Coordinate Transactivation of the Interleukin-2 CD28 Response Element by c-Rel and ATF-1/CREB2*

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The interleukin-2 CD28 response element (CD28RE) acts as a composite enhancer, in conjunction with a 3'-12-O-tetradecanoylphorbol-13-acetate response element (TRE)-like element, to confer CD28 receptor-dependent inducibility to the interleukin-2 promoter in T-cells. When inserted as a single copy upstream of a basal promoter, this composite enhancer, termed the CD28RE-TRE, is both highly active and CD28-inducible in transactivation assays. A multicomponent nuclear protein complex that binds the CD28RE-TRE was isolated by DNase I footprinting from nuclear extracts of mitogen- and CD28 receptor-costimulated human T-cells. Immunological and biochemical analyses of this complex reveal the presence of c-Rel, ATF-1, and CREB2 as major DNA-binding components. Coexpression of c-Rel in combination with ATF-1, CREB2, or ATF-1/CREB2 leads to synergistic transactivation of a CD28RE-TRE reporter plasmid in quiescent Jurkat T-cells. Furthermore, CD28-dependent transactivation of the CD28RE-TRE is specifically inhibited by cAMP response element-binding protein (CREB) dominant-negative expression vectors. Moreover, mutant promoter constructs in which the internal 5'-CD28RE and 3'-TRE-like sequences have been topologically positioned 180° out of phase with one another show loss of mitogen- and CD28-dependent inducibility. Finally, the addition of the CREB-binding transcriptional coactivator p300 leads to a dramatic CREB-dependent increase in both mitogen- and CD28-mediated transactivation of the CD28RE-TRE. These findings demonstrate that full physiological responsiveness to CD28 receptor stimulation in T-cells is dependent on topologically linked sequences within the CD28RE-TRE composite enhancer and provide strong support for a direct role for the CREB family of transcription factors and p300/CREB-binding protein coactivator proteins in cytokine gene induction during T-cell activation.

The extensive characterization of the molecular processes governing immune activation of T-lymphocytes has produced one of the most instructive and informative model systems in the field of signal transduction biology (1–3). A major area of interest to both signal transduction biologists and immunologists has been the elucidation of the mechanisms through which combined signals, derived from the T-cell surface, are integrated to achieve specific biological responses in the activated T-cell. In the case of T-lymphocyte immune activation, the generation of a fully functional immune response is dependent on the integration of dual signals that originate from cell-cell interactions between the antigen-reactive T-cell and the antigen-presenting B-cell or macrophage. The first of these signals is provided by the T-cell receptor, which makes contact with its target antigen in the context of the major histocompatibility complex type II receptor on the surface of the antigen-presenting cell. In cell culture systems, this first antigen receptor-dependent signal can be replaced pharmacologically by mitogenic combinations such as ionomycin and phorbol 12-myristate 13-acetate (PMA) or phytohemagglutinin and PMA (4). The second signal is derived from additional events that are triggered by receptor-mediated interactions with accessory molecules on the T-cell surface. One such accessory molecule is CD28, which interacts with its counter-receptors B7-1 and B7-2 on antigen-presenting cells (5–10). The interaction between the B7 counter-receptors and CD28 can be mimicked to a large extent by activating monoclonal antibodies (e.g. mAb 9.3) specific for the human CD28 receptor (11, 12). Together with mitogenic signals that mimic the occupied T-cell antigen receptor, the CD28 receptor agonists provide a costimulus that leads to full T-lymphocyte activation. Although CD28 stimulation alone is non-mitogenic and does not stimulate T-cells, inhibition or loss of CD28 function in the presence of antigenic stimulation results in blunting of T-cell activation and ultimately can lead to anergy (13).

A large part of these receptor-mediated responses are governed at the level of transcription and, consequently, are mediated by multiple transcription factors that regulate the expression of genes encoding various cytokines responsible for propagating the immune activated state. One such cytokine is the T-cell growth factor interleukin-2 (IL-2) (14). An enhancer within the interleukin-2 promoter that confers inducibility to CD28 costimulation was first identified as a 5'-AGAAT-TCCA-3' sequence located between positions –164 and –154, relative to the start of transcription (10, 15). Subsequent analysis of factors that can bind to and transactivate this sequence has identified a prominent role for the Rel family of transcription factors, in particular c-Rel and RelA, in CD28-mediated transcriptional regulation (16–18). More recently, there have been reports suggesting a role for other transcription factors in governing CD28 responsiveness, including NFAT (nuclear factor of activated T-cells), the high mobility group IV proteins, and an undescribed 35-kDa protein (19–21). These newer find-
ings clearly imply that the precise mechanisms through which CD28 responsiveness is mediated at the interleukin-2 promoter have yet to be elucidated.

This investigation was initiated with the hypothesis that the CD28RE may act physiologically as a composite element functionally linked to a non-consensus 3′-TPA response element (TRE, 5′-AGAGTCA-3′) located at positions −155 to −146. This non-consensus TPA response element (IL-2-TRE or NF-IL-2B) has been previously recognized as a site that is important for induction of AP-1-dependent transcriptional activity at the IL-2 promoter (22–24). This site, like other TREs, can bind in vitro to members of the basic leucine zipper (bZIP) class of transcriptional regulators, including the dimeric Fos/Jun AP-1 family (25, 26) and the related ATF/CREB family of proteins (27–29). The close juxtaposition of these CD28RE and TRE transcriptional regulators, including the dimeric Fos/Jun AP-1 transcription factors complexes. Henceforth, this composite element will be referred to as the CD28RE-TRE enhancer.

We propose that the CD28RE-TRE sequence, which spans positions −174 to −146 in the interleukin-2 promoter, can act individually as a single enhancer element to promote the transcriptional activation of the IL-2 gene. Evidence provided shows that a single copy of the CD28RE-TRE, when linked to a minimal basal promoter, can mediate a dramatically increased mitogen- and CD28-dependent transcriptional activation, which parallels the physiological regulation of interleukin-2 production. A DNA affinity-purified multicomponent complex containing c-Rel, ATF-1, and CREB2 was isolated from nuclear extracts of mitogen-anti-CD28 mAb-activated Jurkat T-cells and binds cooperatively to the CD28RE-TRE. Exogenous expression of c-Rel in combination with ATF-1, CREB2, or ATF-1/CREB2 leads to synergistic transactivation of the CD28RE-TRE in unstimulated quiescent T-cells. Furthermore, anti-CD28 mAb inducibility of the CD28RE-TRE requires phase-dependent alignment of the CD28RE and TRE sequences within the enhancer. CREB dominant-negative expression vectors inhibit endogenous mitogen/anti-CD28 mAb inducibility. Finally, we show that a near 100-fold induction of the CD28RE-TRE by enforced expression of the CREB-binding co-activator p300 is CREB-dependent. These findings establish that the CD28RE-TRE enhancer functions as a composite element in response to CD28 costimulation and provide compelling evidence for a prominent and direct role for the CREB family of proteins and p300/CREB-binding protein in the transcriptional activation of interleukin-2.

EXPERIMENTAL PROCEDURES

Materials—Dissopropyl fluorophosphosphate, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, Hepes, Tween 20, bovine serum albumin, PMA, forskolin, DNA-cellulose, and poly(dI-dC) were purchased from Calbiochem. Cyclosporine was from Sandoz. CD28 mAb inducibility of the CD28RE-TRE half-site (WB5/6), and 5′-AGCTAAGAGTCT-3′ and 5′-TCGAATGACTTCTCTT-3′ for the TRE half-site (WB7/8). Each duplex oligonucleotide contains a HindIII site at the 5′-end and a SalI site at the 3′-end. The oligonucleotides were annealed, phospho-rylated, and ligated as a single copy immediately upstream of the c-fos minimal promoter sequences in the Δ56-CAT vectors (30). The CREB1, CREB2, ATF-1, c-Fos, and p300 expression vectors (RSV-CREB, CM7-CREB2, RSV-ATF-1, pBRK2, and CMV-p300-E2, respectively) have been previously described (30–34). The IL-2-CAT (pI2-2-CAT) and TRE-CAT (pI2-2-CAT) reporter plasmids have been previously described (35, 36). The c-Rel expression vector and 4XCD28RE reporter plasmids (17) were a generous gift from Dr. Tse-Hua Tan (Baylor College of Medicine). CREB dominant-negative expression vectors encoding truncated (bZIP) and acidic extension-modified (aZIP) CREB constructs have been described (37–39).

Cell Culture and Transfection Assays—Jurkat T-cells were cultured in RPMI 1640 medium (Life Technologies, Inc.), 10% fetal calf serum, and 100 units/ml penicillin/streptomycin at 37 °C in 5% CO2. Cells (1–2 × 106) were resuspended in 250 μl of ice-cold phosphate buffer with 2.5–8 μg of reporter plasmid and 1–8 μg of the indicated expression vectors, followed by electroporation (200 V, 1180 microfarads). The cells were then resuspended in 5 ml of complete RPMI 1640 medium at 37 °C. Following transfection and resuspension, the cells were either unstimulated or stimulated with either 1 μg/ml PMA or 1 μg/ml ionomycin and 50 ng/ml PMA plus a 1:1000 dilution of mAb 9.3 as controls. All transfections studies were performed in duplicate (or more) in all cases and are representative of at least two independent experiments. CAT activity in cell extracts was determined as described (40). Quantitation of CAT activity was performed using a Molecular Dynamics PhosphorImager. Results are expressed as the mean ± S.E.

Affinity Purification of the CD28RE—A CD28RE-TRE DNA affinity column was generated by coupling 4 mg of 5′-amino-modified duplex oligonucleotide encoding the CD28RE-TRE sequence (see above) to 4 ml of CNBr-activated Sepharose 4B resin using a previously described procedure (41). Jurkat T-cells were grown to a final density of 8 × 107/ml in 8 liters of RPMI 1640 medium. The cells were then harvested and resuspended in a final volume of 2 liters of RPMI 1640 medium. The resuspended cells were stimulated, for 8 h, by the addition of 1 μg ionomycin, 50 ng/ml PMA, and a 1:1000 dilution of affinity-purified anti-CD28 mAb 9.3 (courtesy of Carl June, Naval Medical Research Institute). Following stimulation, nuclei were isolated, and nuclear extracts were prepared as described previously (42). All subsequent manipulations were performed at 4 °C. The nuclear extract (final volume 10 ml) was dialyzed against 4 liters of buffer containing 10 mM Hepes, 175 mM NaCl, 1 mM sodium azide, 0.05% Tween 20, 4 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin, pH 7.5 (buffer A). Following dialysis, the extract was passed over an 8-ml DNA-cellulose column at 10 ml/h. The eluate was collected and then loaded onto a 4-ml CD28RE-TRE oligonucleotide DNA affinity column at 3 ml/h. The column was then washed successively with 20 ml of buffer A containing 175 mM NaCl, 250 mM NaCl, and 600 mM NaCl. A protein peak was eluted from the column with buffer A containing 1.2 mM NaCl. This peak was assayed for CD28RE-TRE DNA binding activity and then stored at −80 °C. Under these conditions, the affinity-purified CD28RC was stable for at least 24 months with no loss in the quantity or quality of the DNA binding activity.

Antibodies—Antibodies against c-Rel, CREB2, ATF-4 (CREB2), and ATM-1 were obtained from Santa Cruz. The anti-CD28 mAb 9.3 ascites was obtained from Bristol-Myers Squibb Pharmaceutical Research Institute (Seattle, WA).

Electrophoretic Mobility Shift Assays—EMSA analysis and antibody supershift analysis were performed on 4 and 8% polyacrylamide gels at 24 °C as described previously (42). Competition analysis with duplex oligonucleotides was carried out with oligonucleotides that had been normalized for concentration by absorbance at 260 nm, followed by a final adjustment by densitometric scanning of the duplex oligonucleotides analyzed on ethidium bromide-stained gels.

Determination of Interleukin-2 Concentration—Interleukin-2 concentrations were determined by enzyme-linked immunosorbent analysis using the Quantikine interleukin assay kit (R&D Systems) according to the manufacturer’s instructions.

RESULTS

Mitogen- and Anti-CD28 mAb-costimulated Induction of the CD28RE-TRE Parallels the Transcriptional Activation of the
Interleukin-2 Promoter and the Induction of IL-2 Secretion in Jurkat T-cells—As has been shown previously, ionomycin/PMA-induced activation of interleukin-2 production and IL-2 promoter activity in Jurkat T-cells is further up-regulated by costimulation with anti-CD28 mAb (Fig. 1A) (10). This inducibility is similar to the transcriptional activation of the intact IL-2 promoter-chloramphenicol acetyltransferase gene reporter plasmid (IL-2-CAT) in transiently transfected Jurkat T-cells (Fig. 1B, left panel). The activity of the transfected interleukin-2 promoter is closely paralleled by the ionomycin/PMA and anti-CD28 mAb inducibility of a reporter plasmid containing a single copy of the CD28RE-TRE linked to a basal promoter (Fig. 1B). Interleukin-2 production, IL-2 promoter activity, and activation of the CD28RE-TRE enhancer were not induced by pretreatment of quiescent cells with anti-CD28 mAb alone (data not shown; see Refs. 5–10).

An examination of the CD28RE-TRE sequence demonstrates potential binding sites for numerous transcription factors (Table I).2 In an attempt to assess the possible role of specific transcription factors as modulators of CD28-responsive sequences, many earlier studies have relied heavily on experimental designs in which the candidate proteins were overexpressed at excessive levels in vivo or bound to DNA at excessive concentrations in vitro to approximate their potential physiological role in the intact cell. Although a useful approach to assess the gross biochemical feasibility of the involvement of specific factors at targeted cis-regulatory elements, this approach suffers from several limitations. The excessive levels of expressed protein in such experiments limit their interpretation due their extreme deviations from normal physiological conditions and their severe suprastoichiometric levels when compared with other potential endogenous partners, coactivators, or regulators. Moreover, the inherent redundancy of DNA binding sequence specificity within and between different transcription factor families obscures the identification of those

| CD28RE | GGGGTTTAAAGAAATTCCAAAGAGTCATCAGAAGAG |
| CD28RE consensus | GGGGTTTAAAGAAATTCCAAAGAGTCATCAGAAGAG |
| (8/10) xB | GGGGTTTAAAGAAATTCCAAAGAGTCATCAGAAGAG |
| (9/10) c-Rel | GGGGTTTAAAGAAATTCCAAAGAGTCATCAGAAGAG |
| (8/10) RelA | GGGGTTTAAAGAAATTCCAAAGAGTCATCAGAAGAG |
| (6/10) p50 | GGGGTTTAAAGAAATTCCAAAGAGTCATCAGAAGAG |
| (6/10) p50 | GGGGTTTAAAGAAATTCCAAAGAGTCATCAGAAGAG |
| (5/5) HMG a I/Y | GGGGTTTAAAGAAATTCCAAAGAGTCATCAGAAGAG |
| (4/5) Elf-1 | GGGGTTTAAAGAAATTCCAAAGAGTCATCAGAAGAG |
| (4/7) NFAT | GGGGTTTAAAGAAATTCCAAAGAGTCATCAGAAGAG |
| (6/7) AP-1/CREB | GGGGTTTAAAGAAATTCCAAAGAGTCATCAGAAGAG |

* HMG, high mobility group.

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2 Data in Table I were compiled from previous studies (17, 20, 21, 25, 64).

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**Fig. 1.** CD28-dependent transactivation of the CD28RE-TRE parallels induction of the intact IL-2 promoter and is similar to the physiological induction of interleukin-2 secretion in Jurkat T-cells. A, Jurkat T-cells (2 × 10⁷) were either unstimulated or stimulated with either ionomycin (ION)/PMA or ionomycin/PMA plus anti-CD28 mAb for 16 h. The supernatant was retained following centrifugation, and the levels of IL-2 were determined by enzyme-linked immunosorbent assay (see “Experimental Procedures”). B, following electroporation with 4 μg of either IL-2-CAT or CD28RE-TRE-CAT expression vector, Jurkat T-cells (2 × 10⁷) were stimulated as indicated for 16 h and assayed for CAT activity (see “Experimental Procedures”).

**Table I**

Potential transcription factor recognition sites within the CD28RE-TRE

| DNA binding consensus sequences were compiled from previous studies (see “Results”). Numbers in parentheses indicate matches with consensus sequences. |

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**Fig. 2.** A multicomponent affinity-purified complex (CD28RC) binds cooperatively to the CD28RE-TRE and makes extensive contacts with the CD28RE-TRE sequence. Left panel, EMSA analysis of the binding of 300 ng of affinity-purified CD28RC to radiolabeled CD28RE-TRE sequences. The three major complexes (A, B, and C) are indicated. Right panel, dose-response EMSA of the CD28RC with increasing amounts (36.5–300 ng) of purified protein.
factors that may truly be involved in transcriptional regulation at a specific site. These limitations also extend conceptually to those transfection studies that commonly use reporter plasmid vectors containing multimerized copies of the particular cis-elements under investigation. Even though this approach has and will continue to be a useful approach for the investigation of transcriptional activation, the superficial synergy engendered by the presence of tandem copies of test sequences in minimal promoters invariably results in reduced sensitivity to important aspects of regulation and protein specificity.

We have attempted to address these technical and conceptual limitations in transfection studies by using reporter plasmid constructs containing only a single copy of the target CD28RE-TRE sequences. To more accurately identify the possible candidates that may regulate the CD28RE-TRE under physiological conditions, we chose to biochemically analyze the components of a CD28RE-TRE-binding protein complex affinity-isolated from nuclear extracts of mitogen- and anti-CD28 mAb-costimulated Jurkat T-cells (Fig. 2). The natural derivation of this complex, from large-scale preparations of activated T-cell nuclear extracts, offers a more reasonable emulation of the in vivo state.

A DNA Affinity-purified Multicomponent Complex from Ionomycin/PMA- and Anti-CD28 mAb-stimulated Jurkat T-cells Binds Cooperatively to the CD28RE-TRE Sequence—A DNA-binding protein complex was isolated from ionomycin/PMA/anti-CD28 mAb-stimulated Jurkat T-cells by DNA affinity chromatography (Fig. 2). EMSA analysis of the affinity-purified CD28RC reveals the presence of three major complexes of varying mobility, designated A, B, and C (in order of decreasing electrophoretic mobility). Although the appearance of the C complex is variable and could represent aggregation, the differential increase in the formation of these complexes suggests that the B and C complexes may be assembled from more than one component.

FIG. 3. The CD28RE-TRE shows dependence on both the CD28RE and TRE regions for DNA binding affinity and transcriptional activation. A, sequences of the mutant CD28RE-TRE oligonucleotides used in the EMSA and transactivation studies. B, EMSA competition analysis of the binding of 100 ng of CD28RC to the radiolabeled CD28RE-TRE in the presence of either wild-type (WT) or mutant CD28RE-TRE sequences. The positions of the three major complexes (A, B, and C) are indicated. The asterisks indicate two bands that are not competed by wild-type oligonucleotides and represent nonspecific DNA-protein complexes. D, EMSA analysis of the formation of CD28RC complexes on the indicated radiolabeled mutant and wild-type CD28RE-TRE DNAs. The asterisks indicate the positions of visible supershifted complexes.
Fig. 4. The CD28RC contains c-Rel, ATF-1, and CREB2. A: left panel, DNA-bound CD28RC complexes were incubated with antibodies to c-Rel, ATF-1, and CREB2 prior to EMSA analysis (see “Experimental Procedures”). The arrows indicated supershifted antibody-DNA-protein complexes. Right panel, shown are the results from a control EMSA with antibody and the 32P-labeled CD28RE-TRE mixed in the absence of the CD28RC. B: left panel, ~400 ng of affinity-purified CD28RC was separated on an SDS-polyacrylamide gel (10% acrylamide), transferred to nitrocellulose, and stained with colloidal gold. Right panel, shown are the results from immunoblot analysis of 2–4 μg of affinity-purified CD28RC with anti-c-Rel, anti-ATF-1, and anti-CREB2 antibodies. The anti-CREB2 immunoblot was performed on the affinity-purified CD28RC following immunoprecipitation with rabbit polyclonal antibody specific for CREB2, followed by immunoblot analysis with anti-ATF-4/C/CREB2 mAb. Control immunoprecitpitation (IP) with nonspecific sera is also shown. The arrows indicate specific cross-reactive polypeptides that were specifically displaced by glutathione S-transferase-c-Rel or ATF-1-1-encoded peptide (data not shown) or specifically immunoprecipitated by anti-CREB2 mAb. The asterisk indicates the position of the IgG heavy chain. The plus sign indicates the position of polypeptide(s) that are cross-reactive with both monospecific anti-CREB1 and anti-ATF-1 antibodies and could therefore represent CREB1, phosphorylated ATF-1, or both (63). PAGE, polyacrylamide gel electrophoresis. C, shown are the results from Western blot analysis of 6 μg of a crude nuclear extract from ionomycin/PMA- and anti-CD28 mAb-stimulated Jurkat T-cells (starting material) and 6 μg of affinity-purified CD28RC with anti-c-Rel, anti-ATF-1, and anti-CREB2 antibodies (see “Experimental Procedures”).

8- and 9-base transversions in the CD28RE (mCD28RE-TRE) and TRE (CD28RE-mTRE) regions of the composite enhancer (Fig. 3A). Sequence mutations in either the CD28RE or TRE region impair specific competition of the A and B complexes (Fig. 3B). Direct comparison of the ability of the A and B complexes to form on wild-type versus mutant CD28RE-TRE sequences shows that the formation of the A complex has a strong dependence on the integrity of both the CD28RE and TRE regions of the enhancer (Fig. 3C). The formation of the B complex is completely abolished by a mutation in the TRE region of the enhancer sequence; however, a complex that comigrates with the wild-type B complex is still capable of forming on the enhancer sequence with a mutation in the CD28RE region. Thus, both the CD28RE and TRE regions of the CD28RE-TRE composite enhancer are important for sequence-specific DNA binding and formation of DNA-protein complexes formed on the CD28RE-TRE. Interestingly, as shown in Fig. 3E, results from a screen of inhibiting/supershifting antibodies showed that antibodies to c-Rel, ATF-1, and CREB2 caused significant inhibition of the B complex and a coincident formation of supershifted complexes when viewed after longer autoradiographic exposures (Fig. 4A). In contrast, the B complex formed on the mutant mCD28RE-TRE enhancer is inhibited by antibodies to c-Rel and ATF-1, but is not inhibited by anti-CREB2 antibodies. Thus, although a complex resembling the B complex does form on the mutant altered in the CD28RE region, it does not appear to be identical to the B complex formed on the wild-type enhancer.

Affinity-purified CD28RC Is Enriched in c-Rel, ATF-1, and CREB2—As suggested in Fig. 3E, analysis of the affinity-purified CD28RC by EMSA antibody supershift analysis consistently showed the formation of minor, although consistently reproducible antibody-supershifted complexes specific for c-Rel, ATF-1, and CREB2 (Fig. 4A, left panel). The supershifted complexes were seen in multiple experiments and did not appear in control EMSA analysis of mixtures of radiolabeled probe and antibody alone (Fig. 4A, right panel). Western blot analysis of the affinity-purified CD28RC shows the presence of c-Rel, ATF-1, and CREB2 cross-reactive polypeptides at approximate molecular masses of 75, 42–40, and 35 kDa, respectively (Fig. 4B). Moreover, each of these cross-reactive polypeptides is enriched severalfold in comparison to the crude nuclear extract (starting material) (Fig. 4C).

Exogenous Expression of c-Rel and ATF-1 in Quiescent Jurkat T-cells Induces Synergistic Transactivation of the CD28RE-TRE—Comparison of cotransfection of combinations of c-Rel with the c-Fos, CREB, or ATF-1 expression vector and the CD28RE-TRE-CAT reporter plasmid construct showed a higher activity and significantly higher synergy between c-Rel and ATF-1 (Fig. 5, compare normalized A–C). The c-Rel/ATF-1 combination induced transactivation of the CD28RE-TRE that was nearly 25-fold higher compared with c-Rel alone and more than 5-fold higher compared with ATF-1 alone. Mixtures of c-Fos and c-Rel show measurable synergy, but the total activity above c-Rel alone was much lower compared with c-Rel/ATF-1.

Exogenous Expression of CREB2 Alone or in Combination with c-Rel Also Induces Significant Up-regulation of CD28RE-TRE Transactivation—CREB2 also shows moderate synergy with c-Rel (Fig. 6A). Interestingly, single component dose-response curves with increasing amounts of CREB2 show significant transactivation of the CD28RE-TRE (Fig. 6B). This finding is quite provocative since prior studies have described that CREB2 acts as a repressor of transcription for cognate cyclic AMP response elements (33). Finally, combinations of c-Rel, ATF-1, and CREB2 act coordinately and synergistically to induce CD28RE-TRE transactivation nearly 30-fold above background levels (Fig. 6C).

CREB Dominant-negative Expression Vectors Selectively Inhibit CD28RE-TRE Transactivation—Two different dimer-specific CREB dominant-negative expression vectors were used to demonstrate that the physiological activation of the CD28RE-TRE requires CREB family members. The first dominant-neg-
active CREB was generated by truncation of the transactivation domain to leave only the DNA-binding bZIP domain of CREB (38). These bZIP-CREB constructs specifically dimerize with CREB family members including CREB1, CREB2, and ATF-1 to form dimeric hybrid DNA-binding complexes with reduced transactivation potential. Overexpression of these constructs leads to transcriptional repression through competitive quenching of transactivation. The second construct was a truncated CREB leucine zipper modified by an acidic coiled-coil extension (aZIP) in place of the basic DNA-binding domain (37–39). This acidic extension has DNA-mimetic properties and binds with high affinity to the basic DNA-binding domain of endogenous CREB. Dimers formed between these acidic extension leucine zipper constructs (aZIP-CREB) and endogenous CREB are stable hybrid complexes that are incapable of binding DNA and thus have no transactivation potential. Overexpression of the aZIP constructs produces a more avid and selective inhibition of sequence-specific transactivation (39).

Expression of both the aZIP and bZIP CREB dominant-negative constructs in Jurkat T-cells selectively inhibits mitogen/anti-CD28 mAb-induced transactivation of the CD28RE-TRE (Fig. 7A). As predicted, the aZIP-CREB dominant-negative construct is much more effective than the bZIP form. The inhibitory actions of the CREB dominant-negative expression vectors are specific since, although they repress transactivation from CREB-dependent cAMP response element sites (data not shown), they do not inhibit transactivation from an AP-1-dependent TRE expression vector (Fig. 7B). The slightly enhanced transactivation of the TRE by the bZIP vector reflects the lower specificity of the bZIP-CREB versions compared with the aZIP-CREB versions and is probably due to their ability to generate leucine zipper hybrids with AP-1 that retain intrinsic DNA binding activity.

CD28 Inducibility of the CD28RE-TRE Depends on Correct Topological Phasing of Its Internal CD28RE and TRE Sequences—A mutant CD28RE-TRE enhancer (WB3/4) was constructed in which the CD28RE and TRE sequences are separated by an intervening 5 bases that places each sequence 180° out of phase (on opposite faces of the DNA double helix). Transactivation from reporter plasmid constructs bearing this mutant shows nearly complete abrogation of CD28 responsiveness (Fig. 8A). Although, as previously published, an

**Fig. 5.** Enforced coexpression of c-Rel and ATF-1 produces synergistic transactivation of the CD28RE-TRE. A, Jurkat T-cells were cotransfected with 4 μg of CD28RE-TRE-CAT reporter plasmid and 1 μg of c-Rel expression vector (open bar), 4 μg of c-Fos expression vector (hatched bar), or both 1 μg of c-Rel expression vector and 4 μg of c-Fos expression vector (closed bar). B, shown are the results from cotransfection of 4 μg of CD28RE-TRE-CAT with 1 μg of c-Rel expression vector (open bar), 2 μg of CREB1 expression vector (hatched bar), or 1 μg of c-Rel expression vector plus 2 μg of CREB1 expression vector (closed bar). C, shown are the results from cotransfection of 4 μg of CD28RE-TRE-CAT with 1 μg of c-Rel expression vector (open bar), 2 μg of ATF-1 expression vector (hatched bar), or 1 μg of c-Rel expression vector plus 2 μg of ATF-1 expression vector (closed bar).

**Fig. 6.** CREB2 can potently transactivate the CD28RE-TRE and functions synergistically with c-Rel and ATF-1. A, Jurkat T-cells were cotransfected with 4 μg of CD28RE-TRE-CAT and 1 μg of c-Rel expression vector (open bar), 4 μg of CREB2 expression vector (hatched bar), or 1 μg of c-Rel expression vector plus 4 μg of CREB2 expression vector (closed bar). The relative CAT activity shown reflects activity induced above that produced by the reporter plasmid alone. B, Jurkat T-cells were cotransfected with the CD28RE-TRE-CAT reporter plasmid and increasing amounts (0–8 μg) of the CREB2 expression vector. CMV, cytomegalovirus. C, Jurkat T-cells were cotransfected with CD28RE-TRE-CAT and no additions (open bar), 1 μg each of the c-Rel and ATF-1 expression vectors (hatched bar), 4 μg of CREB2 expression vector alone (closed bar), or 1 μg each of the c-Rel and ATF-1 expression vectors plus 4 μg of CREB2 expression vector (cross-hatched bar).
expression vector containing four multimerized tandem copies of the CD28RE is responsive to both mitogen and mitogen/anti-CD28 mAb stimulation (17), the magnitude of its induction is still less than that produced by a single copy of the CD28RE-TRE. Furthermore, reporter plasmid constructs containing a single copy of either of the CD28RE-TRE half-sites (WB5/6 for CD28RE and WB7/8 for the TRE region) are uninducible. Finally, the phase-disrupted CD28RE-TRE (WB3/4) is also only minimally induced by the coexpression of c-Rel and ATF-1. Likewise, c-Rel and ATF-1 coexpression shows very little induction of the multimerized 4XCD28RE, which lacks the TRE sequences (Fig. 8B).

p300 Dramatically Up-regulates Anti-CD28 mAb-stimulated Transactivation of the CD28RE-TRE through CREB—The p300 protein is a member of the coactivator CREB-binding protein family that augments the transcriptional activation of numerous transcription factors by linking them to the basal transcriptional machinery through direct protein-protein interactions (43). As well as showing tight interaction with ATF/CREB family members, p300 has also been recently demonstrated to have binding sites for interactions with Rel family members (43, 44). The exogenous addition of p300 to Jurkat T-cells leads to a near 100-fold increase in transactivation compared with quiescent cells and increases mitogen-stimulated transactivation nearly 30-fold (Fig. 9A). The effect in CD28-stimulated cells is dose-dependent and is detectable with nanogram amounts of the p300 expression vector (Fig. 9B). p300 induction of the CD28RE-TRE is CREB-dependent since it is effectively inhibited by CREB dominant-negative expression vectors (Fig. 9C).

DISCUSSION

We have shown that CD28 inducibility is maximally conferred to the interleukin-2 promoter via a composite enhancer element formed by the linkage of the CD28RE with a 3'-TRE (also known as the NF-IL-2B site) that we refer to as the CD28RE-TRE. This composite enhancer is active in vivo in Jurkat T-cells when linked as a single copy to a minimal promoter. Furthermore, the inducibility of this element parallels the physiological induction of interleukin-2 production in...
response to mitogen and mitogen/anti-CD28 mAb stimulation. Characterization of a DNA affinity-purified complex that binds to the CD28RE-TRE revealed the presence of the transcription factors c-Rel, ATF-1, and CREB2. Exogenous expression of c-Rel in combination with ATF-1 or CREB2 shows synergistic transactivation of the CD28RE-TRE in quiescent T-cells. Moreover, inhibition of the physiological activation of the CD28RE-TRE can be achieved through cotransfections studies using dimer-specific CREB dominant-negative expression vectors. Full CD28 responsiveness of the CD28RE-TRE to physiological stimulation or the action of exogenous combinations of c-Rel and ATF-1 requires the presence and correct phasing of the CD28RE and TRE sequences within the CD28RE-TRE composite element. Finally, the mitogen/anti-CD28 mAb induction of the CD28RE-TRE is dramatically up-regulated by the CREB-binding coactivator protein p300.

Although a role for the Rel-related κB proteins as downstream targets in CD28 signaling has been well established (45–50), a direct role for the CREB family in CD28 responses and/or regulation of interleukin-2 signaling has not been reported. Recent transgenic mouse studies, in which CREB activity was functionally abrogated by the expression of a dominant-negative CREB, report a dramatic loss of interleukin-2 activity within the interleukin-2 promoter at the level of the transcription factors would be expected to exert a cooperative influence within the interleukin-2 promoter at the level of the CD28RE-TRE.

A precise understanding of the molecular nature of the protein complexes that bind the CD28RE-TRE in activated T-cells remains incomplete. Due to the inherent dimeric structure of the Rel and ATF/CREB family members, there is likely to be considerable heterogeneity in the composition of the linked complexes that form on the CD28RE-TRE. The precise stoichiometry of the components of the CD28RC is not known. DNA binding analysis of the CD28RC indicates that both the CD28RE and TRE regions of the enhancer contribute to the specific formation of DNA-binding complexes, and immunological data indicate that the integrity of the DNA-binding B complex is dependent on c-Rel, ATF-1, and CREB2 (Figs. 3E and 4A). Less is known about the biochemical nature of the A complex, although its specific formation correlates well with the transactivation potential of the wild-type enhancer (Fig. 3). Moreover, it cannot be determined at this time whether or not the major complexes seen by EMSA represent the CD28RC holocomplex formed on the CD28RE-TRE enhancer in solution or instead reflect more stable intermediates derived from the assembly or decay of a larger parent complex that does not survive electrophoresis. In view of these points, it would be premature to rule out possible added roles for other Rel or leucine zipper family members as participants in the cross-talk that mediates the transcriptional activation of the CD28RE-TRE.
It has been well established that CD28 costimulation of antigen- or mitogen-activated T-cells results in synergistic increases in interleukin-2 production (5–10). A complete evaluation of this observation requires a careful consideration of the various levels through which the intersection of numerous cellular processes and pathways can result in synergistic increases in interleukin-2 production. CD28 ligation by cellular receptors or activating antibody alone does not induce either IL-2 transcription or CD28 enhancer activation (5–10). Together with mitogen/antigen stimulation, the increased levels of IL-2 RNA are due both to activated transcription and to increased RNA stability (61, 62). This dual effect defines one level of synergy. The synergistic effects of CD28 costimulation on IL-2 RNA are due both to activated transcription and to increases in interleukin-2 production. CD28 ligation by cellular processes and pathways can result in synergistic in-...