Inhibition of activation of transcription factor AP-1 by CD28 signalling in human T-cells

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Co-stimulation of T-lymphocytes by T-cell receptor (TcR) occupancy and activation of the CD28 surface molecule results in enhanced proliferation and interleukin 2 (IL-2) production. The increase in IL-2 gene expression triggered by CD28 involves a κ B-like sequence in the 5'-regulatory region of the IL-2 promoter, called CD28-responsive element. Stimulation of T-cells by agonistic anti-CD28 antibodies in conjunction with phorbol 12-myristate 13-acetate (PMA)- or TcR-derived signals induces the enhanced activation of the transcription factor NF- κ B. Here we

report that CD28 engagement, however, exerts opposite effects on the transcription factor AP-1. Whereas anti-CD28 together with PMA increased the DNA binding and trans-activation activity of NF- κ B, PMA-induced activation of AP-1 was significantly suppressed. The inhibitory effect exerted by anti-CD28 was observed at the level of DNA binding as well as in functional reporter-gene assays. These results suggest that the two transcription factors are independently regulated and may perform different functions during T-cell activation.

INTRODUCTION

Activation of T-lymphocytes requires the presence of two signals which are provided by an antigen-specific stimulus delivered through the CD3–T-cell receptor (TcR) complex and an antigen-independent mechanism referred to as co-stimulation [1,2]. Numerous studies have indicated that TcR engagement in the absence of co-stimulation may induce a state of unresponsiveness (anergy) or programmed cell death (apoptosis) (reviewed in refs. [1,2]). A major co-stimulatory signal is triggered by the activation of the T-cell surface molecule CD28, which is the counter structure of the B7-1 and B7-2 antigen present on activated B cells, dendritic cells and monocytes [3–5]. CD28 is expressed on almost all CD4⁺ and about 50 % of CD8⁺ T-cells. Signalling via CD28 readily synergizes with signals through the CD3–TcR for increased cytokine production and cell proliferation [6–9]. CD28 functions as a regulator of interleukin-2 (IL-2) expression at the level of both gene transcription and mRNA stabilization. The transcriptional stimulation of IL-2 expression by the CD28 co-stimulus is controlled by a κ B-like *cis*-regulatory element (CD28 regulatory element) in the IL-2 promoter that can be occupied by transcription factors of the nuclear factor (NF)- κ B family [10,11]. Co-stimulation of T-cells with agonistic anti-CD28 antibodies together with anti-CD3 or agents that mimic TcR-induced protein kinase C activation results in increased nuclear amounts of NF- κ B proteins [12].

The major biochemical events initiated by CD28 are largely unknown. Several pieces of evidence suggest a signal-transduction pathway that is distinct from the TcR [3]. CD28 signalling is resistant to treatment with the immunosuppressant cyclosporin A and does not involve protein kinase C translocation. Recently, it has been observed that CD28 mediates tyrosine phosphorylation of a 100 kDa protein [13] and activation of phosphatidylinositol-3 kinase [14] and phospholipase C [15]. Activation of NF- κ B by cytokines and other agents has been shown to rely on the intracellular formation of reactive oxygen

intermediates and redox-regulated processes [16–18]. Among other stimuli, CD28-mediated activation of NF- κ B is inhibited by antioxidant compounds, such as *N*-acetylcysteine and dithiocarbamates [19]. A contribution of redox-regulated processes is further supported by the observation that co-stimulation via CD28 leads to a rapid decrease in intracellular thiols [19] (M. Los, W. Dröge and K. Schulze-Osthoff, unpublished work).

Besides NF- κ B, another widely used mediator of immediate-early gene expression is the transcription factor AP-1, which is involved in activation of gene expression associated with various growth and differentiation processes (reviewed in ref. [20]). AP-1 is composed of a heterodimeric complex of the *c-fos* and *c-jun* gene products and is activated by complex mechanisms consisting of transcriptional and post-translational events. We and others have recently shown that alterations of the redox state may exert opposite effects on NF- κ B and AP-1 activation [21,22]. Several antioxidants, which largely inhibited phorbol ester-induced activation of NF- κ B, increased AP-1 activation. This suggests that the two transcription factors may be oppositely regulated by the redox state of the cell. Here we report that, in addition, CD28-triggered signalling differentially affects the two transcription factors. Whereas agonistic anti-CD28 antibodies synergized with phorbol esters for NF- κ B activation, in contrast, DNA binding and trans-activation activity of AP-1 were significantly inhibited.

MATERIALS AND METHODS

Cells and reagents

The human T-cell line Jurkat (subclone JR) was grown in RPMI 1640 supplemented with 10 % (v/v) heat-inactivated fetal-calf serum and antibiotics. CLB-CD28/1, an IgG₁ monoclonal antibody directed against human CD28, was provided by Dr. van Lier (Amsterdam) and used after purification by Protein G–Sepharose chromatography. Phorbol 12-myristate 13-acetate (PMA) and H₂O₂ (perhydrol) were purchased from Sigma.

Abbreviations used: TcR, T-cell receptor; IL-2, interleukin 2; PMA, phorbol 12-myristate 13-acetate; CAT, chloramphenicol acetyltransferase; e.m.s.a., electrophoretic-mobility-shift assay; TBS, Tris-buffered saline; NF, nuclear factor.

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Acetyl-CoA, poly(dI-dC) and [14 C]chloramphenicol were obtained from Boehringer-Mannheim and Amersham respectively.

Oligonucleotides and plasmids

The sequences of the oligonucleotides used to detect the DNA-binding activities of NF- κ B and AP-1 were as follows (the binding sites are underlined): NF- κ B, 5'-AGCTTCAGAGGGGATTTCGAGAGG-3'; AP-1, 5'-AGCTTGATGAGT-CAGCCGGATC-3'. The oligonucleotides were annealed with complementary strands containing 5'-overhanging ends and labelled using Klenow polymerase and [α - 32 P]dCTP. The labelled probe was purified from free nucleotides on push columns (Stratagene). The plasmid TRE2CAT used to determine AP-1-dependent trans-activation activity was generated by insertion of two AP-1-binding sites 5' to the tk promoter of pBLCAT2. This vector contains the HSV basal tk promoter from residues -109 to +51 linked to the chloramphenicol acetyltransferase (CAT) gene [23]. The CAT reporter plasmid termed NF- κ B4CAT has been described previously [17]. It contained four spaced NF- κ B-binding motifs upstream of the tk promoter in pBLCAT2. To detect non-specific effects on CAT expression, transfections with the parent vector pBLCAT2 were performed.

Electrophoretic-mobility-shift assay (e.m.s.a.)

Cells were stimulated at 1×10^6 /ml with PMA (25 ng/ml) or anti-CD28 (1.5 μ g/ml). After the indicated time points, cells were washed with ice-cold PBS and lysed in a high-salt buffer containing 20 mM Hepes, pH 7.5, 400 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 20% (v/v) glycerol, 1% Nonidet P40, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride and 0.01% aprotinin. After 10 min of incubation on ice, extracts were cleared by centrifugation.

Equal amounts of the extracts (about 10 μ g of crude protein; determined by the Bio-Rad assay kit) were incubated with the NF- κ B- and AP-1-specific 32 P-labelled oligonucleotides. Binding reactions were performed in a 20 μ l volume containing 2–4 μ l of extract, 4 μ l of 5 $\times$ binding buffer (20 mM Hepes, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 2.5 mM MgCl₂, 20% Ficoll), 2 μ g of poly(dI-dC) as non-specific competitor DNA, 2 μ g of BSA and 10000–15000 c.p.m. of the labelled oligonucleotide. After 30 min of the binding reaction at room temperature, samples were loaded on a 4% non-denaturing polyacrylamide gel and run in 45 mM Tris-borate, 1 mM EDTA, pH 8.0. For specificity controls a 200-fold excess of unlabelled probe was applied.

Transfections and CAT assays

Transfections were performed by the DEAE-dextran method combined with chloroquine treatment. Briefly, 1.5×10^7 cells were washed in Tris-buffered saline (TBS) and resuspended in 2 ml of TBS containing 200 μ g of DEAE-dextran, 400 μ g of chloroquine and 5–15 μ g of plasmid DNA. Cells were incubated at room temperature with frequent agitation. After 30 min, the cells were washed in TBS and resuspended in 50 ml of culture medium. After 24 h, portions of batch-transfected cells were stimulated for 12–15 h with anti-CD28 (1.5 μ g/ml) or PMA (25 ng/ml). Cell extracts were prepared by three freeze/thaw cycles and measured for protein content. CAT activity was determined essentially as described [24]. Equal amounts of total cell protein were mixed in a 150 μ l volume with 0.53 mM acetyl-

CoA and 2.2 kBq of [14 C]chloramphenicol and incubated at 37 °C for 8–12 h. Acetylated chloramphenicol was extracted in ethyl acetate, resolved on t.l.c. plates and quantified on a t.l.c. linear analyser.

RESULTS

Jurkat T-cells were stimulated with anti-CD28 in the presence and absence of PMA. At 90 min after treatment, cell extracts were prepared and analysed by e.m.s.a. with an NF- κ B- and AP-1-specific oligonucleotide. As shown in Figure 1, PMA induced κ B-specific DNA-binding activity, which was not observed in unstimulated cells. Co-treatment of the cells with anti-CD28 in the presence of PMA induced a significant increase in NF- κ B binding (Figure 1, lane 4). Incubation with anti-CD28 in the absence of PMA, however, resulted in no or only a weak induction of NF- κ B binding (Figure 1, lane 2). These observations confirm previous reports that CD28 signals synergize with the phorbol ester- or anti-CD3-mediated NF- κ B activation [10–12].

As CD28-induced signalling has been reported to rely on the induction of pro-oxidant conditions and to be inhibited by antioxidants [12], we further analysed the above effects in the presence of H₂O₂. Cells treated with H₂O₂ revealed a marked stimulation of NF- κ B binding (Figure 1, lane 5). This DNA-binding activity was slightly enhanced on CD28 stimulation (Figure 1, lane 6). Compared with cells incubated without H₂O₂,

Figure 1 Effect of PMA, anti-CD28 and anti-CD28 + PMA on the activation of NF- κ B

Jurkat T-cells were stimulated for 90 min with PMA (25 ng/ml), with and without anti-CD28 (aCD28; 1.5 μ g/ml). H₂O₂ (100 μ M; lanes 5–8) was added 15 min before cell stimulation. Cell extracts were analysed by e.m.s.a. using a 32 P-labelled κ B-specific oligonucleotide. The position of the NF- κ B–DNA complex is marked by an arrowhead. Specificity of DNA binding was verified using a 200-fold excess of unlabelled oligonucleotide (not shown).

Figure 2 Effect of PMA, anti-CD28 and anti-CD28 + PMA on the activation of AP-1

Cells were stimulated as described in Figure 1. AP-1 DNA binding was analysed in cell extracts with an AP-1-specific oligonucleotide. The position of the AP-1-specific DNA complex is marked by an arrowhead. The weak additional band of low mobility observed in lanes 4, 6 and 7 represents unspecific protein binding or an AP-1 complex of different heterodimer composition.

Figure 3 Time course of induction of AP-1 DNA binding and inhibition by anti-CD28 treatment

Cells were stimulated with PMA and anti-CD28. After the indicated time points, cell extracts were prepared and analysed by e.m.s.a. with the AP-1-specific oligonucleotide. A section of the fluorogram is shown. The arrowhead indicates the position of the AP-1-specific DNA complex.

co-treatment of cells with anti-CD28 and PMA under pro-oxidant conditions resulted in a further enhancement of NF- κ B DNA binding (Figure 1; compare lanes 3 and 4 with 7 and 8). Thus these findings support the idea that PMA- and CD28-induced NF- κ B activation are mediated by redox processes.

The same experimental conditions were used to analyse the

Table 1 Effect of PMA, anti-CD28 and anti-CD28 + PMA on NF- κ B- and AP-1-dependent transactivation

Jurkat T-cells were transfected with NF- κ B4CAT, a CAT reporter plasmid controlled by four NF- κ B-binding sites, or the AP-1-driven CAT plasmid TRE2CAT. At 24 h after transfection, cells were stimulated with the indicated agents as described in Figure 1. After 15 h, cell extracts were prepared and analysed for CAT activity. Controls were performed with the parent plasmid pBLCAT2. The results show a representative experiment out of three independent transfections.

	Acetylation (%)		
	NF- κ B4CAT	TRE2CAT	pBLCAT2
-H ₂ O ₂			
Medium	3.1	5.2	1.1
Anti-CD28	5.7	4.9	1.2
PMA	29.6	52.1	0.9
PMA + anti-CD28	47.1	34.4	1.3
+H ₂ O ₂			
Medium	22.3	18.8	1.1
Anti-CD28	26.8	8.9	0.8
PMA	44.6	26.3	1.4
PMA + anti-CD28	57.6	10.9	1.2

effects on AP-1 activation. AP-1 DNA binding was already detectable in unstimulated cells but was significantly inhibited by anti-CD28 treatment (Figure 2, lanes 1 and 2). Treatment with PMA induced an increase in AP-1 DNA binding (Figure 2, lane 3). In contrast with NF- κ B, however, co-stimulation of the cells with anti-CD28 decreased phorbol ester-induced AP-1 activation to a similar level to that in unstimulated cells (Figure 2, lane 4). Of note is the fact that changes in DNA binding were not observed with an isotype-matched control antibody (results not shown). The inhibitory effect of anti-CD28 was even more pronounced under pro-oxidant conditions. H₂O₂ induced a slight increase in AP-1 DNA binding (Figure 2, lane 5). Treatment of the cells with PMA and H₂O₂ reproducibly resulted in weaker DNA binding than stimulation with PMA alone (Figure 2, compare lanes 3 and 7). In addition, co-treatment of cells with PMA and anti-CD28 under pro-oxidant conditions resulted in an almost complete inhibition of AP-1 DNA binding, which was even lower than in unstimulated cells.

In further experiments the time course of CD28-mediated inhibition of PMA-induced AP-1 activation was analysed. AP-1 DNA binding was greatly activated 1.5 h after PMA treatment, reaching a plateau within 3 h (Figure 3, lanes 1 and 3). Thereafter, DNA-binding activity gradually declined. Anti-CD28-mediated inhibition of AP-1 DNA binding followed a slightly different kinetic course. Inhibitory effects of anti-CD28 were observed 1.5 h after cell treatment, but were maximal after 3 h (Figure 3, lanes 2 and 4).

The effects of CD28 activation on NF- κ B and AP-1 were further investigated in functional trans-activation experiments (Table 1). Jurkat cells were transfected with CAT reporter constructs under the control of either four NF- κ B-binding sites (NF- κ B4CAT) or two AP-1-binding sites (TRE2CAT). When the transfected cells were stimulated with anti-CD28, a slight increase in trans-activation of the κ B reporter construct was induced (Table 1). Incubation with PMA led to a strong enhancement of NF- κ B activation. This increase in CAT activity was further enhanced on co-treatment of the cells with PMA and anti-CD28. Similarly to the results from the e.m.s.a., H₂O₂ triggered NF- κ B activation (Table 1). NF- κ B-dependent gene expression induced under pro-oxidant conditions was further

augmented when the cells were stimulated with PMA and a combination of PMA and anti-CD28.

Under the same conditions, contrasting effects were observed in cells transfected with an AP-1-regulated CAT plasmid (Table 1). PMA induced a more than tenfold increase in CAT activity, whereas anti-CD28 had no effect. However, when cells were treated with a combination of anti-CD28 and PMA, a significant inhibition of AP-1-dependent gene expression was observed in comparison with cells incubated with PMA alone. In agreement with the previous DNA-binding experiments, the inhibitory effect of CD28 stimulation was stronger when cells were kept under pro-oxidant conditions (Table 1). H_2O_2 increased AP-1 activation, which was suppressed by anti-CD28. Also PMA-induced AP-1 activation was markedly inhibited by anti-CD28 under these conditions. To examine non-specific effects on CAT expression, control experiments with the basal reporter plasmid PBLCAT2 were performed. Expression of pBLCAT2 showed virtually no responsiveness towards PMA and anti-CD28 stimulation (Table 1). Therefore both DNA-binding and trans-activation experiments reveal that anti-CD28 selectively potentiates PMA-induced NF- κ B activation but exerts inhibitory effects on the activation of AP-1.

DISCUSSION

The present study shows that stimulation of T-cells by phorbol ester and CD28 engagement exerts distinct effects on the activation of the transcription factors NF- κ B and AP-1. Incubation of Jurkat cells with agonistic anti-CD28 antibodies largely increased PMA-induced activation of NF- κ B, as reported in earlier studies [10–12]. Activation of NF- κ B in response to PMA and anti-CD28 essentially involved post-translational mechanisms and was not suppressed in the presence of transcriptional inhibitors (results not shown). The stimulating effect of both agents has been attributed to the induction of a pro-oxidant state, as NF- κ B activation is efficiently blocked by diverse antioxidant substances [15–18]. In addition, treatment of the cells with anti-CD28 induces a rapid and transient decrease in intracellular thiol concentration, suggesting that oxidative mechanisms are involved [18] (M. Los, W. Dröge and K. Schulze-Osthoff, unpublished work).

Activation of AP-1 by phorbol esters presumably involves a separate pathway from that for NF- κ B [22]. PMA-induced AP-1 activation does not appear to rely on a pro-oxidant effect because it is not inhibited by antioxidants, and is even suppressed under pro-oxidant conditions [22]. Furthermore, we and others have recently reported that diverse antioxidants that inhibit NF- κ B activation even strongly induce AP-1 DNA binding and trans-activation activity [21,22]. Hence it has been suggested that NF- κ B is an oxidant-inducible factor, whereas AP-1 can be regarded as an antioxidant-responsive transcription factor. This study shows that effects similar to those produced by pro-oxidants can be induced by stimulation with anti-CD28. Treatment of the cells with anti-CD28 increased NF- κ B activation, but significantly inhibited AP-1. Therefore these data further suggest that activation of the two transcription factors may be differentially regulated. The inhibition of PMA-induced AP-1 activation could be caused by several candidate mechanisms (reviewed in ref. [20]). Engagement of the CD28 molecule provokes oxidant conditions, which may counteract a signal provided by protein kinase C. For instance, oxidative stress or other CD28-induced downstream signals such as tyrosine phosphorylation could interfere with the dephosphorylation of c-Jun, which mediates AP-1 activation in response to phorbol

esters [25]. Other possible explanations may include CD28-induced changes in AP-1 interaction with inhibitory proteins, inhibition of Fos and Jun synthesis, or increased synthesis of repressing members of the family such as Jun B [20,26]. These mechanisms, however, appear unlikely, because PMA-induced AP-1 activation does not depend on protein synthesis and inhibition by anti-CD28 was already apparent early after cell treatment.

Is there a biological relevance to these observations? Efficient activation of T-cells requires co-stimulatory signals resulting in the increase in IL-2 production and cell proliferation [2]. TcR signalling in the absence of these stimuli may induce a long-lasting state of unresponsiveness (anergy) or programmed cell death (apoptosis). Both processes can be antagonized by anti-CD28, which prevents cell death and restores T-cell responsiveness [2,3,27]. In some ways conflicting with our results, it has recently been shown that anergized cells strongly suppress AP-1 activation, which was suggested to contribute to the anergic state [28]. Other authors, however, could not confirm these data and found no differences in induction of AP-1 on re-stimulation of anergized versus resting cells [29]. In studies with thymocytes and T-cells infected with human immunodeficiency virus, apoptosis induced by growth factor deprivation or incomplete T-cell activation was prevented by CD28 co-stimulation [30]. Apoptosis in T-cells is an active process requiring *de novo* protein synthesis [31,32]. Several papers have reported that early steps of apoptosis are accompanied by transcriptional induction of the AP-1 subunits *c-fos* and *c-jun* [33,34]. In addition, antisense RNA constructs of *c-fos* and *c-jun* prevent or inhibit cell death [34]. Thus it may be conceivable that, in cells undergoing apoptosis, CD28-induced inhibition of AP-1 activation may contribute to the rescuing effect of this co-stimulus. Future experiments therefore need to reveal whether inhibition of AP-1 activation by CD28 interferes with apoptotic processes resulting from incomplete T-cell activation.

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