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Effect of CD28 Signal Transduction on c-Rel in Human Peripheral Blood T Cells

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Optimal T-cell activation requires both an antigen-specific signal delivered through the T-cell receptor and a costimulatory signal which can be delivered through the CD28 molecule. CD28 costimulation induces the expression of multiple lymphokines, including interleukin 2 (IL-2). Because the c-Rel transcription factor bound to and activated the CD28 response element within the IL-2 promoter, we focused our study on the mechanism of CD28-mediated regulation of c-Rel in human peripheral blood T cells. We showed that CD28 costimulation accelerated the kinetics of nuclear translocation of c-Rel (and its phosphorylated form), p50 (NFKB1), and p65 (RelA). The enhanced nuclear translocation of c-Rel correlated with the stimulation of IL-2 production and T-cell proliferation by several distinct anti-CD28 monoclonal antibodies. This is explained at least in part by the long-term downregulation of IκBα following CD28 signalling as opposed to phorbol myristate acetate alone. Furthermore, we showed that the c-Rel-containing CD28-responsive complex is enhanced by, but not specific to, CD28 costimulation. Our results indicate that c-Rel is one of the transcription factors targeted by CD28 signalling.

Antigenic stimulation of T cells requires both recognition by the T-cell receptor (TCR) of the major histocompatibility complex-antigen complex and the interactions of other cell surface molecules (reviewed in reference 59). One such interaction occurs between the accessory molecule CD28 on T cells and its cognate ligands B7-1 (CD80/B7 [33]), B7-2 (2, 16), and B7-3 (BB-1 [7]) on antigen-presenting cells. The CD28 molecule (for reviews, see references 26, 34, and 48) is constitutively expressed as a 44-kDa homodimer which is present on 95% of $CD4^+$ T cells and on approximately 50 to 70% of $CD8^+$ T cells. Blocking CD28 function during T-cell activation with an antigen can drive the T cells into a long-lasting antigen-specific anergic state (18, 53). Conversely, anergy induced by suboptimal stimulation of TCRs can be prevented by ligation of CD28 molecules with anti-CD28 monoclonal antibody (MAb) (23). The CD28 signalling pathway is distinct from the TCR-CD3 signalling pathway (26), as shown by the inability of the immunosuppressant cyclosporin A to block CD28 signalling (5, 27) while completely blocking TCR-CD3 signalling.

CD28 signalling as mimicked by MAbs can cooperate with anti-CD2, anti-CD3, phytohemagglutinin (PHA), and phorbol myristate acetate (PMA) to upregulate T-cell responsiveness. It synergizes with these signals to induce proliferation of T cells and secretion of multiple lymphokines such as interleukin 1 (IL-1), IL-2, IL-3, IL-4, IL-5, gamma interferon (IFN-γ), granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor alpha (34, 55). The combination of anti-CD28 and anti-CD2 MAbs (but not anti-CD3) can both enhance and prolong IL-2 receptor α-chain (IL-2Rα) gene expression (8). Anti-CD28 MAb cooperates with anti-CD3 in the induction of the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) (21, 56) and HIV-1 virus replication (1). The induction of lymphokine, IL-2Rα, and HIV-1 gene expression by CD28 costimulation is mediated by both transcriptional (8, 14, 56, 58, 60) and posttranscriptional (32) mechanisms.

CD28 costimulation activates transcription from the HIV-1 LTR (56) and the IL-2Rα promoter (10) via the κB elements. This suggests that the κB enhancer-binding proteins, the Rel family of transcription factors (reviewed in references 6, 20, and 35), are involved in CD28 signal transduction. CD28 costimulation activates lymphokine gene transcription through a unique conserved motif, the CD28 response element (CD28RE), within several lymphokine promoters (IL-2, IL-3, granulocyte-macrophage colony-stimulating factor, and IFN-γ [9, 14, 15, 58]). The CD28RE sequence is distinct from but related to that of the κB element. Interestingly, three Rel family proteins, p50 (NFKB1), p65 (RelA), and c-Rel, are among the components of the CD28-responsive complex (CD28RC) (17).

κB elements are regulated through posttranscriptional regulation of the Rel family proteins (reviewed in references 6, 20, and 35). These proteins are complexed to ankyrin-containing proteins such as IκBα, p105 (NFKB1 [24, 41, 46]), or p100 (NFKB2 [37]) in the cytoplasm of resting cells. In the case of IκBα, stimulation of the cells leads to its phosphorylation and degradation within minutes of activation. Once released from inhibition, the Rel family proteins translocate into the nucleus and initiate gene transcription. The degradation of IκBα following a variety of different mitogenic signals is transient, and its levels return to normal within 1 h of the onset of stimulation (4, 52; for a review, see reference 3).

Among the Rel family proteins, c-Rel in particular is implicated in the transcriptional regulation of several lymphokine and lymphokine receptor genes, including the IL-1β (25), IL-2 (17), IL-2Rα (11, 54), IL-6 (40), and IFN-γ (49) genes. Most importantly, c-Rel alone (but not NFKB1 p50) binds to the CD28RE site and activates CD28RE-driven chloramphenicol acetyltransferase (CAT) gene expression (17). To demonstrate that c-Rel is a primary target of CD28 signalling, we have

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shown that translocation of c-Rel from the cytoplasm to the nucleus is enhanced, at least partially, by the increased degradation of IκBα in the cytoplasm.

MATERIALS AND METHODS

Cells. Human peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats (Gulf Coast Regional Blood Center, Houston, Tex.) with a Ficoll-Hypaque gradient (Pharmacia). T cells were isolated from PBMCs by negative selection. To do this, PBMCs were stained with L243 (anti-DR; American Type Culture Collection [ATCC], Rockville, Md.), OKM1 (anti-CD11b; ATCC), and LM2 (anti-Mac1; ATCC) at 4°C for 30 min. The cells were then washed and incubated at 4°C for 30 min with magnetic beads conjugated with goat anti-mouse immunoglobulin G (IgG) (10 ml for 2×10^8 cells) (Advanced Magnetics, Cambridge, Mass.) while undergoing occasional gentle mixing. The cells bound to the beads were then removed by consecutive incubation with a magnet. The purified T cells were >99% $CD3^+$.

T-cell proliferation and activation assays. Stimuli included 0.5 μg of PHA (Sigma) per ml, 5 ng of PMA (Sigma) per ml, anti-CD2 MAbs (anti-$T11_2$ and anti-$T11_3$ [38]; a gift from E. Reinherz, Harvard Medical School, Boston, Mass.), and anti-CD28 MAbs. The anti-CD28 panel MAbs (9.3 [22]; CD28.1, CD28.2, CD28.3, CD28.4, CD28.5, and CD28.6 [43]; KOLT2 [30]; CK248 [45]; and M-T281, L293, and 15E8) were provided by the 5th International Workshop on Human Leukocyte Differentiation Antigens. Anti-CD28 MAb 9.3 ascitic fluids were also obtained from C. June (Naval Medical Research Institute, Bethesda, Md.) and J. Ledbetter (Bristol-Meyers Squibb Pharmaceutical Research Institute, Seattle, Wash.). Anti-CD2 and all anti-CD28 MAb ascitic fluids were used at a 1:2,000 dilution.

For proliferation assays, 10^5 T cells in 200 μl of assay medium were stimulated in triplicate for 4 days in a 96-well flat-bottom microtiter plate (Costar). The assay medium consisted of RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 μg of gentamicin sulfate (Gibco) per ml. For the final 24 h, 1 μCi of [^{3}H]thymidine (NEN, Boston, Mass.) in 25 μl of Hanks balanced salt solution (Gibco) was added to each well. Cells were harvested onto filtermats and analyzed by standard liquid scintillation counting techniques. The standard error of the mean was <10% for each sample.

For assays of IL-2 secretion and IL-2Rα expression, 2×10^7 T cells were stimulated for 24 h in 1 ml of assay medium (two wells per stimulus in a 12-well plate). The supernatants were collected and analyzed in two ways: by incubation with the IL-2-dependent cell line CTLL-2 for 24 h in comparison with a recombinant IL-2 standard (see Table 2) or by enzyme-linked immunosorbent assay (ELISA) (Genzyme) (see Table 1 and Fig. 2). For IL-2Rα expression, 5×10^5 cells were stained with anti-IL-2Rα conjugated to fluorescein isothiocyanate (Becton-Dickinson Immunocytometry, Mountain View, Calif.). The percent positive staining for IL-2Rα was quantified by flow cytometry (EPICS PROFILE I; Coulter, Hialeah, Fla.).

Nuclear and cytoplasmic extract preparation. Extracts were prepared by a modification of the procedure of Dignam et al. (12). T cells were stimulated in six-well plates (Fisher), and the cells were collected and washed twice with phosphate-buffered saline. In the experiment whose results are shown in Fig. 2, the supernatant was collected and analyzed for IL-2. The cellular pellet was then rinsed once without resuspension in 70 μl (per 2×10^7 T cells) of buffer A (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 3.3 μg of aprotinin per ml). The pellet was resuspended in 70 μl of buffer A and incubated at 4°C for 15 min with gentle vortexing every 5 min. The lysates were then centrifuged for 3 min at 14,000 × *g*, after which the supernatant was collected as cytoplasmic extract, and the pellet was rinsed again with buffer A without resuspension. The washed pellet was resuspended in 50 μl of buffer C (20 mM HEPES [pH 7.9], 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 1 mM dithiothreitol, 1 mM PMSF, and 3.3 μg of aprotinin per ml) and was incubated at 4°C for 2 to 3 h with occasional vigorous vortexing. The cellular debris was removed by centrifugation for 20 min at 14,000 × *g*, and the supernatant was collected as nuclear extract. Protein concentrations were measured by the Bradford method with bovine gamma globulin as a standard. The typical yield of protein was 15 to 20 μg/10^6 cells for cytoplasmic extracts and 5 to 10 μg/10^6 cells for nuclear extracts.

Western blot (immunoblot) analysis. Samples (15 μg) of total nuclear and cytoplasmic extracts were electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) apparatus. The proteins were then transferred overnight from the gel to a nitrocellulose filter (Hybond C; Amersham) by electroblotting at 50 mA (Trans-Blot Cell; Bio-Rad) in a buffer containing 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) (pH 11.0) and 10% methanol. The blotted filter was blocked for 30 min with 5% nonfat dry milk in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBS-T). The filter was then incubated with rabbit antisera (46) against p50/p105 (Ab1157), p65 (Ab1226), c-Rel (Ab265), or IκBα (Ab1309) in TBS-T plus 5% nonfat dry milk for 2 h at room temperature. These antisera were used at a 1:10,000 (Ab265), 1:5,000 (Ab1157 and Ab1226), or 1:1,000 (Ab1309) dilution. After two washes for 10 min with TBS-T plus 5% nonfat dry milk, goat anti-mouse IgG conjugated to horseradish peroxidase (Amersham) was added at 1:10,000 in TBS-T plus 5% nonfat dry milk and the mixture was incubated for 30 min at room temperature. After three washes as before, the blot was developed with the ECL chemiluminescence detection system (Amersham) according to the manufacturer's instructions and exposed to a film (Hyperfilm NP; Amersham).

Phosphorylation and immunoprecipitation experiments. Purified T cells were washed twice with phosphate-free RPMI 1640 medium (Gibco). T cells (4×10^7) were incubated for 3 h at 37°C in 2 ml of $^{32}P_i$-containing medium (phosphate-free RPMI 1640 supplemented with 1% fetal calf serum, 2 mM glutamine [Gibco], and 500 μCi of $^{32}P_i$ [NEN] per ml). After the cells were labeled for 3 h, the stimuli were added directly to the $^{32}P_i$-containing culture and the cells were stimulated for 5 h.

Nuclear and cytoplasmic extracts were prepared as described above with the addition of 1 μM metavanadate, 15 mM EDTA, 5 mM sodium PP_i, and 200 nM okadaic acid to buffers A and C as phosphatase inhibitors. A double-immunoprecipitation procedure (46) was performed to dissociate the protein-protein complexes. Antiserum against c-Rel, NFKB1 (p50/p105), or p65 (RelA) (2 μl of antiserum per 2×10^7 cell equivalents) was prebound to 50 μl of protein A-agarose beads (Pierce) by a 15-min incubation in 500 μl of 0.5% Nonidet P-40 (NP-40)–50 mM Tris (pH 7.4)–1 mM PMSF. Equivalent amounts of nuclear or cytoplasmic extracts were incubated with the precoated beads for 1 h in a 500-μl total volume of 0.5% NP-40 with 1 mM PMSF. The beads were then washed five times with 0.5% NP-40, and the precipitates were removed by being boiled for 5 min in 50 μl of SDS solubilizing buffer (125 mM Tris [pH 7.5], 3% SDS, 150 mM NaCl, and 5%

2-mercaptoethanol [Sigma]). The boiled immunoprecipitate was diluted to 500 μl with the same immunoprecipitation buffer and precipitated again for 1 h with protein A beads coated with the same antisera as before. These double immunoprecipitates were then subjected to SDS-PAGE on a 10% polyacrylamide gel as described above, dried under a vacuum, and exposed to a film.

Electrophoresis mobility shift assays (EMSA). Samples (8 μg) of nuclear extracts were incubated with the CD28RE oligonucleotide probe in a binding buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, and 2 μg of poly(dI-dC). The binding reactions were performed at room temperature for 20 min. For competition or supershift experiments, nuclear extracts (8 μg) were preincubated with competitor DNAs or antisera for 10 min before the addition of the radiolabeled CD28RE probe. The reaction mixtures (total volume, 20 μl each) were analyzed on 4% nondenaturing polyacrylamide gels in 0.25× Tris-borate-EDTA buffer containing 22.5 mM Tris-borate and 0.5 mM EDTA.

Oligonucleotides. Oligonucleotides were synthesized by the phosphoramidite method on an automated synthesizer (Cyclone Plus; Millipore). The radiolabeled double-stranded oligonucleotide probes were prepared by using the Klenow fragment of DNA polymerase I. The sequences of the oligonucleotides were as follows.

CD28RE (CD28RE site of the IL-2 promoter; positions −168 to −149):

5′-GATCGTTTAAAGAAATTCCAAA-3′
3′-CAAATTTCTTTAAGGTTTCTAG-5′

CD28RE mutant:

5′-GATCGTTTAAAGAAATTggAAA-3′
3′-CAAATTTCTTTAAccTTTCTAG-5′

HIV-κB (NF-κB binding sites of the HIV-1 LTR; positions −106 to −79):

5′-GATCAAGGGACTTTCCGCTGGGGACTTTCCAG-3′
3′-TTCCCTGAAAGGCGACCCCTGAAAGGTCCTAG-5′

RESULTS

Functional response of T cells to anti-CD28 costimulation. Like many signals for T-cell activation, induction of T-cell proliferation with anti-CD28 requires a second stimulus. We used two different stimuli, PMA and a combination of anti-CD2 MAbs, with anti-CD28 to test their relative effectiveness as T-cell mitogens. T-cell stimulation was assessed by four parameters: proliferation at 4 days, IL-2Rα expression, IL-2 secretion, and IFN-γ secretion at 24 h (Table 1). T cells stimulated with anti-CD28 alone had no response by any of these criteria. Also, anti-CD2 MAbs alone did not cause proliferation or IL-2 secretion, although IFN-γ secretion was induced to a small degree. PMA alone caused a small proliferative response and IL-2Rα expression, but neither IL-2 secretion nor IFN-γ secretion was observed. When combined with anti-CD28, PMA and anti-CD2 MAbs caused similarly vigorous proliferations at 4 days. In addition, IL-2, IL-2Rα, and IFN-γ expression was induced by both stimuli at 24 h, but the anti-CD2 plus anti-CD28 response was between 10 and 25% of that of the PMA plus anti-CD28 response for all assays.

Anti-CD28 plus PMA accelerated translocation of Rel family proteins into the nucleus compared with PMA alone. Our previous data showed that nuclear levels of Rel family proteins, p50 (NFKB1), p65 (RelA), and c-Rel, are enhanced by CD28 signalling (17). The mechanism(s) of enhanced levels of nuclear Rel proteins could be at either the transcriptional or the posttranscriptional level. One explanation for this is that CD28 signalling changed the kinetics of nuclear translocation of Rel family proteins. To test this possibility, we incubated cells for various periods with PMA in the presence or absence of the anti-CD28 MAb 9.3. Nuclear and cytoplasmic extracts were examined for NFKB1 (p50/p105), p65 (RelA), and c-Rel proteins by Western blot analysis (Fig. 1). The p50 precursor, p105, was used as an indicator of the integrity of the nuclear-cytoplasmic separation, as it is solely a cytoplasmic protein (24, 41, 46). Control experiments demonstrated that stimulation with anti-CD28 alone did not cause either nuclear translocation or de novo synthesis of c-Rel, p50 (NFKB1), or p65 (RelA) (7a).

We first tested the ability of CD28 signalling to enhance PMA-mediated c-Rel translocation (Fig. 1A). In the nuclei of resting T cells (Fig. 1A, lane 1), only p50 (NFKB1) was detectable. Upon stimulation with PMA alone, levels of c-Rel and p50 (NFKB1) in the nucleus progressively increased; however, p65 (RelA) translocation was not significant (Fig. 1A, lanes 2 to 6). Anti-CD28 MAb stimulation accelerated the nuclear translocation of c-Rel and p65 (RelA), with significant levels of both proteins first visible within 3 h (Fig. 1A, lane 8). In addition, the levels of p50 (NFKB1) and c-Rel in the nucleus were enhanced at later times up to 12 (Fig. 1A, compare lanes 6 and 11) and 24 (7a) h. In contrast, p65 (RelA) translocation was transient, peaking within 3 to 6 h (Fig. 1A, lanes 8 and 9). The late induction of nuclear c-Rel expression correlates with the kinetics of IL-2 secretion (7a).

Examination of cytoplasmic extracts revealed that both PMA alone (Fig. 1A, lanes 13 to 17) and anti-CD28 plus PMA (lanes 18 to 22) induced c-Rel and p50 (NFKB1) expression. In contrast, cytoplasmic p65 (RelA) levels were not induced after activation but, rather, decreased (Fig. 1A, compare lane 18 with lanes 19 to 22) as p65 (RelA) translocated into the nucleus (lanes 8 to 10). When both cytoplasmic and nuclear extracts are considered (Fig. 1A, compare lanes 10 and 22 with lanes 5 and 16), anti-CD28 plus PMA induced more synthesis of p105/p50 (NFKB1) than did PMA alone.

To ensure that CD28-mediated enhanced translocation of Rel family proteins is not unique to the PMA signal, the effects of anti-CD28 MAb on anti-CD28 MAb-mediated translocation were similarly examined by Western blot analysis (Fig. 1B). Anti-CD2 MAbs alone induced only weak translocation of c-Rel with no detectable p65 (RelA) in the nucleus (lanes 2 to

TABLE 1. Proliferation and lymphokine secretion after anti-CD28 costimulation

Stimulus	T-cell proliferation (10^3 cpm)	IL-2R[a]	IL-2 (ng/ml)	IFN-γ (ng/ml)
Medium	0.3	<1.0	<0.1	<0.2
Anti-CD2	0.8	3.6	<0.1	0.2
PMA	4.5	18.1	<0.1	<0.2
PHA	0.3	ND[b]	ND	ND
Anti-CD28	0.1	1.5	<0.1	<0.2
Anti-CD2 + anti-CD28	139.0	20.0	1.8	0.4
PMA + anti-CD28	124.7	87.8	11.0	4.5
PHA + PMA	107.2	83.8	6.2	1.5

[a] For IL-2R expression, data are expressed as percent positive over an arbitrary gate.
[b] ND, not determined.

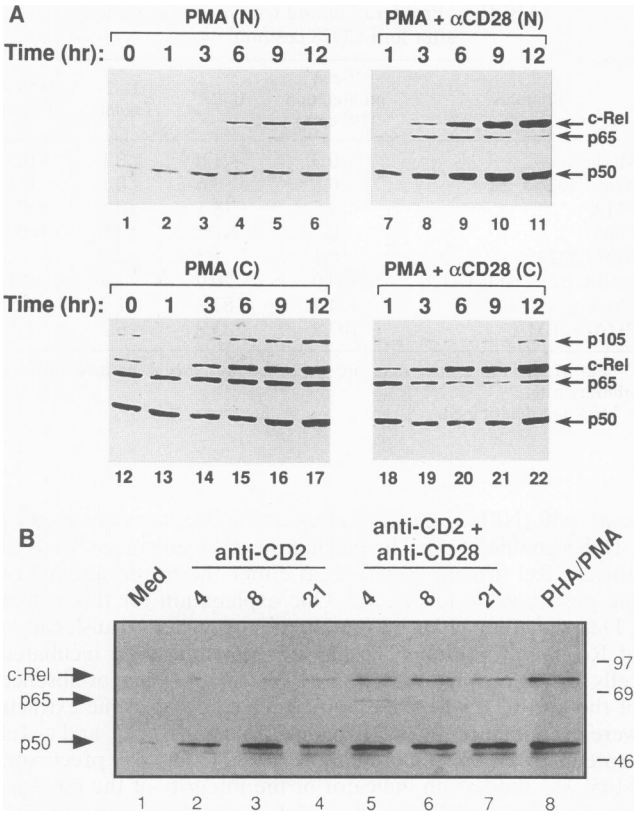

FIG. 1. Enhancement of nuclear translocation of Rel family proteins by anti-CD28 MAb compared with phorbol ester alone. (A) Purified human peripheral blood T cells were stimulated with either PMA alone (5 ng/ml) or PMA plus anti-CD28 MAb 9.3 (1:2,000 dilution). (B) Purified human T cells were stimulated with anti-CD2 ($T11_2$ plus $T11_3$; 1:2,000 dilution each) alone, anti-CD2 plus anti-CD28 (9.3) (1:2,000 dilution), or 0.5 μg of PHA per ml plus 5 ng of PMA per ml. Purified T cells were stimulated for the times (hours) indicated above the lanes and separated into nuclear and cytoplasmic extracts as described in Materials and Methods. Western blots made from these extracts (15 μg per lane) were probed for p50/p105 (NFKB1), p65 (RelA), and c-Rel with antisera Ab1157, Ab1226, and Ab265, respectively. The p105 (NFKB1) band detected in the cytoplasmic extract is an indicator of the integrity of the separation; p105 is an exclusively cytoplasmic protein. Molecular masses (in kilodaltons) are indicated on the right.

4), correlating with their weak ability to induce IL-2Rα expression. Like PMA, anti-CD28 enhanced the nuclear levels of p50 (NFKB1), p65 (RelA), and c-Rel (Fig. 1B, lanes 5 to 7), with the most significant enhancement detected for c-Rel. The p65 (RelA) levels diminished after 4 h, but c-Rel and p50 (NFKB1) levels remained high at all times. In comparison, control stimulation with PHA plus PMA for 21 h induced nuclear levels of c-Rel and p50 (NFKB1) marginally higher than those of anti-CD2 plus anti-CD28 costimulation; however, this time was too late to detect significant levels of nuclear p65 (RelA) (Fig. 1B, lane 8). In contrast to PMA signalling, anti-CD2 with or without anti-CD28 did not induce increased cytoplasmic levels of any of these proteins (7a). Anti-CD28-induced enhancement of nuclear c-Rel levels was reproducible for PMA (seven of seven experiments) and anti-CD2 (five of six experiments) costimulation.

IL-2 secretion correlated with enhanced c-Rel translocation. By using a panel of 10 anti-CD28 MAbs, we tested the

TABLE 2. Enhanced translocation of c-Rel correlates with T-cell activation

Anti-CD28 MAb	Isotype	Proliferation (10^3 cpm)[a]	IL-2 (ng/ml)[b]	Enhanced translocation[c]
None		1.0	0.1	
9.3	IgG2a	30.3	>100.0	Yes
KOLT2	IgG1	27.1	76.0	Yes
CD28.1	IgG1	25.9	64.0	Yes
CK248	IgM	35.2	100.0	Yes
M-T281	IgM	ND	0.1	No
CD28.2	IgG1	20.3	20.0	Yes
CD28.4	IgM	2.6	0.3	No
CD28.6	IgG2a	30.7	>100.0	Yes
L293	IgG1	24.6	37.0	Yes
15E8	IgG1	26.7	15.0	Yes

[a] Data obtained after stimulation for 3 days. ND, not determined.
[b] For IL-2 bioassays, data were collected after stimulation for 24 h.
[c] The presence of enhanced translocation after anti-CD28 stimulation compared with that after stimulation with PMA alone is derived from several experiments, one of whose results are shown in Fig. 2.

correlation between enhanced c-Rel translocation and stimulatory activity in a single donor. In Table 2, the panel of anti-CD28 MAbs in combination with PMA was tested for both T-cell proliferation and IL-2 secretion. PMA alone failed to cause significant cellular proliferation or IL-2 secretion. Among the panel of anti-CD28 MAbs tested, only M-T281 and CD28.4 failed to cooperate with PMA to affect proliferative and IL-2 responses. In several repeated experiments with T cells from different donors, the relative levels of IL-2 secretion among stimulatory anti-CD28 MAbs were different, thus reflecting the biological diversity of human donors. However, M-T281 and CD28.4 were always nonstimulatory.

The correlation between IL-2 secretion and enhanced translocation was tested with some of these anti-CD28 panel MAbs. The capacity of different anti-CD28 panel MAbs to cooperate with PMA in enhancing nuclear levels of c-Rel after 13 h of stimulation was examined by Western blot analysis (Fig. 2). IL-2 levels in the supernatant from the same experiment were measured by ELISAs. PMA alone induced translocation of c-Rel into the nucleus, but only minimal IL-2 secretion was

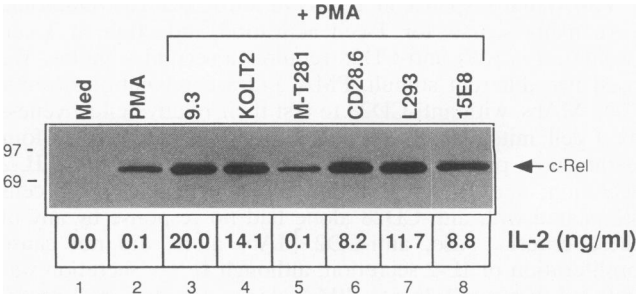

FIG. 2. Correlation of IL-2 secretion with enhanced c-Rel translocation by using the anti-CD28 panel MAbs. Purified T cells were stimulated for 13 h with either 5 ng of PMA alone per ml or PMA plus each of the anti-CD28 panel MAbs provided by the 5th International Workshop of Leukocyte Differentiation Antigens (all at a 1:2,000 dilution). Nuclear extracts were prepared from these stimulated cells and were used for Western blot analysis. The blot was probed with anti-c-Rel antiserum Ab265. At the same time, supernatants from this experiment were assayed for IL-2 activity by ELISA and expressed as nanograms per milliliter. Molecular masses (in kilodaltons) are indicated on the left.

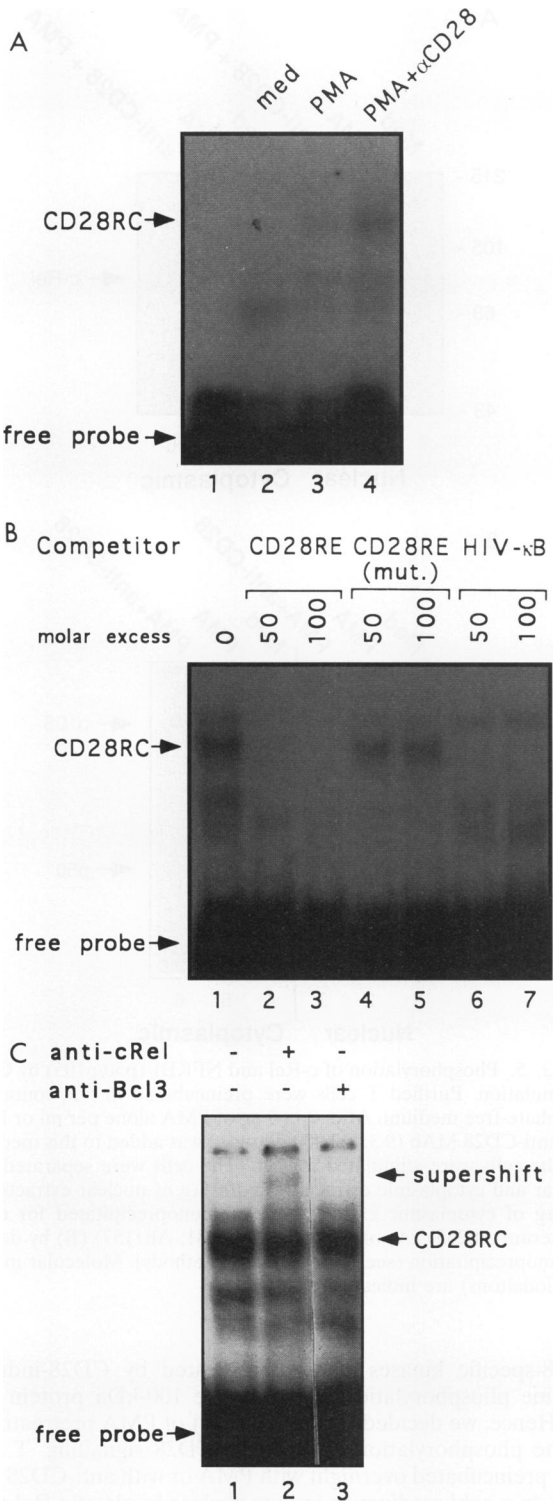

FIG. 3. CD28RC enhanced by, but not specific to, CD28 stimulation. (A) Purified T lymphocytes were stimulated with either 5 ng of PMA alone per ml or PMA plus anti-CD28 MAb (9.3; 1:2,000 dilution) for 40 h. An 8-μg sample of nuclear extracts prepared from these cells was subjected to the EMSA using the labeled CD28RE as a probe (see Materials and Methods). (B) Nuclear extracts prepared from the cells stimulated with anti-CD28 plus PMA were subjected to the EMSA. For the competition experiment, nuclear extracts were incubated for 10 min with excess cold oligonucleotides containing CD28RE, mutated CD28RE, or the HIV-1 κB site before the addition of labeled CD28RE probe. (C) Nuclear extracts prepared from the cells stimulated with anti-CD28 plus PMA were subjected to the supershift experiment. Nuclear extracts were incubated for 10 min with 1 μl of anti-c-Rel (Ab265) or anti-Bcl-3 (Ab1348) before incubation with labeled CD28RE. The supershifted band containing c-Rel is indicated.

observed (Fig. 2, lane 2). Corresponding with the IL-2 secretion, nuclear levels of c-Rel were higher than those induced by PMA alone for all anti-CD28 MAbs (Fig. 2, lanes 3 to 8) with the exceptions of M-T281 (lane 5) and CD28.4 (Table 2) (7a). The lower levels of IL-2 shown in Fig. 2 compared with those in Table 2 are due to the shorter incubation time (13 instead of 24 h). These data showed that enhanced nuclear translocation of c-Rel correlated with effective CD28 signal transduction.

The c-Rel-containing CD28RC is enhanced by, but not specific to, CD28 signalling. The CD28RC has been reported only in the presence of two signals, CD28 and PMA (PMA alone was not sufficient for CD28RC induction [14, 15]). The implication of these studies is that this complex is rate limiting for IL-2 secretion. However, these studies were performed after 2 h of stimulation (14, 15), a point at which nuclear c-Rel was not detectable in the PMA-treated cells (Fig. 1A). We suspected that the accelerated kinetics of nuclear c-Rel levels after anti-CD28 costimulation would exaggerate the relative difference between anti-CD28 plus PMA and PMA at the 2-h time point (Fig. 1A). Therefore, we tested for the presence of the CD28RC by EMSA at a later time point (40 h) (Fig. 3A). At this time, cells incubated in medium alone had no binding activity (lane 2). Both cells stimulated with PMA and those stimulated with anti-CD28 plus PMA contained detectable levels of CD28RC (Fig. 3A, lanes 3 and 4), but the anti-CD28-stimulated cells had higher levels of CD28RC (lane 4). This CD28RC is not just a nonspecific product of the prolonged stimulation, since cells stimulated for the same amount of time with PMA contained no detectable levels of NF-AT, another nuclear factor known to be a rate-limiting factor in IL-2 secretion after T-cell stimulation. However, PHA plus PMA stimulated high levels of NF-AT (7a). In addition, the specificity of this CD28RC was determined by successful competition with an excess of cold CD28RE-containing oligonucleotides (Fig. 3B, lanes 2 and 3). Competition was not observed when mutated CD28RE-containing oligonucleotides were added (Fig. 3B, lanes 4 and 5). Consistent with other results (58), binding of the CD28RC was also efficiently inhibited when the HIV-1 κB-containing oligonucleotide was used (Fig. 3B, lanes 6 and 7).

The presence of c-Rel in the CD28RC was confirmed by incubating anti-c-Rel with the same nuclear extract stimulated with anti-CD28 plus PMA before the EMSA (Fig. 3C). In the absence of antibodies, a strong CD28RC was detected (lane 2). Incubation with anti-c-Rel caused a proportion of the CD28RC band to supershift (Fig. 3C, lane 3), indicating that at least a portion of the CD28RCs contained c-Rel. Incubation with the control antiserum against Bcl-3, an IκB-like protein, did not cause a supershifted band (Fig. 3C, lane 4).

CD28 signalling induces a prolonged downregulation of IκBα. The degradation of IκBα with PMA is a transient phenomenon, since protein levels return to baseline within 1 h (3, 4, 52). One possible explanation for enhanced translocation of c-Rel by CD28 signalling is a prolonged loss of IκBα. To test this possibility, we stimulated cells with PMA or anti-CD28 plus PMA for various periods up to 12 h and probed Western blots prepared from the cytoplasmic extracts with an antiserum against IκBα (Fig. 4). Stimulation with PMA alone did not

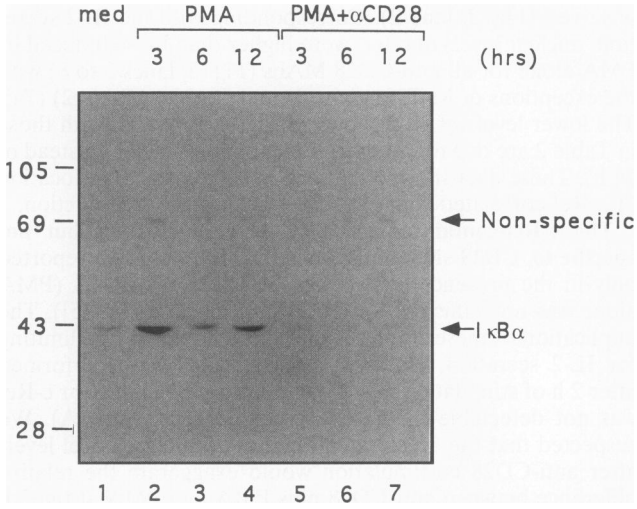

FIG. 4. CD28 costimulation leads to prolonged downregulation of IκBα levels. Purified peripheral blood T lymphocytes were stimulated with 5 ng of PMA alone per ml or PMA plus anti-CD28 MAb (9.3; 1:2,000 dilution) for various periods. Cytoplasmic extracts (15 μg per lane) prepared from the treated cells were subjected to Western blot analysis. The filter was probed with anti-IκBα (Ab1309). Molecular masses (in kilodaltons) are indicated on the left.

affect the levels of IκBα compared with those of the medium control (Fig. 4, PMA lanes). In contrast, the addition of anti-CD28 led to decreased but visible levels of IκBα protein (Fig. 4, PMA+αCD28 lanes).

Anti-CD28 signalling enhances the levels of phosphorylated c-Rel in the nucleus. Although the role of phosphorylation of the Rel family proteins has only been suggested (41), this phosphorylation may influence their interaction with other proteins to create the CD28RC. In addition to the effect of CD28 signalling on overall levels of c-Rel, we assayed its ability to enhance the levels of phosphorylated c-Rel in the nucleus compared with the use of PMA alone (Fig. 5). Cellular pools of ATP were saturated with $^{32}P_i$ for 3 h before the addition of the stimulus. Nuclear and cytoplasmic c-Rel (Fig. 5A) and NFKB1 (p50/p105) (Fig. 5B) were analyzed by a double-immunoprecipitation procedure (46) that eliminates most interacting proteins. Because nuclear c-Rel is not present to a significant degree until several hours after the initiation of stimulation, we chose to stimulate cell cultures for 5 h. At this time, stimulation with both PMA and anti-CD28 plus PMA induced nuclear expression of c-Rel and p50 (NFKB1) (Fig. 1).

Stimulation of T cells with PMA or anti-CD28 plus PMA induced the phosphorylation of c-Rel detected by the anti-c-Rel antiserum Ab265 (Fig. 5A). In the cytoplasm, anti-CD28 costimulation did not appreciably change the levels of phosphorylated c-Rel (Fig. 5A, compare lanes 5 and 6). However, CD28 costimulation increased the levels of phosphorylated c-Rel in the nucleus (compare lanes 2 and 3). In light of the fact that there is more nuclear c-Rel in the cells treated with anti-CD28 plus PMA than in PMA-treated cells (Fig. 1), it is likely that the phosphorylation of c-Rel per mole of protein has not changed. In contrast to c-Rel, p50 (NFKB1) phosphorylation was not detectable at the same 5-h time point (Fig. 5B). The cytoplasmic p50 precursor, p105, was phosphorylated after PMA treatment (Fig. 5B, lane 5), but the levels of p105 phosphoprotein decreased after the addition of anti-CD28 (lane 6).

The requirement for overnight pretreatment to induce

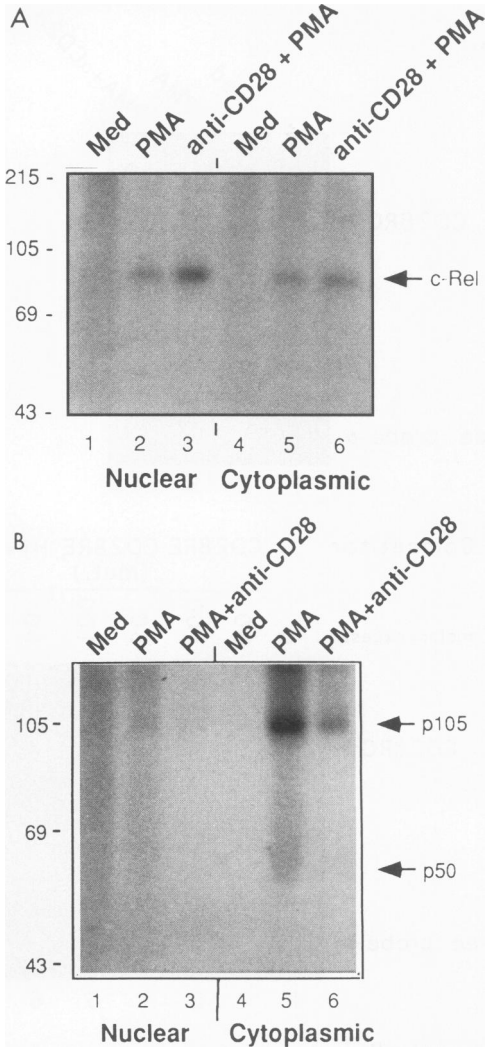

FIG. 5. Phosphorylation of c-Rel and NFKB1 (p50/p105) by CD28 costimulation. Purified T cells were preincubated in $^{32}P_i$-containing phosphate-free medium. After 3 h, 5 ng of PMA alone per ml or PMA plus anti-CD28 MAb (9.3; 1:2,000 dilution) was added to this medium, and the cells were stimulated for 5 h. The cells were separated into nuclear and cytoplasmic extracts, and 100 μg of nuclear extracts and 200 μg of cytoplasmic extracts were immunoprecipitated for c-Rel (antiserum Ab265) (A) or p50/p105 (NFKB1; Ab1157) (B) by double immunoprecipitation (see Materials and Methods). Molecular masses (in kilodaltons) are indicated on the left.

CD28-specific kinases was demonstrated by CD28-induced tyrosine phosphorylation of a putative 100-kDa protein (36, 57). Hence, we decided to test the effect of PMA pretreatment on the phosphorylation of c-Rel by CD28 signalling. T cells were preincubated overnight with PMA or with anti-CD28 plus PMA in a cold medium to induce nuclear levels of c-Rel (Fig. 6). In two of the samples preincubated with PMA, anti-CD28 was added immediately before the last hour (lanes 2 and 4). These treated cells were labeled with radioactive $^{32}P_i$ immediately before the last 4 h. Nuclear and cytoplasmic extracts were immunoprecipitated for c-Rel by using anti-c-Rel (Ab265) as described above. Under these conditions, the levels of phosphorylated c-Rel in the nuclei of cells treated with anti-CD28 plus PMA (Fig. 6, lane 3) were 30% higher than those of PMA-treated cells (lane 1). In contrast, the levels of phosphor-

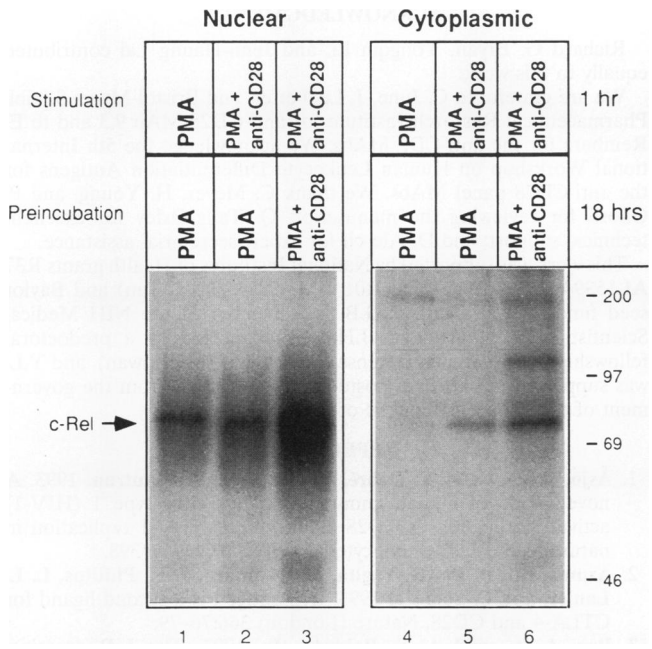

FIG. 6. Phosphorylation of c-Rel by CD28 costimulation in cells preincubated with PMA. Purified T cells were preincubated for 15 h in cold medium with 5 ng of PMA per ml in the presence or absence of anti-CD28 MAb. At the end of the incubation, the cells were then washed in phosphate-free medium with 10% dialyzed fetal calf serum and stimulated for an additional 4 h with the same stimulus in the medium containing $^{32}P_i$. In one of the wells, anti-CD28 MAb was added to the culture for the last hour (lanes 3 and 6). Nuclear and cytoplasmic extracts were prepared and immunoprecipitated for c-Rel as described for Fig. 5. In lane 6, anti-c-Rel Ab265 also precipitated three additional phosphoproteins with unknown identities; however, they were not consistently detected in repeat experiments. Molecular masses (in kilodaltons) are indicated on the right.

ylated c-Rel in the cytoplasm were quite low in PMA-treated cells (Fig. 6, lane 4). Cytoplasmic phosphorylation of c-Rel occurred to a significant degree only when anti-CD28 was added, either throughout the culture period (Fig. 6, lane 6) or immediately before the last hour (lane 5). The changes in the levels of phosphorylated c-Rel (compare lanes 3, 5, and 6 with lanes 1 and 4) may simply reflect the changes in the steady-state levels of c-Rel (Fig. 1). However, it is still possible that CD28 signalling induces the phosphorylation of c-Rel on residues different from those phosphorylated by PMA.

DISCUSSION

A major challenge in the study of CD28 signalling is the lack of a detectable response with the treatment of soluble bivalent anti-CD28 alone (reviewed in references 26 and 34). No calcium flux, inositol phosphate release, or significant tyrosine kinase activity occurs unless CD28 is super-cross-linked (31, 42, 43). Although a direct study of CD28 signalling has not been possible, two distinct events stimulated by CD28 ligation can be detected in the presence of a primary signal such as PMA: the tyrosine phosphorylation of several proteins (36, 57) and the induction of the CD28RC in the nucleus (14, 58). However, the tyrosine-phosphorylated proteins remain unidentified, and no signalling steps between the surface and the nucleus have been elucidated.

Two observations point to the Rel family proteins as important mediators of the CD28 signalling pathway. First, Rel family proteins are present in the CD28RC (17) (Fig. 3C). Second, NF-κB plays an important role in the CD28-mediated enhancement of transcription from the HIV-1 LTR (21, 56) and the IL-2Rα promoter (10). These observations suggest that CD28 signal transduction directly affects the posttranslational regulation of the Rel family proteins. Because c-Rel binds to and activates the CD28RE within the IL-2 promoter (17), we focused this study on c-Rel.

Our data show that in human peripheral blood T cells, CD28 costimulation with anti-CD28 MAb plus PMA enhanced and accelerated the nuclear translocation of p50 (NFKB1), p65 (RelA), and c-Rel compared with that of PMA treatment alone. This enhanced translocation correlated with the ability of CD28 costimulation to stimulate T-cell proliferation, IL-2 secretion, IFN-γ secretion, and IL-2Rα surface expression (Table 1). For c-Rel and p50 (NFKB1), the enhancement had late kinetics, with nuclear levels increasing progressively up to 24 h. Higher levels of p50 (NFKB1) and c-Rel were also observed in cytoplasmic extracts after stimulation with PMA and anti-CD28 plus PMA, with kinetics comparable to their induction in the nucleus. In contrast, the enhancement of p65 (RelA) translocation with anti-CD28 peaked transiently from 3 to 6 h. The decline in p65 (RelA) levels in the cytoplasm suggests that p65 (RelA) was either degraded in the cytoplasm or quantitatively translocated to the nucleus without replenishment of the cytoplasmic p65 (RelA) levels by de novo protein synthesis. When combined with anti-CD2, CD28 signalling also enhanced the translocation of Rel family proteins, particularly c-Rel, correlating with the ability of CD28 signalling to cooperate with CD2 signalling to induce T-cell activation. Thus, enhancement of translocation with CD28 signalling is not unique to PMA but occurs with other types of mitogenic signals.

Although enhancement of p50 (NFKB1), p65 (RelA), and c-Rel correlated with T-cell stimulation, the relative contribution of each in the assembly of the CD28RC is not clear. Because c-Rel activates the CD28RE-driven reporter gene expression in a cotransfection assay (17), we thought that enhanced translocation of c-Rel would correlate closely with functional CD28 signalling. To address this issue, we tested a panel of anti-CD28 MAbs that differed in their ability to stimulate T-cell proliferation at a time (15 h) optimal for c-Rel translocation. We found that the ability of the anti-CD28 MAbs to stimulate IL-2 secretion in combination with PMA correlated with their ability to enhance the nuclear translocation of c-Rel. Indeed, there exists a further correlation between the late kinetics of nuclear c-Rel translocation and late IL-2 accumulation in the medium (7a).

The regulation of Rel family protein translocation may be explained by our observation that IκBα was absent from the cytoplasm for up to 12 h following CD28 signalling, in contrast to the results with PMA signalling alone. Previous studies have found that cytoplasmic IκBα is degraded within minutes of cellular stimulation, thus allowing NF-κB to move into the nucleus (3, 4, 52). However, IκBα protein in the cytoplasm rapidly reappears, returning to prestimulation levels within 1 h. These observations are consistent with the lack of change in IκBα levels that we saw following stimulation with PMA alone for 3 h or more. CD28 signalling led to the long-term loss of IκBα, allowing more of the Rel family proteins to move into the nucleus. This could be explained in at least three ways: (i) CD28 signalling activates a kinase that phosphorylates IκBα, leading to IκBα degradation; (ii) CD28 signalling activates a protease that degrades IκBα; or (iii) CD28 signalling prevents the reappearance of IκBα after its initial degradation by downregulation of IκBα synthesis. In addition, the decreased

levels of p105 (NFKB1) after CD28 stimulation (Fig. 1 and 6) suggest that CD28 can decrease the levels of more than one ankyrin-containing protein.

The mere presence of c-Rel and p50 (NFKB1) in the nucleus was not sufficient for IL-2 secretion, since PMA alone stimulated significant nuclear levels of c-Rel in the absence of an IL-2 induction. Indeed, the CD28RC was seen in the nucleus of cells stimulated with PMA alone, albeit at low levels. Therefore, a functional modification of c-Rel (or c-Rel-interacting proteins) must play an important role in CD28 signalling. Although the role of the phosphorylation of Rel family proteins is not well characterized, phosphorylation can affect their function, perhaps through regulation of protein-protein interactions (39, 41). We have demonstrated that CD28 signalling, besides enhancing the translocation of c-Rel, also enhanced the levels of phosphorylated c-Rel in the nucleus. Two mechanisms could explain this observation. First, CD28 signalling may induce the phosphorylation of c-Rel on residues different from those phosphorylated by PMA. Second, the increased phosphorylation may reflect the increased overall levels of the c-Rel protein in the nucleus.

One result of CD28 signalling is the appearance of the CD28RC in the nucleus. This nuclear factor binds to the IL-2 promoter at a unique CD28RE site that is related to the κB site. Previous studies have shown that CD28RC is not seen in the absence of CD28 signalling (14, 15). In contrast, our study showed that small amounts of CD28RC were induced following PMA stimulation alone, indicating that CD28RC is enhanced by, but not specific to, CD28 signalling. There are two major differences between these two studies. First, our studies were done with human peripheral blood T cells instead of the Jurkat T-cell line (for a review, see reference 26). Second, we used a late time point of 40 h instead of 2 h, the time used in previous studies. We chose this late point for two reasons. First, the highest levels of c-Rel were detected at 40 h (7a), a time at which nuclear factor inductions in PBMCs are at a peak (19). Second, the early time points may mask the presence of a PMA-induced CD28RC. The accelerated kinetics of c-Rel translocation induced by anti-CD28 plus PMA, as opposed to that induced by PMA alone, is most evident at the 2-h time point. Thus, the difference between the CD28RC levels induced by PMA and those induced by PMA plus anti-CD28 would be similarly exaggerated at the 2-h time point. Others have also shown that CD28RC is not specific to CD28 signalling; anti-CD3 plus PMA can also induce CD28RC and transcription from CD28RE linked to a reporter gene (9). These results suggest that CD28RE may mediate other signalling pathways in addition to the CD28 signal transduction pathway.

Our results indicate that c-Rel is a target of CD28-mediated signal transduction. In light of this, it is interesting that Rel family proteins also interact with other classes of transcription factors such as Fos/Jun (50), C/EBP family proteins (51), ATF family proteins (13, 28), HMG I(Y) proteins (13), Sp1 (44), TATA-binding protein (29, 61), and TFIIB (61). We are also studying the potential roles of other Rel family proteins, including p50 (NFKB1) and p65 (RelA), in mediating CD28 signal transduction. Our identification of c-Rel as the transcription factor at the receiving end of CD28 signalling should provide an important initial step towards future identification of other nuclear factors and kinases or phosphatases that are involved in the c-Rel-mediated CD28 signal transduction pathway.

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