Activation of Interleukin-2 Gene Transcription via the T-cell Surface Molecule CD28 Is Mediated through an NF-kB-like Response Element*

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Production of interleukin-2 (IL-2) by human T-lymphocytes can be augmented by costimulation via CD28. It has been reported that signaling via CD28 acts by stabilization of lymphokine mRNAs (Lindsten, T., June, C. H., Ledbetter, J. A., Stella, G., and Thompson, C. B. (1989) Science 244, 339–343). Here we demonstrate that costimulation via CD28 also provides a signal which activates transcription of the IL-2 gene. A CD28-responsive element (CD28RE) in the IL-2 enhancer at position −162 to −152 serves as a NF-κB enhancer motif. In vitro binding studies have demonstrated that the via CD28-induced signal synergizes with either phorbol myristate acetate or anti-CD3 for the induction of a nuclear factor that binds CD28RE and the human immunodeficiency virus (HIV-1) NF-kB motif. The significance of the sequence similarity of CD28RE with the NF-κB enhancer motif was demonstrated by cross-competition studies using un-labeled CD28RE, HIV-1 NF-kB binding site, and a mutated version of the NF-κB motif. In addition, we found that NF-κB-dependent reporter gene expression was induced by costimulation via CD28. These results indicate that besides an effect on lymphokine mRNA stabilization, stimulation via CD28 acts at the level of transcription via coinduction of an NF-kB-like activity.

Interaction of the T-cell receptor (TCR)-CD3 complex with the combination of antigen and self-histocompatibility molecules on the surface of antigen-presenting cells initiates a cascade of biochemical events, including activation of protein kinase C and an increase in intracellular calcium, followed by transcriptional activation of a variety of genes, among them the gene for IL-2 (1–4). Physiological triggering of the TCR-CD3 complex can be mimicked by binding the TCR-CD3 complex with anti-CD3 monoclonal antibodies in combination with phorbol myristate acetate (PMA). Besides the TCR a number of additional surface molecules have been implicated to control IL-2 production and T-lymphocyte activation. One of them is the homodimeric glycoprotein CD28 expressed on the surface of most mature T-cells (5, 6). Binding of anti-CD28 monoclonal antibody causes augmentation of IL-2 production and T-cell proliferation in conjunction with stimulation by monoclonal antibody directed against the TCR-CD3 complex (7–9). The B-cell activation antigen B7/BB1 has been identified as the natural ligand for CD28 (10, 11). Hence, T-cell activation is expected to be regulated via CD28 at sites of B-cell activation.

A number of observations indicate that the CD28 signal is different from the TCR-induced pathway (reviewed in Ref. 12). Recent work has shown that signaling via CD28 acts by stabilization of mRNAs for the lymphokines IL-2, interferon-γ, tumor necrosis factor-α, and granulocyte-macrophage colony-stimulating factor (13). Interestingly, the combination of anti-CD28 and PMA results in IL-2 production. Since the IL-2 gene is not transcribed in quiescent cells and neither anti-CD28 nor PMA alone induces detectable IL-2 gene transcription, the production of IL-2 is not likely to be accounted for by an effect on mRNA stability alone (14).

Therefore, we examined if CD28 costimulation also affects IL-2 gene transcription. Regulation of IL-2 gene transcription is controlled by a transcriptional enhancer extending from −52 to −319 relative to the transcriptional start site (15–17). Specific regulatory sequences within the IL-2 enhancer that bind the nuclear factors AP-1, NF-kB, Oct-1, and NFAT-1 have been implicated in regulation of IL-2 gene expression (reviewed in Ref. 18). We used the T-lymphoma Jurkat as a model for resting T-cells. Jurkat cells express TCR-CD3 and CD28 and respond to stimulation via these receptors (19–22).

In contrast to Lindsten et al. (13) who did not detect an effect on IL-2 gene transcription in peripheral blood T-cells we demonstrate that costimulation via CD28 increases transcription driven by the IL-2 enhancer/promoter. Furthermore, we describe a so far unidentified element at position −162 to −152 in the IL-2 enhancer that serves as a CD28-responsive element.

MATERIALS AND METHODS

Cell Culture, Transfection, and CAT Assay—The human Jurkat T-cell leukemia line was cultured in Iacove's medium supplemented with 5% human serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2·10−4 M β-mercaptoethanol. Transfection of Jurkat cells, 15 μg of DNA/1·10⁶ cells, was done as previously described (17). At 40 h after transfection cells were stained as indicated with monoclonal anti-CD28 (CLB-CD28/1) at 1 μg/ml (23), monoclonal IgE anti-CD3 (CLB-T3/4/E) at 1 μg/ml (23), or PMA at 5 ng/ml or combinations of these stimuli. Cells were harvested for assay of CAT expression 8 h after stimulation. CAT activity was determined by thin-layer chromatography (24) or by a mixed-phase assay (25).

Nuclear Extracts and Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared essentially as described by Ohlson and Edlund (26). Nuclear proteins were finally precipitated with 0.3 M (NH₄)₂SO₄ and dissolved in 50 mM Hepes, pH 7.8, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride,

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and 10% glycerol. Binding reactions contained 8 μg of nuclear extracts, 10 mM Tris-Cl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, and 5 μg of poly(dI-dC) and were incubated for 10 min at room temperature prior to incubation with about 5000 cpm of a 32P-end-labeled double-stranded oligonucleotide for 30 min at room temperature in a total volume of 15 μl. As probes we used CD28RE 5′GATCAGAAATTCCAAA3′ (-162 to -150) or HIV-1 KB 5′GAGGGAGCTTCCG3′ oligonucleotides. Complexes were separated on 4% polyacrylamide gels with 45 mM Tris borate, 1 mM EDTA, pH 8.0 buffer.

RESULTS

CD28 Costimulatory Activation of IL-2 Enhancer Activity—Disconnection of the IL-2 enhancer/promoter region from the IL-2 gene allows the analysis of transcriptional regulation of the IL-2 enhancer without interference of IL-2 mRNA stability effects. Therefore we used a construct termed “pIL-2/CAT” containing the IL-2 enhancer/promoter region from −319 to +47 linked to the CAT reporter gene in combination with the human T-lymphoma Jurkat (17). As anticipated, the combination of anti-CD28 and anti-CD3 led to IL-2 production by Jurkat cells although neither anti-CD28 nor anti-CD3 by itself was sufficient for this production (Fig. 1A). Furthermore, the CD28 signal up-regulates IL-2 production from cells stimulated with anti-CD3 and PMA.

Jurkat cells were transfected with pIL-2/CAT and subsequently stimulated with different combinations of reagents. In transfected cells treated with the anti-CD3 plus anti-CD28 CAT activity was 6-fold above levels induced by anti-CD3 or anti-CD28 alone (Fig. 1B). Stimulation with anti-CD3, PMA, and anti-CD28 together gave a 24-fold rise in CAT activity while the combination of anti-CD3 and PMA without anti-CD28 gave a 17-fold enhancement. No increase in CAT activity was measured for the Rous sarcoma virus enhancer/promoter upstream of the CAT gene in response to anti-CD28 costimulation which observation excludes nonspecific effects of anti-CD28 on CAT mRNA or protein (data not shown). From the synergy of anti-CD3 and anti-CD28 on IL-2 enhancer activity we conclude that stimulation via CD28 has an effect on IL-2 gene transcription.

Identification of a CD28-responsive Site within the IL-2 Enhancer.—To identify the CD28-responsive element within the IL-2 enhancer we tested a series of internal deletion/substitution mutants directing transcription of CAT (27) (Fig. 2). Jurkat cells were transfected with the mutant constructs and subsequently stimulated with anti-CD3 in the absence or presence of anti-CD28 (Fig. 2). Three of the mutants tested didn’t respond to costimulation with CD28. The deleted sequences in two of these mutants contained a functional binding motif for NFAT-1 (−279 to −263) and Oct-1 (−82 to −73), respectively. Since both the Oct-1 and NFAT-1 sites are known to be T-cell receptor-responsive elements the approach taken didn’t allow detection of an effect by anti-CD28 costimulation on CAT activity (18, 27, 28). However, we could exclude NFAT-1 and Oct-1 as CD28-responsive elements since anti-CD28 costimulation didn’t lead to increased expression of the CAT gene driven by three and four copies of the sequence from −290 to −263 (NFAT-1) (27-29) and −93 to −63 (Oct-1) (27), respectively (data not shown). The third mutant that didn’t respond to CD28 costimulation has the sequence 5′AATTCAGAC3′ (from −159 to −151) replaced by the XhoI linker sequence 5′CCTGAGAC3′.

Induction of an NF-κB-like Sequence-specific DNA Binding Activity upon Costimulation via CD28.—The region from −159 to −151 is part of the sequence 5′AGAAATTCCAC3′ (−162 to −152) which reads on the complementary strand 5′TGGAATTTTC3′. Comparison of this sequence with binding

![Fig. 1. Effects of stimulation via CD28 on IL-2 production and IL-2 enhancer activity by Jurkat cells. A, Jurkat cells were cultured at 6.7 × 10⁶ cells/ml in the presence of medium alone, soluble monoclonal IgE anti-CD3 (CD3), soluble monoclonal anti-CD28 (CD28), PMA, or combinations of these stimuli. After 8 h supernatant was harvested, and IL-2 production was quantitated using the method described by Gillis et al. (36). Data presented are the mean of three independent experiments. B, Jurkat cells were transiently transfected with pIL-2/CAT and stimulated as described above. The cells were then harvested, and CAT activity was measured in cell lysates incubated for 15 h at 37°C (24). Relative CAT activity is the mean of three independent determinations of percent acetylation relative to the medium control. Error bars indicate the standard error of the mean.](image1)

![Fig. 2. Identification of a CD28-responsive element by analyzing IL-2 enhancer mutants on their ability to induce CAT activity upon costimulation via CD28. Jurkat cells were transfected as described in Fig. 1B. The IL-2 enhancer with the known functional protein-binding sites is presented schematically. The transfected constructs had the IL-2 promoter/enhancer and variants thereof containing internal deletions linked to the CAT gene (27). Each pool of transfected cells was divided into two groups which were stimulated for 8 h with anti-CD3 or a combination of anti-CD3 and anti-CD28, respectively. The factor of stimulation is the CAT activity induced by the combination of anti-CD3 and anti-CD28 divided by the activity induced with anti-CD3 alone. Data are the mean ± S.E. from three independent experiments.](image2)
Regulation of IL-2 Gene Transcription via CD28

The latter deviant is also present in the murine IL-2 receptor-α and tumor necrosis factor-α NF-κB motifs (30, 31). To examine if the sequence from -162 to -152 is involved in binding with anti-CD28-inducible nuclear proteins, we performed gel mobility shift assays with a double-stranded oligonucleotide containing the sequence from -162 to -150, called CD28RE, which stands for CD28 response element (Fig. 3A). Inducible electrophoretically retarded DNA-protein complexes were detected with nuclear extracts from Jurkat cells treated for 8 h with anti-CD3 plus anti-CD28. A similar result was obtained with nuclear extracts from cells stimulated with PMA in combination with anti-CD28. No complexation was observed with extracts from cells stimulated with anti-CD28, anti-CD3, or PMA alone.

Next we used as probe an oligonucleotide containing the 3′-NF-κB binding sequence in the HIV-1 LTR which is identical to the NF-κB binding site in the k-light chain enhancer. As noted earlier this sequence in the LTR has been shown to interact with members of the NF-κB family (30, 32). In accordance with others we observe a one-signal requirement for induction of nuclear factors binding this motif. In similarity with the CD28RE probe anti-CD28 synergizes with either anti-CD3 or PMA for induction of binding activity analogous with the combination of PMA and anti-CD3 (not shown).

To examine whether the sequence similarities between the HIV-1kB motif and CD28RE were functionally significant cross-competition studies were performed using nuclear extract from cells stimulated with anti-CD28 and anti-CD3 and 32P-labeled CD28RE and HIV-1kB oligonucleotides as probes, and unlabeled CD28RE, HIV-1kB, and an oligonucleotide containing a mutated NF-κB binding motif were added as competitor. As shown in Fig. 3B a 500-fold molar excess of HIV-1kB sequences blocked protein binding to CD28RE. In contrast, oligonucleotides containing a mutated NF-κB binding motif had no effect on the complex formation. CD28RE oligonucleotides were similarly tested for inhibitory effects on the binding of inducible nuclear proteins to 32P-labeled HIV-1kB. Competition with a 500-fold molar excess of unlabeled CD28RE almost completely inhibited the formation of 32P-labeled HIV-1kB-protein complexes. No effect on DNA-protein interaction was observed upon addition of the mutated NF-κB oligonucleotide as competitor. Taken together these results indicate that the NF-κB sequence similarity proved significant and that proteins interacting with CD28RE are also involved in binding the HIV-1kB motif and vice versa.

To compare binding affinities of the via CD28- and CD3-induced factor to the CD28RE and HIV-1kB oligonucleotides we performed competition titration experiments with graded amounts of CD28RE, HIV-1kB, and mutant NF-κB oligonucleotides as competitor (Fig. 3B). These competition studies indicate that the induced DNA binding activity has a preference for binding the HIV-1kB motif relative to the CD28RE.

**Induction of NF-κB Transcriptional Activity via CD28**—We then examined the ability of anti-CD28-induced factors to activate transcription directed by NF-κB binding sites. For this purpose we used a construct designated HIV-κb/CAT that is dependent on NF-κB for activation of transcription of the CAT gene (33). Stimulation with anti-CD28 was shown to activate NF-κB-dependent transcription (Fig. 4). Anti-CD28 by itself gave a 3-fold increase in CAT activity compared with control levels induced by medium alone. Stimulation with anti-CD28 plus anti-CD3 resulted in a 2.8-fold enhancement in CAT activity compared with levels induced by anti-CD3 alone. Furthermore, we see that anti-CD28 costimulation increases NF-κB transcriptional activity induced by either PMA or PMA plus anti-CD3 2.7- and 1.3-fold, respectively. These effects on NF-κB transcriptional activation are in accordance with the effects of these stimuli on binding activity (Fig. 3A).

**Discussion**

In the present study we have demonstrated that signaling via CD28 induces IL-2 enhancer activity in the T-cell lymphoma Jurkat. Evidence that this mechanism also applies for peripheral T-cells is provided by the observation that anti-CD28 synergizes with PMA to induce IL-2 gene expression in

**Fig. 3. Inducibility of an NF-κB-like binding activity in Jurkat cells stimulated via CD28.** A, electrophoretic mobility shift assays were performed with nuclear extracts from unstimulated Jurkat cells (lanes 2 and 9), Jurkat cells stimulated for 8 h with anti-CD28 (lanes 3 and 10), anti-CD28 plus PMA (10 ng/ml, lanes 4 and 11), PMA alone (lanes 5 and 12), anti-CD28 plus anti-CD3 (lanes 6 and 13), and anti-CD3 (lanes 7 and 14). As probes we used end-labeled CD28RE (lanes 1–7) and HIV-1 kB (lanes 8–14). B, binding reactions with nuclear extracts from PMA plus anti-CD28-stimulated Jurkat cells performed with graded amounts of either unlabeled HIV-1kB, CD28RE, or mutant NF-κB oligonucleotides. The mutant oligonucleotide has the sequence 5′GACTCTTGTTCGCAAGC3′ (GGG to CTC substitution). As probes we used 32P end-labeled HIV-1kB (top) and CD28RE (bottom). Only the retarded protein-DNA complexes are shown.
peripheral T-cells. Since neither of these stimuli alone induces IL-2 gene transcription an enhancement of the IL-2 mRNA half-life is not sufficient to account for the IL-2 expression (14). However, Lindsten et al. (13) were not able to detect an effect on IL-2 transcription upon anti-CD28 stimulation in a run-on transcription assay using peripheral T-cells. We believe that the effect on transcription was not detected because of the rather short time (i.e. 3 h) after stimulation that was chosen to isolate the nuclei. This explanation is supported by the finding that the via CD28-induced IL-2 enhancer activity is at the earliest detectable at 4 h after stimulation (data not shown). In accordance with this observation June et al. (12) reported that at 6 h after stimulation of peripheral T-cells IL-2 mRNA levels appear to be enhanced by a CD28-dependent increase in transcription as well as mRNA stability. Stimulation via CD28 was shown to synergize with activation of PKC for the induction of an activity that binds the thymidine kinase promoter directly directed transcription of the CAT gene. Cells were stimulated as described in Fig. 1. CAT activity was determined by a mixed-phase assay (25). The stimulation via CD28 didn't affect the ability of the thymidine kinase promoter to direct the CAT expression. Relative CAT activity is the mean of three independent determinations of CAT activity expressed relative to the medium control. Error bars indicate the standard error of the mean.

NF-kB is explained by superinduction of an NF-kB-like activity.

The observed preference for binding the HIV-1kB motif relative to CD28RE might be an obvious consequence of small differences between the two sequences. However, it cannot be excluded that heterogeneity in NF-kB is in part responsible for this observation. Recently, Greene and co-workers (32) have demonstrated that NF-kB consists of a family of at least four inducible and differentially expressed binding proteins in human T-cells. Some of the proteins showed a preference for binding with particular NF-kB motifs. Currently we are characterizing the proteins constituting the via CD28-inducible HIV-1kB protein and CD28RE-protein complexes.

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