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The interleukin 2 CD28-responsive complex contains at least three members of the NF κB family: c-Rel, p50, and p65

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ABSTRACT **Optimal activation of T cells requires at least two signals. One signal can be delivered by the antigen-specific T-cell receptor, and the second signal is provided by the costimulatory molecule(s) delivered by the antigen-presenting cell. CD28 is a T-cell surface molecule and stimulation through this protein plays an important role in delivering the second activation signal. In this report, we show that in human peripheral blood T cells, CD28-mediated signal transduction involves the rel family proteins—c-Rel, p50, and p65. Treatment of peripheral blood T cells with phorbol 12-myristate 13-acetate (PMA) and anti-CD28 monoclonal antibody (mAb) results in augmentation of nuclear c-Rel, p50, and p65, and this augmentation can occur in the presence of the immunosupressant cyclosporin A. It is also shown in this report that, in response to PMA/anti-CD28 mAb or anti-CD3/anti-CD28 mAb, c-Rel, p50, and p65 are associated with CD28-responsive element present in the promoter of the human interleukin 2 gene. The functional significance of c-Rel involvement in the CD28-responsive complex is demonstrated by transient transfection analysis, where cotransfection of c-Rel augments the level of expression of a chloramphenicol acetyltransferase reporter gene linked to the CD28-responsive element.**

A key activation pathway in the T-cell-mediated immune response involves the T-cell receptor (TCR)–CD3 complex. Although the specificity of T-cell activation is determined by the TCR, TCR signaling alone is not sufficient for T-cell proliferation and production of lymphokines [e.g., interleukin 2 (IL-2), interferon γ, etc.] (1, 2). Accessory cell-derived costimulatory signals are also required for optimal activation of T cells (3, 4). In the absence of these costimulatory signals, induction of a state of clonal anergy has been proposed (3, 5–7). A T-cell surface molecule, CD28, plays an important role in the costimulation process (8, 9), and it has been shown recently that induction of anergy in T-cell clones can be blocked by the CD28-mediated costimulatory signal (10).

Activation of T cells and subsequent production of IL-2 via CD28 cell surface molecule triggering is distinct and different from the TCR-mediated pathway (9, 11, 12). A natural ligand of CD28, B7/BB1, has recently been identified (13), and cellular interaction mediated by CD28 and the B-cell activation antigen B7/BB1 may represent an important functional interaction between T and B lymphocytes (14–16). However, the molecular mechanisms involved in the CD28-mediated signal transduction pathway have yet to be fully characterized. Recently, a CD28-responsive element (CD28RE) in the IL-2 gene promoter has been identified and shown to have enhancer activity (17). The nucleotide sequence of the CD28RE has been found in 5′ upstream regions of many lymphokine genes (17). CD28-mediated protein tyrosine phosphorylation has recently been shown by Vandenberghe *et al.* (18). However, the transcription factor(s) involved in the CD28-mediated signal transduction pathway has not been fully characterized. In this manuscript, we demonstrate that treatment of freshly purified human peripheral blood T cells with phorbol 12-myristate 13-acetate (PMA) and anti-CD28 monoclonal antibody (mAb) results in increased nuclear c-Rel as well as the NF κB p50 and p65 subunits, and this increase in nuclear protein levels is resistant to cyclosporin A (CsA). Furthermore, we show that in response to PMA/anti-CD28 mAb or anti-CD3/anti-CD28 mAb, c-Rel, p50, and p65 are all associated with the CD28RE. Finally, in transient transfection analysis we demonstrate that cotransfection of a c-Rel expression plasmid can upregulate chloramphenicol acetyltransferase (CAT) expression 6-fold in a CAT vector containing the CD28RE.

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MATERIALS AND METHODS

Preparation of T-Cell Nuclear Extracts and Western Blot Analysis. Freshly purified human peripheral blood T cells (>95% $CD3^+$) were treated with medium alone (RPMI 1640 medium containing 2% fetal calf serum), PMA (10 ng/ml), PMA/anti-CD28 mAb (100 ng/ml), PMA/anti-CD28 mAb/CsA (100 ng/ml), PMA/ionomycin (1 μg/ml), or PMA/ionomycin/CsA for 7 hr (cells were pretreated with CsA for 30 min before other stimuli were added). Nuclear extracts were prepared according to the procedure of D. G. Kelvin, G. White, D. L. Longo, and D. F. Ferris (personal communication). The extracts were analyzed by SDS/PAGE (10% acrylamide) followed by immunoblotting with rabbit anti-c-Rel/anti-p50/anti-p65 antibodies. Immunoreactive bands were visualized by using horseradish peroxidase-coupled goat anti-rabbit immunoglobulin and the enhanced chemiluminescence detection system (Amersham).

Antisera. The antisera used in Western blot and immunoprecipitation experiments are described elsewhere (19). In brief, anti-human c-Rel antiserum 265 was raised against a C-terminal peptide (amino acids 573–587), anti-human-p50 antiserum 1141 was raised against an N-terminal peptide (amino acids 2–15), and anti-p65 antiserum 1226 was raised against a C-terminal peptide (amino acids 537–550). Each antiserum is specific for its protein and does not cross-react with other rel family members (19).

DNA-Binding Assay. A bacterial expression vector for human c-Rel, pKKrel, was used to express c-Rel protein in *Escherichia coli* (20), and the bacterial lysate was prepared as described (21). Control bacterial lysate was prepared from *E. coli* containing only the expression vector. Electrophoretic

Abbreviations: TCR, T-cell receptor; IL-2, interleukin 2; CD28RE, CD28-responsive element; PMA, phorbol 12-myristate 13-acetate; mAb, monoclonal antibody; CsA, cyclosporin A; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility-shift assay; CD28RC, CD28RE-binding protein complex.

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mobility-shift assay (EMSA) with bacterial extracts was performed as described (22, 23). The buffer used in EMSA with T-cell nuclear extracts has also been described (17).

Oligonucleotides used in EMSA were as follows: 1, CD28RE (−164 to −149 of the IL-2 gene), AAAGAAATTC-CAAAGA; 2, CD28RE mutant, AAAGAAGCCTCAAAGA; 3, IL-2 receptor α κB (−271 to −257 of the NF κB-binding site of the IL-2 receptor α promoter), GATCAGGGGAATC-TCCC; 4, Sp1 (−80 to −44 of Sp1-binding sites of the human immunodeficiency virus type 1 long terminal repeat), GATCGGGAGGCGTGGCCTGGGCGGGACTGGG-GAGTGGCGA.

UV Crosslinking and Immunoprecipitation. The bromouridine-substituted CD28RE oligonucleotide was synthesized on a DNA synthesizer (Applied Biosystem, model 392). UV crosslinking and immunoprecipitation were performed according to the procedure described by Kochel and Rice (24) with the following modification: UV irradiation was performed on the gel after EMSA. The complex was excised and protein was eluted according to the described procedure (25). After precipitating the proteins with acetone, the crosslinked products were analyzed by SDS/PAGE. To characterize the crosslinked products by immunoprecipitation, the eluted products from EMSA gel were used as a starting material for immunoprecipitation as described (24).

RESULTS

It has been demonstrated that the CD28 costimulatory signal can act through the κB element (26). However, the CD28RE of the IL-2 gene has been mapped to a distinct sequence that is different from the previously identified κB site of the IL-2 promoter (17). To determine whether NF κB family proteins (p50, p65, and c-Rel) are involved in the CD28 signaling pathway, nuclear extracts from purified human peripheral blood T cells were treated with PMA alone, anti-CD28 mAb alone, or PMA plus anti-CD28 mAb and were analyzed by Western blotting using anti-c-Rel antiserum (Fig. 1*a*), anti-p50 antiserum (Fig. 1*b*), or anti-p65 antiserum (Fig. 1*c*). Treatment with medium alone or with anti-CD28 mAb alone had no effect on the level of nuclear c-Rel (Fig. 1*a*, lanes 1 and 3), while PMA did upregulate the levels of nuclear c-Rel (lane 2). However, maximum induction of nuclear c-Rel was observed after the combined PMA/anti-CD28 mAb treatment (lane 4). Similar results were obtained for NF κB p50 and p65, as maximum inductions were observed after combined PMA/anti-CD28 mAb treatment (Fig. 1 *b* and *c*, lane 4). To determine whether augmentation of the PMA response is specific to anti-CD28 mAb, the experiment was repeated with PMA with or without anti-CD2 mAb. Unlike the result with anti-CD28 mAb, anti-CD2 mAb did not augment the PMA response (data not shown). Thus, the effect is specific to anti-CD28 mAb and is not reproduced with another T-cell reactive antibody.

Induction of T-cell proliferation and IL-2 expression by PMA plus anti-CD28 mAb is resistant to CsA (27), whereas the major transcriptional stimulation of IL-2 gene expression mediated through the TCR complex is sensitive to CsA (28). To determine whether the upregulation of nuclear c-Rel, p50, and p65 was mediated through the CD28-signaling pathway, T cells were treated with PMA/anti-CD28 mAb in the presence and absence of CsA, and nuclear extracts were examined by Western blot analysis. As shown in Fig. 1 *d–f*, the increase in nuclear c-Rel, p50, and p65 in response to PMA/anti-CD28 mAb was resistant to CsA, consistent with involvement of the CD28-signaling pathway. In a control experiment, the upregulation of nuclear c-Rel, p50, and p65 by PMA/calcium ionophore (ionomycin) treatment of T cells was found to be sensitive to CsA as expected (Fig. 1*g*; data not shown). Although CsA treatment did not block the upregulation of nuclear rel family proteins completely, no IL-2 production was observed after this treatment. One possible explanation is that the level of rel family proteins that remained after PMA/ionomycin/CsA treatment was not sufficient for gene transcription (see *Discussion*).

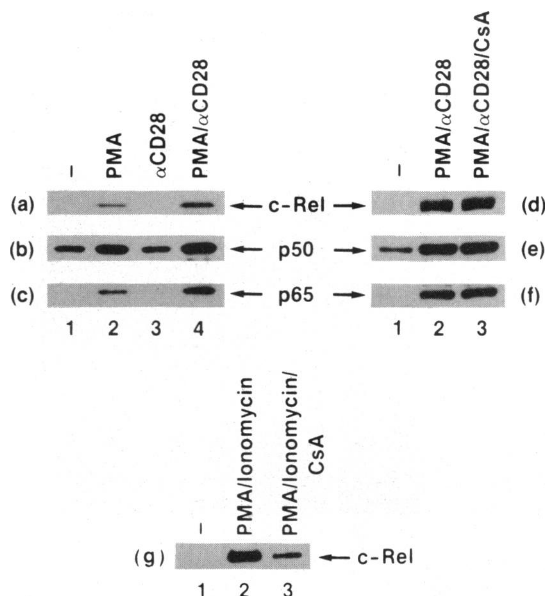

FIG. 1. Upregulation of nuclear c-Rel, p50, and p65 in peripheral blood T cells. Nuclear extracts (6 μg of protein) from purified peripheral blood T cells were analyzed by Western blot using anti-c-Rel antiserum 265 (*a*), anti-p50 antiserum 1141 (*b*), and anti-p65 antiserum 1226 (*c*). Lanes: 1, extract from cells treated with medium alone; 2, extract from PMA-treated cells; 3, extract from cells treated with anti-CD28 mAb alone; 4, extract from PMA plus anti-CD28 mAb-treated cells. (*d–f*) Effects of CsA on treatments. Nuclear extracts from T cells treated with medium alone (lane 1) or PMA/anti-CD28 mAb in the absence (lane 2) or presence (lane 3) of CsA were analyzed by Western blot using the same anti-c-Rel (*d*), anti-p50 (*e*), and anti-p65 (*f*) antisera. (*g*) Sensitivity of upregulation of nuclear c-Rel by PMA/ionomycin to CsA is shown. Lane 1, extract from cells treated with medium alone; lane 2, extract after PMA/ionomycin-treated cells; lane 3, extract after PMA/ionomycin/CsA treatment.

As it has been reported that the CD28RE forms specific protein–DNA complexes after stimulation through the CD28 pathway (17), we next sought to determine whether the members of the NF κB family that were upregulated by PMA/anti-CD28 mAb treatment could interact with the CD28RE. An EMSA of the CD28RE using a bacterial extract containing c-Rel revealed the formation of a DNA–protein complex (Fig. 2*A*, lane 3). The DNA–protein complex was blocked by competition with unlabeled CD28RE (lanes 4 and 5) and an IL-2 receptor α κB oligonucleotide (lanes 6 and 7), but not by the nonspecific oligonucleotides Sp1 (lane 8), tax responsive element (data not shown), or an oligonucleotide corresponding to the silencer region of the interferon γ gene (data not shown). To determine whether the CD28RE is also recognized by NF κB p50, an EMSA was performed with affinity-purified, bacterially expressed p50 (a generous gift from R. Roeder, The Rockefeller University). In this assay, labeled IL-2 receptor α κB oligonucleotide was used as a probe while unlabeled CD28RE oligonucleotide was used as a competitor (Fig. 2*B*). While the DNA–protein complex was blocked by competition with unlabeled κB oligonucleotide (lane 2), no competition was observed with the unlabeled CD28RE oligonucleotide (lane 3). This result indicates that bacterially expressed NF κB p50 does not recognize the CD28RE sequence. When an EMSA was performed with NF κB p50 and a labeled CD28RE oligonucleotide, no DNA–

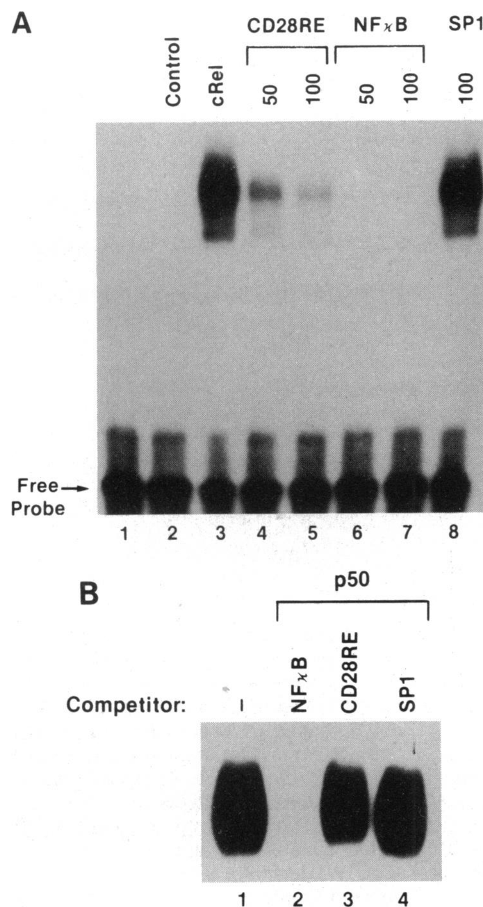

FIG. 2. Recognition of CD28RE by bacterially expressed c-Rel, but not p50. (*A*) EMSA using 3 μg of total protein from control bacterial lysate (lane 2) or lysate from cells expressing c-Rel protein (lanes 3–8). A double-stranded ^{32}P-labeled oligonucleotide (1 ng) consisting of the CD28RE site (AAAGAAATTCCAAAGA; −164 to −149) of the IL-2 gene (17) was used as a probe. Competition of the DNA–protein complex was with unlabeled CD28RE oligonucleotide (lanes 4 and 5), IL-2 receptor α κB site (GATCAGGGGAATCTCCC; ref. 29) (lanes 6 and 7), and Sp1 (lane 8). Lane 1, free probe. (*B*) EMSA using affinity-purified, bacterially expressed p50. Probe was a ^{32}P-labeled IL-2 receptor α κB site. Unlabeled CD28RE (lane 3), IL-2 receptor α κB (lane 2), and Sp1 (lane 4) oligonucleotides were used as competitors (200-fold molar excess).

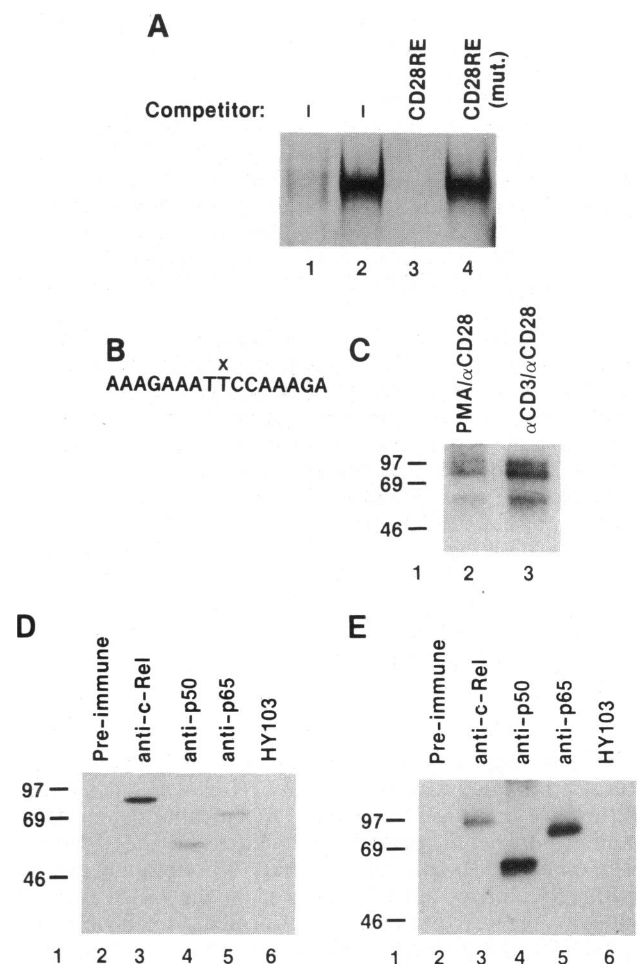

FIG. 3. Characterization of CD28RC. (*A*) EMSA of T-cell nuclear extract after treatment of cells either with medium alone (lane 1) or with PMA/anti-CD28 mAb (lanes 2–4). Assay was performed with ^{32}P-labeled CD28RE oligonucleotide. Competitors (100-fold molar excess) were unlabeled CD28RE (lane 3) and a mutant of CD28RE (AAAGAAGCCTCAAAGA) (lane 4). (*B*) Sequence of bromouridine-substituted CD28RE probe, where X indicates position of bromouridine substitution. (*C*) UV crosslinking analysis of CD28RC. Probe used was the ^{32}P-labeled oligonucleotide shown in *B*. Lanes: 1, molecular size markers (kDa); 2, nuclear extract from T cells treated with PMA and anti-CD28 mAb; 3, nuclear extract from T cells treated with anti-CD3 and anti-CD28 mAb. (*D* and *E*) Immunoprecipitation of UV-crosslinked CD28RC using extract from PMA/anti-CD28 mAb-treated cells (*D*) and from anti-CD3/anti-CD28 mAb-treated cells (*E*). For immunoprecipitation experiments, the UV-crosslinked product eluted from EMSA gel was boiled in 1% SDS, diluted, and immunoprecipitated with the respective antiserum. Lanes: 1, molecular size markers (kDa); 2, preimmune serum; 3, c-Rel antiserum 265; 4, p50 antiserum 1141; 5, p65 antiserum 1226; 6, irrelevant antiserum HY103.

protein complex was obtained (data not shown), indicating that the CD28RE can be recognized by bacterial-derived c-Rel but not p50.

We next characterized the inducible CD28RE-binding protein complex (CD28RC) present in nuclear extracts of PMA/anti-CD28 mAb-treated human peripheral blood T cells (Fig. 3*A*, lane 2). In an EMSA with the CD28RE oligonucleotide, this complex (CD28RC) was blocked by competition with unlabeled CD28RE oligonucleotide (lane 3) but not by the mutant oligonucleotide (lane 4). This complex was not induced after treatment of cells with anti-CD28 mAb alone, but it was induced (although to a lesser extent) by treatment with PMA alone (data not shown). A similar pattern of induction was observed in T-cell extracts from 10 separate donors. When a gel-shift analysis was performed with CD28RE using a PMA/ionomycin-treated T-cell nuclear extract, an inducible DNA–protein complex was obtained that was similar to that following PMA treatment alone but in a much reduced level compared to PMA/anti-CD28 mAb treatment (data not shown).

To characterize the inducible protein–DNA complex, UV crosslinking was performed with a bromouridine-substituted probe (Fig. 3 *B* and *C*). Three major protein bands were obtained that migrated with molecular masses slightly higher than 85, 65, and 50 kDa (Fig. 3*C*, lane 2). To determine whether a physiologically relevant stimulus also can induce these proteins, anti-CD3 mAb OKT3 was used instead of PMA. A similar result was obtained with a nuclear extract from cells treated with anti-CD3 plus anti-CD28 mAb (lane 3). PMA treatment or anti-CD3 mAb treatment alone also induced these proteins, but the level of induction was lower than with PMA/anti-CD28 mAb or anti-CD3/anti-CD28 mAb treatment, respectively, just as in EMSA (data not shown). The effect of anti-CD3 plus anti-CD28 mAb treatment of T cells was similar to PMA/anti-CD28 mAb treatment with

Table 1. Cotransfection of c-Rel in Jurkat cells augments expression of a CAT plasmid containing four copies of CD28RC-binding site

Constructs	Induction, -fold
pBLCAT2 + pRSVcREL	1
4xCD28RE-CAT + pSRSPA	1
4xCD28RE-CAT + pRSVcREL	6

Jurkat cells were cotransfected by electroporation with 20 μg of reporter plasmid plus 10 μg of expression vector. Cells were harvested after 48 hr and CAT ELISA was performed according to the manufacturer's protocol (Boehringer Mannheim). Results are the average of six different experiments (range from 4x to 7.2x). Reporter plasmids were 4xCD28RE-CAT, containing four copies of the CD28RE (one copy, AAAGAAATTCC) and pBLCAT2, the parental CAT plasmid (20). Expression plasmids were pRSVcREL, which contains the human *REL* gene and the Rous sarcoma virus long terminal repeat and pSRSPA, the parental expression vector (20).

respect to nuclear translocation of rel family members and induction of CD28RC (data not shown).

To identify the proteins crosslinked to the CD28RE, the UV-treated samples were boiled in SDS and immunoprecipitated with anti-c-Rel, anti-p65, and anti-p50 antisera. The results of this experiment are shown in Fig. 3*D*. No protein band was obtained when preimmune serum was used (lane 2). The c-Rel antiserum precipitated a protein that migrated slightly slower than c-Rel (lane 3), consistent with its being bound to the oligonucleotide. Similarly, p50 (lane 4) and p65 (lane 5) were detected by the anti-p50 and anti-p65 antisera, respectively. No protein was obtained when an irrelevant antiserum (HY103) was used for immunoprecipitation (lane 6). By the same procedure, we also characterized the DNA–protein complex obtained after treatment of T cells with anti-CD3 and anti-CD28 mAb. As with the extract from PMA/anti-CD28 mAb-treated cells, c-Rel, p50, and p65 were present in the CD28RC (Fig. 3*E*, lanes 3–5).

The *in vivo* significance of the association between CD28RE and c-Rel was tested by cotransfection studies with a c-Rel expression vector and a reporter plasmid containing the CAT gene linked to four copies of CD28RC-binding site. The core-binding sequence of CD28RE (−164 to −154) was used to construct CD28RE-CAT plasmid. The result of transient cotransfection into the Jurkat T-cell line is presented in Table 1. Cotransfection of c-Rel enhanced reporter gene expression by ≈6-fold. This demonstrates a functional interaction between c-Rel and CD28RE, consistent with the direct interaction observed in UV crosslinking and immunoprecipitation experiments.

DISCUSSION

These findings strongly suggest that NF κB family proteins are involved in CD28-mediated signaling and play an important role in T-cell gene expression. We demonstrated that stimulation of normal T cells with a combination of PMA and anti-CD28 mAb results in increased nuclear rel, p65, and p50. Nuclear extracts contained proteins able to bind to the CD28RE, and UV crosslinking and immunoprecipitation experiments showed that rel, p65, and p50 are among these proteins. Similar results were obtained after stimulation of cells with a combination of anti-CD3 and anti-CD28 mAb. The functional significance of c-Rel involvement in the CD28RC was demonstrated by transient transfection analysis, where cotransfection of c-rel increased the level of CAT expression through a CD28RE. These findings confirm an earlier report that signaling via the CD28 molecule is mediated through an NF κB-like element (26, 30).

The sequence of the CD28RE, as defined by mutational analysis (17), is AAGAAATTCC, which differs at two positions from the consensus κB site GGGRNNYYCC (31). Recently, Kunsch *et al.* (32) have used random oligonucleotides and bacterially expressed rel family proteins in binding site selection experiments. They demonstrated that homodimers of rel, p65, and p50 prefer slightly different binding sites and that rel has the least stringent requirements. In fact, their consensus rel site NGGNNA/TTTCC differs at only one position from the CD28RE, while the consensus p50 site GGGGATYCCC differs at five positions. Thus, it is not surprising that our bacterial rel bound CD28RE while purified p50 did not. In spite of this lack of p50 binding to the CD28RE, we detected p50 in the CD28RC. The presence of p50 could be due to a CD28-signaling-specific modification of p50 that allows DNA binding. Alternatively, p50 may heterodimerize with rel or p65 and thereby come close enough to the DNA to be crosslinked after UV irradiation. It should be noted that our experiments do not define which specific homo- or heterodimers bind the CD28RE.

While maximum nuclear translocation and complex induction occurred when PMA was used in conjunction with anti-CD28 mAb, treatment with PMA alone resulted in significant translocation and complex formation. This is somewhat surprising since PMA alone does not activate T cells. It is well known, for example, that PMA does not induce IL-2 production. One possible explanation is that during T-cell purification some of the T cells were preactivated by interaction with B cells containing B7, the ligand for CD28. In these cells, PMA would act as the required second signal and would induce activation. However, we routinely monitored interferon γ, and we found that it was produced when cells were treated with PMA plus anti-CD28 mAb, or anti-CD3 plus anti-CD28 mAb, but never with PMA alone. This suggests that nuclear translocation and complex formation in response to PMA alone cannot be explained by preactivation of the cells. There are several other possible explanations. First, it is possible that nuclear levels of critical proteins do not reach high enough levels in response to PMA and that activation requires the higher levels induced by the second signal. Such an effect has been documented by Fiering *et al.* (33), who showed that expression controlled by the transcription factor NF-AT requires a certain threshold level of the factor. A second possibility is that additional, as yet undefined, proteins are induced following anti-CD28 mAb treatment and that these proteins are required for active complex formation. Third, anti-CD28 mAb treatment might result in some type of posttranslational modification of one or all of the CD28RE-binding proteins, resulting in functional complex formation. A recent report demonstrating CD28-mediated protein tyrosine phosphorylation supports this hypothesis (18).

Recently, Fraser and Weiss (34) reported that the CD28RC contains three polypeptides of approximately 35, 36, and 44 kDa. There are several differences between our protocol and theirs that could contribute to the different results. First, they used a T-cell line for their studies, while we used purified peripheral blood T cells. Second, they stimulated cells for 2 hr, as opposed to 7 hr in our case. Third, their oligonucleotide was derived from the granulocyte–macrophage/colony-stimulating factor promoter, not the IL-2 promoter, and there are minor differences between them. Lastly, we, but not they, used a bromouridine-substituted oligonucleotide in the crosslinking studies.

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