Functional Disparity of Distinct CD28 Response Elements toward Mitogenic Responses*

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Activation of T cells through the antigen-specific T-cell receptor in combination with a costimulatory signal results in efficient cytokine gene transcription. The CD28-induced signal represents a major costimulatory signal for T cells. A CD28 response element, named CD28RE, was first identified in the interleukin-2 (IL-2) promoter region. Here we demonstrate that the NF-κB sequence in the IL-6 promoter functions as a CD28 response element. Mutations in this sequence rendered the IL-6 promoter unresponsive to CD28 costimulation. Moreover, this element could replace the IL-2 CD28RE in conferring CD28 responsiveness to the IL-2 promoter. In analogy to the known CD28 response elements IL-2 CD28RE, IL-8 CD28RE, and the human immunodeficiency virus-1 (HIV-1) NF-κB motif, the IL-6 NF-κB motif efficiently bound c-Rel, c-Rel/NFκB1, and the recently identified inducible T-cell factor NF-MATp35. However, the IL-6 NF-κB sequence together with the IL-8 CD28RE and HIV-1 NF-κB sequence differed from the IL-2 CD28RE in the binding of NF-κB/Rel family proteins. Although the IL-2 CD28RE exerted selective binding with c-Rel and c-Rel/NFκB1, the other CD28REs allowed efficient binding of a wide range of NF-κB/Rel family proteins. The difference in binding specificity correlated with the capacity of the distinct CD28 response elements to function in the context of the IL-6 promoter in response to T-cell activation. Domain swapping experiments revealed that the IL-8 CD28RE and HIV-1 NF-κB motif conferred similar responsiveness as the genuine IL-6 NF-κB motif in the transcriptional activation of the IL-6 promoter upon CD28 costimulation. In contrast, replacement of the IL-6 NF-κB sequence by the IL-2 CD28RE motif strongly reduced the responsiveness of the IL-6 promoter. These data indicate that despite the sequence similarity, two different classes of CD28 responsive elements exist that differ in their NF-κB binding capacity and the ability to confer CD28 costimulatory responsiveness toward a heterologous promoter.

Activation of T cells through the antigen-specific T-cell receptor in combination with a costimulatory signal results in the coordinate expression of a number of cytokine genes (1). The CD28 receptor represents a major costimulatory molecule present on most T cells (2, 3). Two ligands for CD28, i.e. CD80 and CD86 expressed on antigen-presenting cells, have been identified (2, 3). The combined triggering of the T cell receptor in combination with CD28 stimulation leads to T cell proliferation and a high level of cytokine production. A number of cytokines, among them IL-2, 1 IL-3, IL-6, IL-8, interferon-γ, and GM-CSF are being produced upon CD28 costimulation (4–7). The coordinate production of these cytokines is a determining factor in the control of cell proliferation and differentiation and the functioning of lymphoid cells and nonlymphoid cells during an immune response.

The mechanism by which CD28 regulates the production of cytokines by T cells involves activation of cytokine gene transcription (6–11) and stabilization of mRNA (12). We and others have demonstrated that costimulation of T cells through CD28 in combination with T cell antigen receptor triggering enhances IL-2 enhancer activity 5- to 6-fold (8–10). The increase in IL-2 gene transcriptional activity was shown to require a sequence between nucleotides −162 and −153 within the IL-2 promoter named CD28 response element (CD28RE) (8–10). Until now uncertainty exists about proteins that are involved in the IL-2 promoter activity mediated via CD28RE. The observation that the CD28RE sequence revealed 80% similarity to the consensus NF-κB binding sequence prompted speculation about involvement of NF-κB/Rel family proteins in CD28-mediated IL-2 gene transcription (9). Evidence exists that NFκB1, RelA, and c-Rel can interact with CD28RE (13–15). Analysis of c-Rel knockout mice suggested a critical role of c-Rel in CD28-induced IL-2 gene regulation (16). Binding of high mobility group protein HMG-I (Y) is believed to be essential for function and c-Rel binding to the CD28RE (17). There is also evidence that the CD28RE represents a new nuclear factor of activated T cells site (15, 18, 19). More recent studies have also shown a role for other transcription factors in governing CD28 responsiveness, including ATF-1 and CREB2 (20). In addition, we and others have reported that the predominant protein species that appeared after UV-cross-linking was an approximately 35-kDa protein, which is clearly different from the known NF-κB/Rel family members (7, 21). This apparently novel inducible protein was called NF-MATp35 (nuclear factor of mitogenic-activated T cells). Similar to induction of IL-2 production, induction of NF-MATp35 was dependent on de novo protein synthesis and required a mitogenic combination of T cell stimuli, not necessarily including CD28 signaling. Induction of NF-

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1 The abbreviations used are: IL, interleukin; CD28RE, CD28 response element; NF-MAT, nuclear factor of mitogenic-activated T cells; luc, luciferase; CAT, chloramphenicol acetyltransferase; BrdU, bromodeoxyuridine; GM-CSF, granulocyte-macrophage colony-stimulating factor; HIV, human immunodeficiency virus; LTR, long terminal repeat; wt, wild type.
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Results and Discussion

In this study, we now show that the NF-κB motif within the IL-6 promoter functions as a CD28 response element.

Together, the known CD28REs (Table I) are conserved and display only limited sequence heterogeneity. In favor of a functional relevance of their sequence variation, comparative analysis of the different CD28REs revealed that the IL-6 NF-κB sequence together with the IL-8 CD28RE and HIV-1 NF-κB motif differ from the IL-2 CD28RE in the binding of NF-κB/Rel proteins. The differences in binding specificity correspond with functional disparity of the distinct CD28REs in the context of the IL-6 promoter. These data provide evidence for the existence of two functional classes of CD28REs.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The wild-type IL-6 promoter-luciferase plasmid (wt-pIL6luc) and a variant IL-6 promoter construct containing mutant NF-κB sequence (5'-AATATTTCGCC-3') (pIL6(-κB)luc) were kind gifts of Dr. Shizuo Akira and have been described elsewhere (28). Site-directed mutagenesis of the NF-κB site in the IL-6 promoter was carried out using a two-step PCR mutagenesis technique. The NF-κB site (GGGTATTTCGCC) in the IL-6 promoter was replaced by the TGGGATTTTCCC sequence instead of the TGGGATTTTCCC sequence (5'-gatcTTTGGAATTTCTT-3'). These constructs were designated as pIL6(HIV-1NF-κB)luc, pIL6(IL6CD28RE)luc, and pIL6 (IL8CD28RE)luc, respectively. The pIL2(IL6NF-κB)CAT construct was constructed by insertion of the IL-6 NF-κB sequence instead of the CD28RE site in the IL-2 promoter (29).

Cell Culture—The human T-cell leukemia line Jurkat was cultured in Iscove’s medium supplemented with 10% fetal calf serum, 50 μM β-mercaptoethanol, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Cells were cultured at a density of 0.7 × 10^6 cells/ml as indicated and dissolved in 50 mM HEPES, pH 7.8, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol. Nuclear proteins were finally precipitated with (NH₄)₂SO₄ (0.3 g/ml) and dissolved in 50 mM HEPES, pH 7.8, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol. Binding reactions containing 4 μg of nuclear protein, 10 μl Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, and 2.5 μg of poly(dI-dC) were incubated for 10 min at room temperature before incubation with about 10,000–50,000 cpm of a 32P-labeled double-stranded probe for 30 min at room temperature in a total volume of 15 μl. As probes we used IL-6 NF-κB (5'-gatcTGG-GATTTCGCC-3'), IL-8CD28RE (5'-gatcTGGGATTTTCCC-3'), IL-2CD28RE (5'-gatcTGGGATTTTCCC-3'), NF-κB palindromic (5'-gatcGGGACGTCCC-3'), HIV-1 NF-κB (5'-gatcGGGACGTCCC-3'), and mutant NF-κB oligonucleotides. The mutant oligonucleotide has the sequence 5'-GACTCAATTTCACC-3' (GGG to CTC substitution).

Fig. 1. Activity of wt and variant IL-2 and IL-6 promoters in Jurkat cells upon T-cell activation. Jurkat cells were transiently transfected with either wt-pIL6luc, mutant pIL6(-κB)luc (A), wt-pIL2CAT, or the mutant pIL2(IL6NF-κB)CAT (B). After 40 h, the cells were harvested, and the luciferase or CAT activity was determined. The MATp35 was also sensitive to cyclosporin A. Hence the CD28REs can be viewed as composite binding elements that interact with at least six factors, i.e. c-Rel, HMG-I (Y), NF-AT, ATF-1, CREB2, and NF-MAT, which have been implicated in mediating the CD28-induced transcriptional response.

Besides the IL-2 gene, other cytokine genes, including the ones encoding IL-3, IL-8, interferon-γ and GM-CSF, and the HIV-1 LTR, were shown to be transcriptionally regulated by CD28 signal transduction (6, 7, 9). Functional studies revealed that the regulatory regions of these cytokine genes and the HIV-1 LTR contained NF-κB-like sequences resembling the IL-2 CD28RE sequence that served a role as CD28 response elements (6, 7, 9). An NF-κB consensus binding sequence has also been found in the promoter region of the IL-6 gene (22–27), suggesting that this cytokine might be regulated in a similar way in response to CD28 costimulation. In this study, we now show that the NF-κB motif within the IL-6 promoter functions as a CD28 response element.
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The nucleotide sequence changes in the IL-2 and IL-6 promoters are indicated in boldface.

| Constructs | IL-2 promoter from −152 to −162 | IL-6 NF-κB (−72 to −62) |
|------------|--------------------------------|-------------------------|
| wt-pL2CAT  | pIL2(IL6NFκB)CAT 5’-TGGGATTCTCTC-3’ |                           |
| pIL6-κB    |                                  | gGgCgTTTCCGc-3’           |
| pIL6(CD28RE)κB |                              |                           |
| pIL6HIV-κB |                                  |                           |

The nucleotide sequence changes in the IL-2 and IL-6 promoters are indicated in boldface.

Oligonucleotides were synthesized on a DNA synthesizer (Millipore Expedite nucleic acid synthesis system). The following probes were used (thymidines tagged with an asterisk were substituted by bromodeoxyuridine):

| Promoter | Sequence |
|----------|----------|
| IL-6 NF-κB | 5’-gatcTTGGGATCTCTTGCGc-3’ |
| IL-8CD28RE | 5’-gatcTTGGGATCTCTTGCGc-3’ |
| IL-2CD28RE | 5’-gatcTTGGGATCTCTTGCGc-3’ |
| HIV-1 NF-κB | 5’-gatcTTGGGATCTCTTGCGc-3’ |

Transfection and CAT and Luciferase Assay—Jurkat T cells were transfected with 10 μg of DNA/10⁷ cells as described previously (29). At 48 h after transfection cells were stimulated for 6 h. Subsequently, cells were harvested, and CAT (31) or luciferase activity was determined. The plasmid pRSV/luciferase (29) was co-transfected with pL2CAT or pL2(IL6NFκB)CAT constructs to control for transfection efficiency. Cells transfected with the pIL6uc construct or the pIL6uc construct containing HIV-1 NF-κB, IL-8CD28RE, or IL-2CD28RE sequences instead of the IL-6 NF-κB were harvested and resuspended in 50 μl of 0.1 M sodium phosphate buffer, pH 7.8, containing 1 mM dithiothreitol and lysed by three cycles of freezing-thawing. Luciferase activity was measured using a Lumat model LB9501 luminometer. The plasmid pCMVCAT was cotransfected with the luciferase constructs to control for transfection efficiency. Transfections were performed at least two times, and representative results are shown here.

RESULTS

Identification of a CD28RE within the IL-6 Promoter—Transient transfection studies in Jurkat T cells using the promoter region of the IL-6 gene (−225 to +13) directing the expression of the luciferase reporter gene demonstrated that the IL-6 promoter is responsive upon CD28 costimulation in combination with anti-CD3 (Fig. 1A). Mutation analysis revealed that the NF-κB site at position −75 to −63 was required for CD28-induced activation of the IL-6 promoter (Fig. 1A).

To examine whether the IL-6 NF-κB sequence was able to confer CD28 responsiveness to the IL-2 promoter, we replaced the CD28RE sequence of the IL-2 promoter with the IL-6 NF-κB sequence (Table I). Transfection studies with pIL6(IL6NFκB)CAT revealed that IL-6 NF-κB motif, in analogy to the endogenous CD28RE motif, was able to confer constitutary activity to the IL-2 promoter.

Binding of NF-κB/Rel Family Proteins to Distinct CD28REs—Using electrophoretic mobility shift assay we compared the ability of the IL-6 NF-κB sequence, the HIV-1 NF-κB sequence, and known CD28 response elements, i.e. IL-2 CD28RE and IL-6 CD28RE, to form complexes with recombinant NF-κB/Rel proteins expressed in COS cells. As shown in Fig. 2A, all the NF-κB/Rel family proteins tested, i.e. NFκB1, c-Rel, NFKB1/RelA, and NFKB1/c-Rel, formed complexes with the IL-6 NF-κB motif. The usage of specific antibodies directed against individual NF-κB/Rel family members in supershift analysis confirmed the specificity of the NF-κB/Rel-mediated protein-DNA complexes (data not shown).

The oligonucleotides used were synthesized on a DNA synthesizer (Millipore Expedite nucleic acid synthesis system). The following probes were used (thymidines tagged with an asterisk were substituted by bromodeoxyuridine):

| Promoter | Sequence |
|----------|----------|
| IL-6 NF-κB | 5’-gatcTTGGGATCTCTTGCGc-3’ |
| IL-8CD28RE | 5’-gatcTTGGGATCTCTTGCGc-3’ |
| IL-2CD28RE | 5’-gatcTTGGGATCTCTTGCGc-3’ |
| HIV-1 NF-κB | 5’-gatcTTGGGATCTCTTGCGc-3’ |

The nucleotide sequence changes in the IL-2 and IL-6 promoters are indicated in boldface.
UV Cross-linking Analysis of CD28RE-protein Complexes—To characterize the proteins that contact DNA in the CD28RE-protein complex that is formed when using nuclear extracts of Jurkat cells that received a mitogenic combination of stimuli, we performed photoaffinity cross-linking analysis. For that purpose we used BrdU-substituted probes in combination with nuclear extracts derived from Jurkat cells that had been stimulated for 8 h with a combination of anti-CD3 and anti-CD28. As shown in Fig. 4, three major bands with apparent molecular masses of 35, 50, and 70–85 kDa became visible after SDS-polyacrylamide gel electrophoresis analysis of UV-cross-linked products when using the IL-6 NF-κB, IL-8 CD28RE, and HIV-1 NF-κB oligonucleotide probes. We previously identified the protein moieties in the 50- and 70–85-kDa cross-linked HIV-1 NF-κB/protein products as NFKB1 and RelA/c-Rel, respectively (21).

The pattern of cross-linked protein-DNA products obtained with the IL-2 CD28RE motif was different from that obtained with IL-6 NF-κB, IL-8 CD28RE, and HIV-1 NF-κB. Using the BrdU-substituted IL-2 CD28RE, UV-cross-linking analysis revealed 35- and 70-kDa protein-DNA products named NF-MAT (nuclear factor of mitogenic-activated T cells)-p35 and NF-MATp70, respectively. The 35-kDa protein-DNA complex observed with the IL-6 NF-κB, IL-8 CD28RE, and HIV-1 NF-κB were identical in mobility to that formed with the IL-2 CD28RE probe. Therefore, the CD28-induced protein moiety that is present in these complexes is likely to represent NF-MATp35.

These results indicate a difference in binding preference in nuclear extracts of stimulated Jurkat cells between the CD28RE from the IL-6 and IL-8 gene promoters and HIV-1 LTR on one hand and the IL-2 CD28RE on the other hand. Whereas the IL-6 NF-κB motif, like the IL-8 CD28RE and the HIV-1 NF-κB motif, exhibits binding to both NF-κB/Rel family proteins and NF-MAT, the IL-2 CD28RE reveals selective binding to NF-MAT.

Effect of Distinct NF-κB/CD28RE Motifs on the CD28-induced IL-6 Promoter Activity—To determine to what extent the observed differences in binding capacity between the above-
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mentioned NF-κB/CD28RE binding elements influence CD28-induced gene expression, we performed sequence-swapping experiments. Therefore, we generated a series of constructs of the IL-6 promoter in which the 10-base pair NF-κB sequence (Fig. 1A). From these findings we conclude that the costimulatory signal acts through a CD28-specific response. Studies with c-Rel−/− mice, which revealed deficient GM-CSF and IL-2 production, clearly supported a role for c-Rel in the regulation of these cytokines (16). Moreover, it has been demonstrated that CD28 signaling leads to phosphorylation and increased nuclear expression of c-Rel (34). However, our results as well as those of others show that the IL-2 CD28RE-protein complexes from Jurkat cells do not contain a significant amount of c-Rel proteins (7, 21, 32). Recently, it became apparent that optimal binding of c-Rel to CD28REs and subsequent transactivatory potential increases in the presence of HMG-I(Y), which binds the CD28RE core sequence, represented by a xB-like element called CD28RE. Our results presented here demonstrate that the NF-κB motif within the IL-6 promoter functions as a CD28 response element.

The capacity of the elements to bind NF-κB/Rel family proteins strongly suggested that these proteins are involved in CD28-mediated signaling. However, although some of the CD28REs may be classified as genuine NF-κB/Rel binding motifs, others bind poorly to NF-κB/Rel proteins. Based on binding studies of the IL-6 NF-κB motif with recombinant NF-κB/Rel proteins, the IL-6 NF-κB motif can, in analogy to the IL-2 CD28RE and HIV-1 NF-κB, be categorized as a CD28RE that binds the whole spectrum of NF-κB/Rel proteins. The IL-2 CD28RE and GM-CSF CD28RE sequences show low NF-κB/Rel binding capacity (7, 21, 32). In this study we found that IL-2 CD28RE preferentially bound recombinant c-Rel and c-Rel/NFκB1 heterodimers. This finding is not surprising since the IL-2 CD28RE differs at only one position from the consensus c-Rel binding site NGGNN(A/T)TTCC (33). The poor binding to NFκB1 homodimers and NFκB1/RelA heterodimers is explained by differences between the IL-2 CD28RE sequence and reported NFκB1 and RelA consensus binding sites.

The ability of c-Rel to bind CD28RE is believed to be critical for generating a CD28-specific response. Studies with c-Rel−/− mice, which revealed deficient GM-CSF and IL-2 production, clearly supported a role for c-Rel in the regulation of these cytokines (16). Moreover, it has been demonstrated that CD28 signaling leads to phosphorylation and increased nuclear expression of c-Rel (34). However, our results as well as those of others show that the IL-2 CD28RE-protein complexes from Jurkat cells do not contain a significant amount of c-Rel proteins (7, 21, 32). Recently, it became apparent that optimal binding of c-Rel to CD28REs and subsequent transactivatory potential increases in the presence of HMG-I(Y), which binds the CD28RE core sequence, represented by a xB-rich motif, in the minor groove (17). Since poly(dI-dC) is known to compete for the interaction of HMG-I(Y) with DNA, usage of poly(dI-dC) as a nonspecific competitor in gel retardation analysis may

FIG. 4. UV-cross-linking analysis of BrdU-labeled CD28RE and Jurkat-derived nuclear proteins. UV-cross-linking of nuclear proteins from Jurkat T cells stimulated with anti-CD3 plus anti-CD28 to the bromodeoxyuridine-substituted IL-2 CD28RE (lane 1), IL-6 NF-κB (lane 2), IL-8 CD28RE (lane 3), and HIV-1 NF-κB (lane 4) probe.

FIG. 5. Activity of wt and variant IL-6 reporter gene constructs toward T-cell activation signals. The NF-κB site in the wt-pIL6-luciferase-promoter construct (wt-pIL6luc) is changed into the NF-κB binding site of the HIV-1 LTR (pIL6 (HIV-1 NFκB)luc) or the CD28RE site of IL-2 (pIL6 (IL2CD28RE)luc) or IL-8 (pIL6 (IL8CD28RE)luc) promoter. Jurkat T cells were transfected with pIL6luc constructs as indicated. Cells were stimulated with anti-CD3 and anti-CD28. Data for the mutants are presented as the average percent value relative to the induced response of the wild-type IL-6 promoter (shown as 100%).

![Probes:](Image 89x497 to 256x729)

![activity (%):](Image 309x462 to 552x729)
account for low or undetectable c-Rel in CD28RE-protein complexes (35–36).

Recent evidence suggested a functional role for the nuclear factor of activated T cells, which has weak homology to the Rel DNA binding domain, in CD28RE-mediated IL-2 gene transcription (15, 18, 19). Another factor implicated in CD28RE-mediated effect on transcription is a nuclear protein, referred to as NF-MAT (7, 21). Previously, we demonstrated that nuclear appearance of NF-MAT is correlated with IL-2 expression. UV-cross-linking experiments revealed that this protein interacts with the whole spectrum of CD28REs tested. Hence the CD28REs can be viewed as composite binding elements that interact with at least three factors, i.e. c-Rel, HMG-I (Y), and NF-MAT, implicated in mediating the CD28-induced transcriptional response.

Substitution of the IL-2 CD28RE motif within the IL-2 promoter by the IL-6 NF-κB motif rendered the IL-2 promoter equally responsive to T cell activation. This result provided further evidence for assignment of the IL-6 NF-κB motif as CD28RE. Although the IL-6 NF-κB motif and CD28RE are mutually interchangeable within the IL-2 promoter without impairment of CD28-coinduced transcriptional activity, motif-swapping experiments within the context of the IL-6 promoter gave a different result. Swapping experiments whereby the IL-6 κB motif was replaced by heterologous CD28REs revealed that all the full-spectrum NF-κB/Rel binding CD28REs do function as CD28 response elements in the context of the IL-6 promoter, whereas the IL-2 CD28RE, which reveals restricted binding capacity, is not functional in this setting. This suggests that NF-κB/Rel proteins play a significant role in CD28-induced transactivation of the IL-6 promoter.

Furthermore, these data strongly indicate that the capacity of the two distinct classes of CD28RE to function as a target site for CD28-coinduced transcriptional activation is largely determined by the context of the promoter. An explanation for this observation could be that NF-κB proteins have a direct effect on transcription. Alternatively, the presence of binding elements that cooperate with the κB-like CD28RE motifs may be critical. In this respect it is worth mentioning the presence of a NF-IL6 binding element in the vicinity of the IL-6 NF-κB sequence. Functional cooperation between NF-IL6 and NF-κB proteins has been shown when these elements are in close proximity (28). It has been proposed that such interactions may be crucial for transcriptional activity of the IL-6 promoter. Moreover, the IL-8 promoter and HIV-1 long terminal repeat contain a NF-IL6 binding element adjacent to their NF-κB binding sequences (28, 37). Therefore, the interaction of NF-IL6 with NF-κB proteins bound to the NF-κB may provide a molecular explanation for the disparity between IL-6, IL-8, and HIV-1 NF-κB elements on one hand and IL-2 CD28RE on the other hand in response to CD28 costimulation.

In conclusion, our results indicate that two functionally different classes of CD28RE can be assigned that coincide with differences in their NF-κB binding capacity. Hence, the findings reported in this study may point to the existence of two molecular mechanisms operating in the CD28-induced transcriptional regulation of cytokine gene expression via so-called CD28REs.

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