX and ANIS as indicated. CsA and CHX/ANIS were added 10 and 30 min, respectively, before induction with ionomycin. RNase mapping of TNF-α and γ-actin mRNA was carried out as described in the legend to Fig. 1. (Left) Positions of the TNF-α and γ-actin protected fragments. CsA or CHX plus ANIS have no effect on constitutive TNF-α mRNA expression (see Fig. 1, lanes 1 and 2 and 10 and 11).

Identification of Promoter Elements Necessary for Transcriptional Induction and CsA Sensitivity

TCR Stimuli. To identify the promoter elements required for the induction and CsA sensitivity of TNF-α gene expression, a human TNF-α 5′ promoter deletion series fused to the CAT reporter gene (described in reference 16) was trans-
Figure 3. (A) αCD3 induction of human TNF-α/CAT gene fusions in Ar-5 T cells. Autoradiogram shows results of CAT assays of extracts prepared from Ar-5 cells transfected with 5' deletion constructs of the human TNF-α/CAT reporter gene fusions diagrammed in the figure. Cells were transfected and mock induced (−) or induced with αCD3 (+) for 18 h in the presence or absence of CsA as indicated. CsA was added 10 min before αCD3. A promoterless CAT construct (Pocat) showed no activity. (Diagram) Positions of three NF-kB binding sites (k1, k2, and k3) relative to the deletion endpoints and the transcription start site. (B) A histogram depicting the percent conversion of [14C]chloramphenicol to its acetylated forms quantified by a beta-scope (Betagen, Waltham, MA) of the experiment displayed in (A). The experiment displayed is representative of four independent transfection experiments.
ed into Ar-5 cells and the cells were then stimulated with αCD3 in the presence and absence of CsA. This series of plasmids contains progressive deletions of each of the κB-like binding sites (κ1, κ2, and κ3) in the TNF-α promoter (diagram, Fig. 3 A), and thus can be used to evaluate the functional contribution of these sites to TCR-mediated activation and CsA sensitivity of the gene. The TNF-α CAT fusion construct containing -600 nt relative to the TNF-α mRNA cap site was highly inducible by αCD3 and this induction was effectively blocked when the cells were pretreated with CsA (Fig. 3 A, lanes 1–3). Deletion of κ1 which lies between the sequences spanning -600 and -576, or deletion of κ2, which lies between the sequences -242 and -199 nt, had little effect on the promoter's inducibility by αCD3 or its sensitivity to CsA (Fig. 3 A, lanes 4–12). Deletion of the sequences between -199 and -118 nt reduced the absolute values of both basal and induced CAT activity (Fig. 3 A, lanes 13–15). However, the promoter's inducibility is not affected by the deletion of these sequences, and the -118 deletion construct was still sensitive to CsA (see Fig. 3 B). We conclude that the sequences between -199 and -118 contain a positive regulatory region that is required for maximal levels of induction of a linked CAT reporter gene by αCD3.

In contrast, deletion of the sequences between -118 and -80 nt, which removes the κ3 element, almost eliminated αCD3 inducibility and CsA sensitivity of the TNF-α gene (Fig. 3 A, lanes 13–15, and see Fig. 3 B). Further deletion of the TNF-α promoter to -52 nt relative to the mRNA cap site had no additional effect (Fig. 3, lanes 19–21), indicating that the sequences lying between -52 and +89 nt relative to the TNF-α mRNA cap site are sufficient for basal activity and minimal inducibility. In conclusion, the sequences between -118 and -80 contain a CsA-sensitive promoter element that is involved in αCD3 inducibility of the gene.

Ca²⁺ Ionophore. The same TNF-α 5' promoter deletion series was tested in cells induced with calcium ionophore alone. As shown in Fig. 4, the -199 TNF-α CAT fusion construct was highly inducible by ionomycin and this induction was blocked by CsA (lanes 1–3). The level of induction with ionomycin was roughly equivalent to the level of induction obtained with αCD3 (compare Fig. 4, lanes 13–15 to lanes 1–3). As previously noted with αCD3, deletion of the sequences between -199 and -118 resulted in a marked decrease in the absolute levels of CAT activity, however, the decrease in activity was much more pronounced in the case of ionomycin induction than with αCD3 (compare Fig. 4, lanes 1–6 with Fig. 3 A, lanes 10–15). Nevertheless, the -118 construct was clearly induced by ionomycin treatment and this induction was blocked by CsA (Fig. 4, lanes 4–6). When the sequences between -118 and -80, which contain the κ3 site, were deleted, induction by ionomycin was no longer detectable (lanes 7–9). Deletion of the sequences between -600 and -199 had no effect on the ionomycin inducibility of the TNF-α promoter (data not shown). In conclusion, these results indicate that the sequences between -199 and +89 nt relative to the TNF-α start site mediate induction of the gene by both TCR ligands and calcium ionophore, and that the sequences between -118 and -80 are also required for inducibility and CsA sensitivity in the case of both inducers.

The κ3 Site Functions as an Inducible, CsA-sensitive Transcriptional Element In Vivo within the Context of the TNF-α Promoter.

The significant drop in inducibility and CsA sensitivity of the TNF-α promoter seen upon deletion of the sequences between -118 and -80 suggested that these sequences contained an αCD3 and calcium-inducible, CsA-sensitive promoter element. The region defined by this deletion contained the previously characterized κB-like binding site, κ3, and its surrounding sequences (spanning -106 to -87 nt relative to the TNF-α transcription start site) (18). To determine the importance of these sequences, (referred to as κ3 or the κ3 element throughout this paper), and to evaluate its in vivo function within the context of the TNF-α promoter during T cell activation, TNF-α promoter CAT fusion genes with base substitution mutations in the κ3 site were constructed. These mutations named 5’, middle, 3’, and 3’ flank are depicted in Fig. 5 C. They were created in the context of the -199 TNF-α promoter (WT) and are all isogenic except for the base changes noted.

TCR Stimuli. Mutation of any of the nucleotides within the NF-κB consensus motif of the κ3 site caused a reduction in the relative induction of CAT activity in response to αCD3 stimulation of transiently transfected Ar-5 cells. Mutations in the 5' or 3' ends of the site reduced the induction of CAT activity to ~28% (5' M) and 44% (3' M), respectively, of the level of induction seen with the WT plasmid (Fig. 5 A). The effect of mutating the middle nucleotides (MM), was more modest, decreasing induction to ~70% of WT levels. In contrast, mutation of the nucleotides flanking the 3' end of the κ3 site (3' flank M), did not decrease induction of CAT activity, but rather caused a slight increase in the average level of induction (~116% of WT induced levels). These results
containing mutations in the K3 or Spl sites (diagrammed in Fig. 5 A). The K3 site participates in CsA-sensitive induction of the human TNF-α promoter in Ar-5 cells. Cells were transfected with the WT and mutated TNF-α promoter/CAT fusion construct as described in Materials and Methods. (A) The K3 site is required for CsA-sensitive induction of the -199 TNF-α CAT fusion construct in response to αCD3. Inspection of the sequences between -61 and -27 n relative to the TNF-α cap site revealed an Sp1 binding motif (5'-CCCCGCC-3') between -53 and -46 n relative to the TNF-α transcription start site (16). To determine whether these sequences were involved in the regulation of the gene by αCD3, the core nucleotides of this site within the context of the -199 TNF-α gene promoter were substituted with the bases displayed in Fig. 5 C. Mutation of the Sp1 motif did not decrease basal or induced levels of CAT activity, but rather, resulted in an increase in the average levels of induction (~140% of WT levels). Therefore, the decreased inducibility and relative CsA sensitivity displayed by the 3'M, 3'M', and MM constructs was specific to these base substitutions.

**Calcium Ionophore.** Mutation of the k3 site had a much more pronounced effect on the ionomycin-mediated induction of the TNF-α promoter. As displayed in Fig. 5 B, mutations in either the 5' or 3' sequences of the k3 site (3'M and 3'M') essentially abrogated induction by ionomycin, whereas mutation of sequences in the middle of the k3 site (MM) significantly decreased inducibility to ~17% of WT levels. Mutation of the nucleotides flanking the 3' sequences of the k3 site (3' flanked M) had a greater effect on ionomycin-mediated induction than on αCD3-mediated induction, causing a decrease of ~30% in the absolute level of induction of CAT activity. Mutation of the Sp1 sequences did not decrease the inducibility or CsA sensitivity of the promoter, but rather increased induced levels of CAT activity as previously observed for αCD3. Thus, although promoter sequences in addition to k3 are involved in the induction of the -199 TNF-α promoter/CAT fusion by αCD3, k3 plays a major role in its CsA-sensitive induction in response to ionomycin.

**Multiple Copies of the k3 Promoter Element Confer CsA-sensitive Inducibility on a Minimal TNF-α Promoter**

To further characterize the function of the k3 element and to determine whether k3 could function as a discrete regulatory element, Ar-5 cells were transfected with a construct in which multimers of k3 were placed upstream of a minimal TNF-α promoter fused to CAT. The control construct containing the minimal TNF-α promoter (~61 to +89 n) was minimally inducible by αCD3 (approximately twofold) (Fig. 6 A, lanes 1 and 2). In contrast, a construct that contained six copies of the k3 element upstream of ~61 TNF-α CAT was clearly inducible by αCD3 (approximately sevenfold) and this augmented induction was blocked by CsA (Fig. 6 A, lanes 4–6). The k3 multimers also conferred ionomycin inducibility on the minimal TNF-α promoter (from ~3-15-fold) performed as in (A), except that the cells were induced with 1 μM ionomycin in the presence or absence of CsA as indicated. The results of three independent transfection experiments are shown. (C) A diagram of the base substitutions introduced into the k3 or Sp1 sites. (Boxes) The k3 and Sp1 sites.
fold) that was blocked by CsA (Fig. 6 B, lanes 4–6). In conclusion, this analysis indicated that k3 sequences are sufficient to mediate CsA-sensitive transcriptional induction in response to αCD3 or ionomycin. When the k3 multimers were fused to a heterologous promoter (-128 β-globin), they did not confer either inducibility or CsA sensitivity on the CAT reporter gene (data not shown). Therefore, the k3 element behaved in T cells as an αCD3, calcium-inducible, and CsA-sensitive transcriptional promoter element in the context of its own minimal TNF-α gene promoter.

Rapid Protein Synthesis-independent Induction of a CsA-sensitive k3 Binding Factor(s) in Stimulated T Cells

TCR Stimuli. To further characterize the k3 element and to test whether k3 could bind to an inducible, CsA-sensitive nuclear factor in stimulated T cells, nuclear extracts were prepared from Ar-5 cells that had been stimulated with αCD3 in the presence and absence of CsA for the various times indicated in Fig. 7 A. Using the k3 oligonucleotide spanning the sequences from -106 to -87 n relative to the TNF-α...
mRNA cap site in an EMSA, two inducible complexes (Fig. 7, upper and lower) were detected. These complexes were apparent within 15 min of activation by αCD3 (Fig. 7, lanes 1 and 3), and were maximally induced at 30 min after stimulation (Fig. 7, lane 5). By 4 h after αCD3 induction, the upper complex was barely detectable (Fig. 7, lane 11), whereas the intensity of the lower complex was significantly decreased (Fig. 7, lane 11). Induction of both inducible complexes was completely blocked at all time points by pretreatment of the cells with CsA (Fig. 7, lanes 4, 6, 8, and 10).

To determine whether induction of the k3 binding factor(s) required prior protein synthesis, nuclear extracts from Ar-5 cells that had been induced with αCD3 for various times in the presence and absence of protein synthesis inhibitors were tested. As shown in Fig. 7 B, de novo protein synthesis is not required for induction of k3 binding activity since cycloheximide and anisomycin did not prevent or delay the appearance of k3 binding activity after αCD3 induction (Fig. 7, lanes 3 and 4). We conclude that the k3 element specifically binds to an αCD3-inducible, CsA-sensitive, protein synthesis-independent factor(s) that is induced within minutes of T cell activation. Thus, the inducible binding characteristics of the k3 binding factor(s) are entirely concordant with the requirements for induction of TNF-α gene transcription.

As an internal control for specificity of binding and protein loading in the experiments displayed in Fig. 7, we tested the same nuclear extracts in an EMSA using an oligonucleotide spanning the sequences −61 to −26 n relative to the TNF-α mRNA cap site (−61 to −27 n) as a probe. As expected from the functional data demonstrating that these sequences were not primarily involved in the αCD3 CsA-sensitive induction of the TNF-α gene, the expression of a constitutive factor binding these sequences was not increased by αCD3, or affected by treatment with CsA or protein synthesis inhibitors (data not shown). Therefore, the inducible CsA-sensitive binding activity in these nuclear extracts was detected specifically using the k3 oligonucleotide as a probe. Furthermore, the SP1 binding motif that occurs within the sequences spanning −61 to −27 n was shown to be involved in the constitutive binding to the −61 to −27 n probe as an excess of nonradiolabeled wild-type oligonucleotide competed for binding, whereas an oligonucleotide containing a

Figure 8. Ionomycin induces the k3 binding complexes in Ar-5 nuclear extracts. Gel shift experiments were performed using the k3 and −61 to −27 n oligonucleotides as probes. (A) Ar-5 cells were mock (−) induced or induced with 1 μM ionomycin or 100 nM ionomycin as indicated for 30 min and nuclear extracts were prepared. Treatment with 1 μM ionomycin results in the appearance of the upper and lower k3 binding complexes (compare lanes 1 and 2). 100 nM of ionomycin results in the appearance of the lower complex but not the upper complex (lane 3). The −61 to −27 n probe binds to a constitutive factor that is not induced by ionomycin (lanes 4–6). (B) Ionomycin induction the k3 binding complex is blocked by CsA and does not require de novo protein synthesis. Nuclear extracts were prepared from Ar-5 cells that were mock induced (−), treated with CsA alone, or CHX/ANIS alone, or induced with 1 μM ionomycin in the presence or absence of CsA or CHX/ANIS as indicated. The nuclear extracts were bound to the k3 oligonucleotide or the −61 n to −27 n oligonucleotide as indicated.
The mutaion in the Sp1 site did not (data not shown). Thus, the sequences spanning -61 to -27 n specifically bind to 2). A lower concentration of ionomycin (100 nM) slightly for 15 min were also tested for the presence of g3 binding was confirmed by using the same extracts to bind to the - 61 induction by ionomycin.

Furthermore, ionomycin-mediated induction of the upper and lower k3 binding complexes was apparent in nuclear extracts from uninduced cells (Fig. 8, lanes I and 2). A lower concentration of ionomycin (100 nM) slightly induced the lower complex (Fig. 8, lane 3). The equivalence of protein loading and the specificity of ~c3 inducible binding was confirmed by using the same extracts to bind to the -61 to -27 n probe (Fig. 8, lanes 4-6).

Characterization of the k3 Binding Factor(s)
The k3 Binding Factor Is Distinct from NF-kB but May Resemble NFAT. To establish that the inducible k3 binding complexes bound specifically to the k3 sequence, the ability of nuclear extracts from stimulated T cells to bind to the radiolabeled k3 oligonucleotide in the presence of an excess of unlabeled k3 competitor oligonucleotide was tested. Unlabeled k3 oligonucleotide competed for binding to both the upper and lower complexes (Fig. 9, lanes 1-2), whereas oligonucleotides corresponding to the two other NF-kB consensus sites in the TNF-α promoter, x1 and k2, which are not involved in the induction and CsA sensitivity of the gene (see Fig. 3), did not compete for binding (Fig. 9, lanes 3 and 4).

Previous studies (16) employing human B cell nuclear extracts had demonstrated that k1, k2, and k3 specifically bound a complex that could be competed by an oligonucleotide matching the murine Ig k chain enhancer NF-kB site, but not by an oligonucleotide that was mutated in three guanine residues absolutely required for NF-kB binding. Therefore, whereas k1, k2, k3, and the Ig k chain enhancer NF-kB site are all capable of binding to the same protein in B cells, the k3 binding factor appears specific for the k3 site alone, consistent with the unique role of this site in the induction of the TNF-α gene in activated T cells. Subsequent studies established that k3 does not form any DNA-protein complex with purified p50/p65 NF-kB (data not shown), indicating that the k3 binding factor is distinct from the p50 homodimer or the p50/p65 NK-kB heterodimer. The k3 site can bind to proteins in cellular extracts from Ntera cells transfected with c-rel and p50 (54, 55), however, the complex formed migrates with different mobility from the k3 binding complex (data not shown). Moreover, antibodies to c-rel, p50, and p65 (56) do not react with the k3 binding complex detected in nuclear extracts from Ar-5 cells stimulated with αCD3 or ionomycin or from Ar-5 cytosolic extracts (data not shown). We conclude that the k3 binding complex in activated T cells does not contain c-rel, p50, or p65, and is thus distinct from NF-kB.

Other studies employing nuclear extracts from stimulated T cells showed that an NFAT-like factor bound to the Ig k chain enhancer NF-kB site, and this binding involved residues at the 3’ end of the kB site (49). To determine whether the k3 binding factor might be related to this NFAT-like factor, a competition analysis was done with Ig NF-kB and NFAT oligonucleotides. Oligonucleotides matching the wild-type Ig k chain enhancer NF-kB site (WT), and the NF-kB site from the IFN-β gene (PRDII) competed for binding of the k3 binding factor. However, an Ig NF-kB oligonucleotide that was mutated in three guanine residues absolutely required for NF-kB binding (27) also competed for binding to the k3 oligonucleotide (M3', Fig. 9, lane 7), whereas an oligonucleotide mutated in the 3’ residues was no longer able to compete for binding (M3', Fig. 9, lane 8). This pattern of competition was identical to that seen for the binding of the NFAT related factor to the Ig NF-kB site (49), and suggested that the k3 binding factor was related to NFAT. Consistent with this, an oligonucleotide matching the distal NFAT site from the murine IL-2 gene promoter competed for binding to the k3 binding factor (Fig. 9, lane 9).
Methylation Interference Analysis of the Upper and Lower κ3 Binding Complexes Suggests that the Same Sequences Required for In Vivo Activity Participate in the Formation of Both Complexes.

To determine whether the upper and lower κ3 binding complexes contracted different bases of the κ3 oligonucleotide, a methylation interference analysis using nuclear extracts from ionomycin-stimulated Ar-5 cells was performed. Both the 5' and 3' sequences of the site contacted DNA-binding proteins (Fig. 10). Specifically, methylation of the three guanine residues in the 5' end of the κ3 element (relative to the coding strand) substantially interfered with binding of the upper complex (Fig. 10, compare lane 2 with lanes 1 and 3), whereas methylation of two of the three guanines in the 3' end of the site eliminated binding of the upper complex (Fig. 10, compare lane 7 to lanes 6 and 8). For the lower complex, methylation of the same 5' and 3' nucleotides resulted in partial interference with binding (Fig. 10, compare lane 4 with lanes 3 and 5 and lane 9 with lanes 8 and 10). Similar results were obtained using nuclear extracts that had been stimulated with αCD3 (data not shown). Methylation also interferes with binding to sequences which flank the 5' nucleotides (see noncoding strand, ATGAG), indicating that these sequences may also be involved in the binding of the upper and lower complexes. In conclusion, the same 5' (TGGG) and 3' (CTCC) nucleotides that are crucial for the TCR- and ionophore-mediated induction of the TNF-α promoter are also crucial for the binding of the upper and lower κ3 binding complexes to the κ3 oligonucleotide. Moreover, this analysis indicates that both the upper and lower κ3 binding complexes bind with similar recognition properties to the κ3 oligonucleotide. These complexes may therefore contain the same inducible nuclear factor, and the difference in mobility between the two complexes may be due to differential modification and/or association with a different nuclear factor.

The κ3 Binding Factor Preexists in Unstimulated T Cells

The rapid induction of the κ3 binding factor and its independence from de novo protein synthesis suggested that it is present in unstimulated Ar-5 cells. Since specific κ3 binding activity was not detected in nuclear extracts from unstimulated Ar-5 cells (Fig. 7, A and B, lanes 1 and 2), cytosolic extracts from unstimulated cells were tested for κ3 binding activity. As shown in Fig. 11, the κ3 oligonucleotide specifically bound to a protein(s) in cytosolic extracts from unstimulated Ar-5 cells. The radiolabeled κ3 oligonucleotide formed two specific complexes that migrated similarly to those seen when nuclear extracts from stimulated T cells were used (compare to Figs. 7 and 9). Thus, the κ3 binding factor preexists in unstimulated Ar-5 cells, either sequestered in the cytosol or in an inactive form that can be extracted from the nuclei of unstimulated cells under our extraction conditions.

Furthermore, the level of κ3 binding factor proteins in the cytosolic extracts decreased by >65% after stimulation with ionomycin, and pretreatment of the cells with CsA blocked this decrease (Fig. 11 B, lanes 1–3). However, when we employed the −61 to −27 n probe and tested its binding to these extracts, we also detected a decrease in binding after induction by ionomycin (Fig. 11, lanes 4–6), whereas a probe matching the AP1 site from the murine collagenase gene bound a protein that did not decrease after induction (Fig. 11, lanes 7–9). The binding complexes formed by the −61 to −27 n and AP1 probes may represent proteins that are leached from the nucleus during the preparation of cytosolic extracts. This is commonly observed for a variety of nuclear proteins including rb (57) and ets proteins (58). Therefore, although...
Figure 11. (A) The κ3 binding factor is present in cytosolic extracts from unstimulated T cells. Unstimulated Ar-5 cells were lysed in hypotonic buffer containing NP-40, and the soluble proteins were concentrated by ammonium sulfate precipitation. After resuspension and dialysis, the proteins were assayed in an EMSA for binding to the κ3 oligonucleotide. Two specific complexes, designated upper and lower (arrows), bind to the κ3 oligonucleotide. The binding of these complexes was competed by an excess of unlabeled κ3 (lane 2), but not by κ1 or κ2 (lanes 3 and 4). (B) The κ3 binding factor decreases in cytosolic extracts from cells stimulated by ionomycin. Cells were unstimulated (UN) or stimulated with 1 μM ionomycin (+) in the presence or absence of CsA, and cytosolic extracts were prepared as described in (A). The proteins were assayed in an EMSA for binding to the κ3 oligonucleotide (lanes 1-3), the −61 to −27 n oligonucleotide (lanes 4-6) and the AP1 oligonucleotide (lanes 7-9).

we have demonstrated that the κ3 binding factor is detected in the cytosolic fraction of unstimulated T cells and appears rapidly in nuclear extracts upon stimulation, we cannot conclude definitively that there is a translocation of the κ3 binding factor from the cytosol to the nucleus in stimulated T cells. Immunofluorescence experiments using antibodies to purified κ3 binding protein will be required to resolve this question.

Discussion

Induction of the Endogenous TNF-α Gene in Murine T Cells. We have demonstrated that the TNF-α gene is one of the earliest genes induced in T cells activated by TCR ligands. TNF-α mRNA levels increase strikingly within 15–30 min of exposure of a murine T cell clone to activating antibodies against the TCR-CD3 complex. This increase reflects an increase in TNF-α gene transcription as judged by the commensurate increase in CAT activity in transient transfection assays using human TNF-α promoter CAT fusion plasmids. The transcriptional activation of the TNF-α gene is not dependent on de novo protein synthesis, since pretreatment of the cells with protein synthesis inhibitors has no effect on the kinetics or magnitude of TNF-α mRNA expression. Thus, in contrast to genes encoding most other lymphokines and lymphokine receptors, including IL-2, whose transcription is dependent upon de novo protein synthesis (for reviews see references 59 and 60), the TNF-α gene behaves as an immediate early gene in activated T cells.

Induction of the TNF-α gene in Ar-5 T cells is mediated by a calcium-dependent pathway since it occurs in T cells stimulated with calcium ionophore alone. Moreover, induction does not require the α and β isozymes of protein kinase C, since it occurs efficiently in cells that have been depleted of these isozymes by prolonged treatment with the phorbol ester PDBu (data not shown). These data again differentiate the induction requirements of the TNF-α gene from those of other activation-related genes such as IL-2, whose transcription requires both the protein kinase C and calcium-mediated signals (36, 61) and is abolished by protein kinase C depletion (46, and Goldfeld, A. E., P. G. McCaffrey, and A. Rao, unpublished observations). However, the induction of TNF-α gene transcription resembles the induction of other lymphokine genes in that it is completely blocked by the immunosuppressive agents CsA and FK506.

The κ3 Element Is an αCD3 and Ionophore-inducible Promoter Element in Activated Murine T-cells. The region between −118 and −87 n of the human TNF-α gene promoter contains a previously identified NF-kB consensus sequence, κ3. Site-directed mutagenesis of κ3 within the context of the −199 TNF-α promoter decreased but did not obliterate αCD3 in-
duction, suggesting that although k3 is a key element, other sequences participate in TCR-mediated induction of the TNF-α promoter. In contrast, ionomycin induction appeared to be primarily mediated through the k3 element as mutation of the 5' or 3' nucleotides almost entirely obliterated the promoter's inducibility. The specificity of this effect is confirmed by the observation that the mutation of other sequences within the promoter did not decrease the promoter's inducibility or CsA sensitivity.

A Preexisting k3 Binding Factor Is a Target for CsA. The protein(s) binding to the k3 element, the k3 binding factor, preexists in unstimulated T cells. Although its location cannot be definitively established in the absence of antibody reagents, it is absent from nuclear extracts of unstimulated T cells, but is present in cytosolic extracts from unstimulated T cells. This indicates that it either normally resides in the cytoplasm of unstimulated cells or that it is extracted from the nucleus of unstimulated cells under our extraction conditions. In either case, binding of the k3 binding factor to the k3 element in nuclear extracts requires a modification event that occurs upon T cell activation. It is rapidly detected (within 15 min) in nuclear extracts from T cells stimulated by aCD3 or ionomycin. Furthermore, induction of the k3 binding factor is completely concordant with induction of TNF-α gene expression itself: neither process requires de novo protein synthesis and both can be totally blocked by pretreatment with CsA. Taken together, these data suggest that TCR stimulation and the ensuing increase in intracellular calcium activate the k3 binding factor by causing its posttranslational modification and/or translocation to the nucleus, and that the nuclear k3 binding complex participates in TNF-α gene induction. The rapid disappearance of the k3 binding factor from nuclear extracts after induction may reflect its recycling back to the cytoplasm, inactivation, or degradation. The k3 element thus represents a novel cyclosporin-sensitive regulatory element and the first example characterized in a gene other than IL-2.

Relationship of the k3-Binding Factor to Other Transcription Factors. Despite the resemblance of the k3 element to an NF-κB binding site (16), the p50/p65 NF-κB heterodimer and/or c-rel are not part of the k3 binding complex. First, the k3 element does not bind to purified p50/p65 NF-κB in an EMSA. Second, the appearance of the p50/p65 NF-κB heterodimer in nuclear extracts of activated Ar-5 T cells is not inhibited by CsA at 30 min after activation, and is only partially inhibited at 2 h (49, and McCaffrey, P. G., and A. Rao, unpublished results). In contrast, the nuclear induction of the k3 binding complex is completely blocked by CsA in all time points tested. Third, an oligonucleotide that is mutated in the 5' guanines crucial for binding of the p50/p65 NF-κB heterodimer competes for binding to the k3 binding factor. Finally, antibodies to p50, p65, or c-rel have no effect on the mobility of the k3 binding complex. Thus, the k3 binding factor is distinct from p50/p65 NF-κB and does not contain c-rel.

The k3 binding factor is clearly distinct from the factors oct-1 and OAP-40 (44), which bind to the NFIL2A site of the IL-2 promoter. Although the induction of multimers of the NFIL2 site is blocked by CsA and FK506 (37), their maximal induction requires stimulation with both phorbol ester and ionophore, whereas the k3 multimer constructs are inducible by ionomycin alone. The oct-1 protein is constitutively expressed in nuclear extracts of unstimulated T cells and its binding is not affected by CsA or FK506 (37). Furthermore, the octamer-associated protein OAP-40, although inducible in a gel shift assay, requires protein synthesis for its induction (44).

The relationship of the k3 binding factor to the factor binding to the NFAT site of the IL-2 promoter remains to be elucidated. This factor, also termed NFAT, consists of a preexisting subunit (NFATp) present in cytosolic extracts of unstimulated T cells (41-43, 62), which associates with fos and jun proteins in the nuclei of activated T cells (42). The preexisting NFATp subunit resembles the k3 binding factor in that its appearance in nuclear extracts of activated T cells can be induced with calcium ionophore, does not require de novo protein synthesis, and is completely blocked by CsA (41-43, 62-64). Previous studies have shown that NFATp is a substrate for calcineurin, the serine/threonine phosphatase whose activity is inhibited by CsA-cyclophilin and FK506-FKBP complexes (43). The data presented here indicate that the k3 binding factor may also be a calcineurin substrate.

The NFAT site shares no clear sequence similarity with the k3 element, other than being pyrimidine rich. However, factors related to NF-ATp are capable of binding to several NF-κB sequence motifs (49), and we have shown that the murine IL-2 NFAT oligonucleotide is able to compete with k3 for binding. It is therefore possible that the k3 binding factor may be a member of a closely related family of transcription factors resembling NF-ATp, that undergo a shared calcium-dependent, CsA-sensitive posttranslational modification step. Alternatively, NFATp itself could be a part of the k3 binding complex.

In conclusion, the TNF-α gene represents a new model system for the study of those signal transduction events after T cell activation that results in CsA-sensitive inducible gene transcription. The protein product of the TNF-α gene cooperates in the differentiation and proliferation of B and T cells (4, 5, 65), the generation of dendritic Langerhans cells (66), and is capable of inducing latent HIV-1 into a productive infection (7, 67). Recent studies have demonstrated that TNF-α plays a critical role in superantigen-triggered lethal shock mediated by T cells (68), and elevated TNF-α levels are associated with renal and cardiac allograft rejection (69, 70) and several infectious diseases (71). Therefore, in addition to providing insight into the molecular events that follow lymphocyte activation, and mechanisms of inducible gene transcription, these studies could have a practical impact upon the modulation by immunosuppressants of TNF-α levels in transplant medicine, lymphoproliferative disorders, and other disease states.
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