The EMBO Journal vol.13 no.18 pp.4370–4381, 1994

AP-1 transcriptional activity requires both T-cell receptor-mediated and co-stimulatory signals in primary T lymphocytes

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Communicated by R.A.Flavell

The transcription factor AP-1 contributes significantly to the regulation of interleukin-2 gene transcription during T-cell activation and may play a role in thymocyte development. To study the regulation of AP-1 transcriptional activity in primary T-cells, reporter transgenic mice were generated that express luciferase gene under the control of AP-1 binding sites. Here, we demonstrate that while protein kinase C activation is sufficient to induce DNA-binding activity, an additional intracellular calcium increase is required to induce transcriptional activity of AP-1 in primary mouse T-cells. Furthermore, transcriptional, but not DNA-binding, activity of AP-1 is cyclosporin sensitive and requires tyrosine phosphorylation. This dissociation between DNA-binding and transcriptional activity is likely due, at least partially, to post-translational modifications of the AP-1 complex required for transcriptional activity. Moreover, in addition to these two signals delivered by ligand binding to the T-cell receptor (TcR) AP-1 transcriptional activity absolutely requires the presence of a co-stimulatory signal that can be mediated by the interaction of CD28 with its ligands B7-1 and B7-2. Thus, TcR-mediated and co-stimulatory signals required for T-cell activation appear to be integrated, in part, at the level of the regulation of transcriptional activity of AP-1.

Key words: AP-1/CD28/phosphorylation/T-cells/transcription factors

Introduction

Interaction of the T-cell receptor (TcR) complex with antigen in association with the major histocompatibility complex (MHC) results in tyrosine kinase-mediated phospholipase C activation, which leads to an increase in intracellular calcium concentration and protein kinase C (PKC) activation (Weiss *et al.*, 1986; Perlmutter *et al.*, 1993). However, stimulation of the TcR alone is insufficient to activate most T-cells and a second signal, provided by antigen-presenting cells (APC), is also required and is referred to as the co-stimulatory signal (Mueller *et al.*, 1989). B7-1 is one of the co-stimulatory molecules expressed on APC and it is the counter-receptor for two ligands expressed on T lymphocytes (Freeman *et al.*, 1989). The first ligand, CD28, is constitutively expressed on T-cells and, after ligation, induces interleukin 2 (IL-2) secretion and proliferation. The second ligand, CTLA4, is homologous to CD28 and appears on T-cells after activation, but its role in T-cell activation remains to be determined (Linsley and Ledbetter, 1993). Recently, a second counter-receptor has been identified for both CD28 and CTLA4, termed B7-2 or B70, also expressed on APC (Azuma *et al.*, 1993; Freeman *et al.*, 1993). Other studies have shown that in the absence of co-stimulatory signals, stimulation of T-cells through the TcR leads to clonal inactivation, or anergy (Jenkins and Schwartz, 1987; Mueller *et al.*, 1989). A key regulatory step in the decision between T-cell proliferation or the induction of anergy lies in the regulation of the expression of the IL-2 gene since no IL-2 gene transcription is observed in the absence of a co-stimulatory signal (Mueller *et al.*, 1989). Little is known about the nature of the co-stimulatory signal and how it is co-integrated with the signals delivered by TcR to result in expression of the IL-2 gene. Initially, it was reported that CD28 mediates an increase in stability of IL-2 as well as other cytokine mRNAs (Lindstein *et al.*, 1989). Further, it was described that the CD28-mediated signal participates directly in the transcription of the IL-2 gene (Fraser *et al.*, 1991; Fraser and Weiss, 1992). However, the mechanisms by which co-stimulation regulates IL-2 gene transcription are still unclear.

Several regulatory elements recognized by ubiquitous and T-cell-specific transacting factors comprise the minimal inducible promoter region of the IL-2 gene (Crabtree, 1989; Ullman *et al.*, 1990).These include two Oct-1 binding sites called NFIL-2A and NFIL-2D, proximal and distal AP-1 and NFAT binding sites, and a single NFκB binding site. Each one of these transcription factors is regulated specifically. Thus, the ubiquitous Oct-1 that binds to the NFIL-2D element is constitutive in unstimulated T-cells (Kamps *et al.*, 1990). NFAT, an activated T-cell-specific factor, requires both PKC and calcium signal pathways (Shaw *et al.*, 1988), and its activity is sensitive to cyclosporin A (CsA), which blocks the calcium-dependent pathway by inhibiting the protein phosphatase calcineurin (Emmel *et al.*, 1989; O'Keefe *et al.*, 1992; Jain *et al.*, 1993). NFκB activity requires only PKC stimulation and is partially CsA resistant (Hoyos *et al.*, 1989). The AP-1 transcription factor is a complex mixture of different members of the Fos (c-Fos, FosB, Fra-1, Fra-2 and FosB2) and Jun (c-Jun, JunB and JunD) family of proteins (Cohen and Curran, 1988; Halazonetis *et al.*, 1988; Nakabeppu *et al.*, 1988; Hirai *et al.*, 1989; Ryder *et al.*, 1989; Zerial *et al.*, 1989). Each of these members contains a 'leucine zipper' which permits its dimerization with other members of the Fos/Jun family. Homodimers of Jun, but not of Fos, bind to 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive elements (TREs). However, the DNA-

binding affinity is notably higher for heterodimers of Fos−Jun. AP-1 activity is regulated by *de novo* synthesis of Jun and Fos members, as well as by post-translational modification such as phosphorylation and dephosphorylation (Pulverer *et al.*, 1991; Lin *et al.*, 1992). In contrast to NFAT activity, AP-1 activity has been described to be, and is generally believed to be, inducible by PKC activators alone, notably phorbol esters, and therefore to be CsA resistant (Angel *et al.*, 1987; Lee *et al.*, 1987; Ullman *et al.*, 1993).

Most regulation studies concerning the activity of these nuclear factors have been based upon tumor cell lines, such as Jurkat or EL-4 cells, in which the regulation of the IL-2 promoter is uncoupled from cellular proliferation, which thus places a limit on the physiological significance of the response. Further studies have been performed using T-cell hybridomas, or T-cell clones, that also do not reflect the naive T-cells which are the target for the initial antigenic stimulus *in vivo*. However, little is known about the regulation of DNA-binding activity and, more importantly, the transcriptional activity of these factors in primary mouse or human T-cells due to an enormous difficulty in transfecting these cells.

We have developed a model to analyze AP-1 transcriptional activity in primary mouse T-cells by generating reporter transgenic mice that express the firefly luciferase under the control of four TREs. This model makes possible the study of the regulation of AP-1 transcriptional activity not only during T-cell activation, but also in thymic development. In the present report, we describe the signaling requirement for AP-1 induction. We demonstrate, for the first time, a dissociation between the signals required for DNA-binding or transcriptional activity of AP-1. PKC activation and intracellular Ca^{2+} increase are required for transcriptional activity, while stimulation of PKC alone is sufficient to induce DNA-binding of AP-1. In addition, AP-1 transcriptional activity, as well as IL-2 gene expression, in primary mouse T-cells require the co-stimulatory signal which can be provided by the interaction between CD28 and its ligands expressed on APC.

Results

Production and characterization of AP1−luciferase transgenic mice

To generate reporter transgenic mice for AP-1, we microinjected the firefly luciferase gene driven by four human collagenase TREs (Angel *et al.*, 1987; Lee *et al.*, 1987) in the context of the minimal rat prolactin promoter (AP1−luc; Figure 1A). This TRE, as well as the human metallothionein TRE, has been used in numerous T-cell studies due to its high affinity for AP-1 complex. In correlation with prior work, transient transfection of this construct in Jurkat T-cell line results in a 3- or 15-fold induction of relative luciferase activity upon phorbol 12-myristate 13-acetate (PMA) or PMA plus ionomycin activation, respectively (data not shown). Five positive founders were identified by slot-blot analysis and sucessfully bred to produce stable lines of transgenic mice. To determine luciferase gene expression in these transgenic mice, we stimulated total spleen cells for 24 or 48 h with PMA plus ionomycin to obtain optimal AP-1 activation, and luciferase activity was analyzed as described in

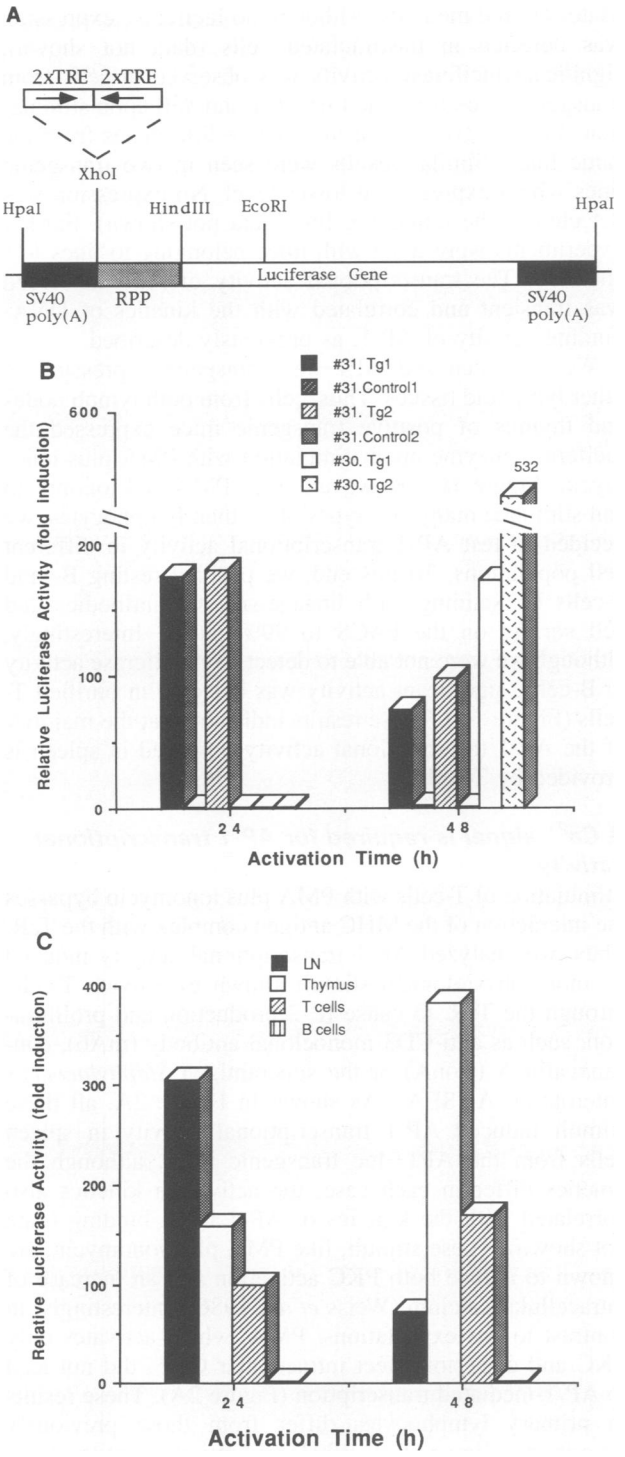

Fig. 1. Generation of AP-1−luciferase reporter transgenic mice. (**A**) Structure of the AP1−luciferase (AP1−luc) fusion gene used for microinjection.The 2.8 kb *HpaI* digestion fragment microinjected contained the luciferase coding region fused to an SV40 splice and polyadenylation site, and driven by the rat minimal prolactin promoter (RPP) and two copies of the AP-1 oligunucleotide (2×TRE) inserted at the *XhoI* site. (**B**) Luciferase activity in cells from spleen from two independent transgenic mice of line #31 (Tg1 and Tg2), two transgenic mice of line #30 (Tg1 and Tg2) and two negative littermates of line #31 (Control 1 and 2). (**C**) Luciferase activity in lymph nodes (LN), thymus, purified T-cells and purified B-cells from transgenic mice of line #31. Cells were unstimulated or stimulated with PMA (5 ng/ml) plus ionomycin (250 ng/ml) for 24 or 48 h, and luciferase activity was analyzed as described in Materials and methods. Luciferase values are expressed as fold activation over the value obtained from unstimulated cells.

Materials and methods. Although no luciferase expression was detected in unstimulated cells (data not shown), significant luciferase activity was observed in cells from transgenic mice from the lines #31 and #30 upon stimulation (Figure 1B), but not in negative littermates from the same lines. Similar results were seen in two transgenic lines which expressed at lower level. No expression was detected in the remaining line (data not shown). Further experiments were done with mice belonging to lines #31 and #30. The transcriptional activity of AP-1 observed was transient and correlated with the kinetics of DNA-binding activity of AP-1, as previously described.

We also analyzed AP1−luc transgene expression in other lymphoid tissues. Thus, cells from both lymph nodes and thymus of positive transgenic mice expressed the luciferase enzyme upon stimulation with PMA plus ionomycin (Figure 1C). However, since PMA and ionomycin can stimulate many cell types other than lymphocytes, we decided to test AP-1 transcriptional activity in different cell populations. To this end, we purified resting B- and T-cells by staining with lineage-specific antibodies and cell sorting on the FACS to 99% purity. Interestingly, although we were not able to detect any luciferase activity in B-cells, significant activity was detected in purified T-cells (Figure 1C). These results indicated that the majority of the AP-1 transcriptional activity observed in spleen is provided by T-cells.

A Ca^{2+} signal is required for AP-1 transcriptional activity

Stimulation of T-cells with PMA plus ionomycin bypasses the interaction of the MHC antigen complex with the TcR. Thus, we analyzed AP-1 transcriptional activity induced by more physiological stimuli known to activate T-cells through the TcR, to cause IL-2 production and proliferation, such as anti-CD3 monoclonal antibody (mAb), concanavalin A (ConA) or the superantigen *Staphylococcus* enterotoxin A (SEA). As shown in Figure 2A, all these stimuli induced AP-1 transcriptional activity in spleen cells from the AP1−luc transgenic mice, although the kinetics differ in each case; the activation kinetics also correlated with the kinetics of AP-1 DNA-binding (data not shown). These stimuli, like PMA plus ionomycin, are known to induce both PKC activation and an increase of intracellular calcium (Weiss *et al.*, 1986). Interestingly, in contrast to our expectations, PMA, which activates only PKC and does not affect intracellular Ca^{2+}, did not lead to AP-1-mediated transcription (Figure 2A). These results in primary lymphocytes differ from those previously described (Ullman *et al.*, 1993) and our own observations with this construct (see above), where activation of PKC alone by PMA was sufficient to induce AP-1-mediated transcription in the Jurkat tumor cell line. Increasing the PMA concentration even to extremely high doses did not result in an induction of AP-1 transcriptional activity, whereas low doses of PMA in combination with ionomycin induced a high level of AP-1 transcriptional activity (Figure 2B). Likewise, ionomycin alone had no effect on AP-1 transcriptional activity. Similar results were obtained in spleen cells from the other line (#30) of transgenic mice (Figure 2C), indicating that the difference that was observed between murine T-cells and Jurkat cells is not a consequence of non-specific regulation of AP-1 due to

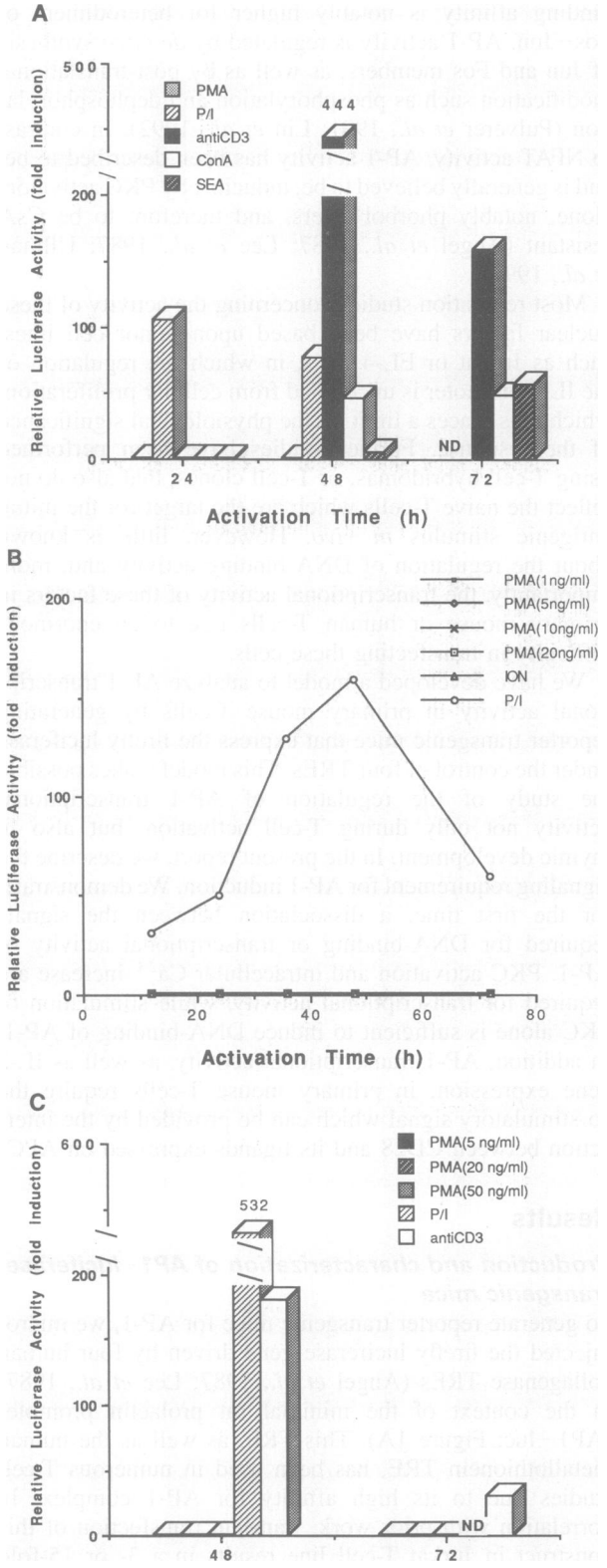

Fig. 2. AP-1 transcriptional activity in response to different stimuli. Spleen cells from transgenic mice were incubated in the presence of PMA (5 ng/ml), PMA (5 ng/ml) plus ionomycin (250 ng/ml) (P/I), anti-CD3 mAb (1 μg/ml), Con A (3 μg/ml) or SEA (1 μg/ml) (**A**) or in the presence of PMA alone at 1, 5, 10, 20, 50 ng/ml, ionomycin alone (250 ng/ml) (ION) or PMA (5 ng/ml) plus ionomycin (250 ng/ml) (P/I) (**B**) and (**C**). After the indicated periods of time luciferase activity was analyzed. (A) and (B), transgenic mice from line #31; (C) transgenic mice from line #30. ND, not demonstrated. Results shown are representative of three independent experiments.

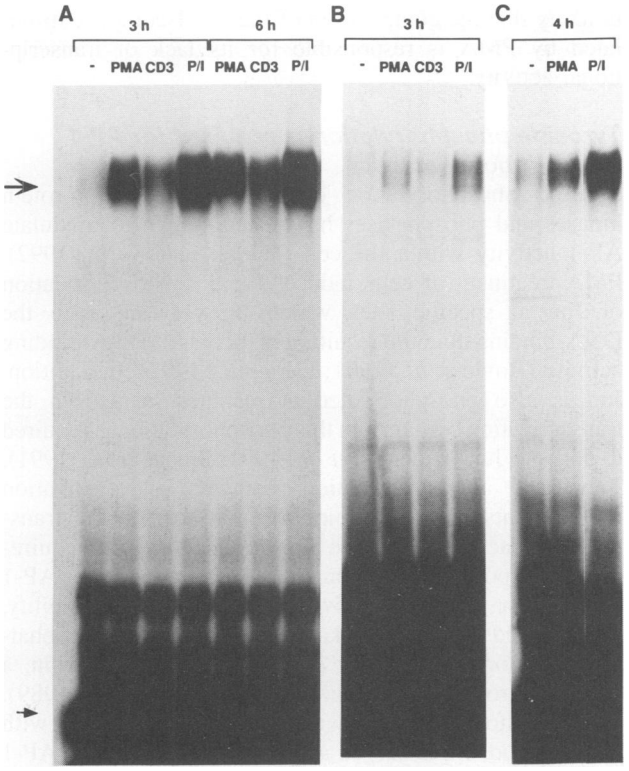

Fig. 3. Analysis of AP-1 DNA-binding activity. Electrophoretic mobility shift (EMS) analysis of nuclear extracts from splenocytes (**A** and **B**) or purified T-cells (**C**) unstimulated (−) or stimulated with PMA (5 ng/ml), PMA plus ionomycin (250 ng/ml) (P/I) or anti-CD3 mAb (1 μg/ml) (CD3) for different periods of time. For each assay, 2.5 μg of nuclear extracts were incubated with an ^{32}P end-labeled 20 nucleotide oligomer containing the human collagenase TRE (A and C) or a 21-nucleotide oligomer containing the AP-1-proximal site from the IL-2 promoter (B). The large arrow shows AP-1 complex, while the small arrow indicates free probe.

the transgene integration site. Purified T-cells also required PMA and ionomycin to activate AP-1, and again no transcriptional activity was observed in the absence of ionomycin (data not shown).

To correlate the transcriptional activity of AP-1 with the presence of TRE DNA-binding complexes, nuclear extracts were prepared from unstimulated spleen cells or cells stimulated for different periods of time with either PMA, PMA plus ionomycin or anti-CD3 mAb. The analysis of DNA-binding proteins by electrophoretic mobility shift assay revealed that PMA stimulation was sufficient to induce AP-1 binding to the collagenase TRE, although the level of binding was enhanced considerably by the addition of ionomycin (Figure 3A). Strikingly, however, the level of DNA-binding complexes induced with anti-CD3 mAb was similar to or lower than that seen with PMA alone, despite the fact that transcriptional activity was induced with anti-CD3 mAb and not with PMA. In all cases, the complex was specific for AP-1, as demonstrated by competition with an excess of double-stranded unlabeled oligonucleotide that contained a consensus AP-1 site (data not shown) as well as with an antibody against Jun family members (see below). Similar results were obtained when a probe containing the proximal AP-1 site from the IL-2 promoter was used instead of the collagenase TRE (Figure 3B), although this oligonucleotide shows a lower apparent affinity for the AP-1 complex, as previously described (Jain *et al.*, 1992). In order to rule out the possibility that the DNA-binding activity observed was provided by different T-cell populations present in spleen, the inducibility of AP-1 DNA-binding in a purified T-cell population was analyzed. As shown in Figure 3C, PMA alone was also able to induce DNA binding of AP-1 in purified T lymphocytes, despite the fact that it could not activate transcription driven by AP-1.

AP-1 transcriptional acticity is sensitive to CsA

CsA is an immunosuppressive drug which blocks calcium-dependent signaling via the TcR by inhibiting the Ca^{2+}/calmodulin-dependent phosphatase, calcineurin (O'Keefe *et al.*, 1992), and inhibits both DNA binding and transcriptional activity of NFAT (Emmel *et al.*, 1989; Jain *et al.*, 1993). Previous experiments performed in Jurkat cells show that AP-1 DNA binding as well as transcriptional activity are resistant to CsA (Emmel *et al.*, 1989; Ullman *et al.*, 1993). However, since in primary T-cells AP-1 transcriptional activity required calcium, we decided to analyze the regulation of AP-1 by CsA. CsA also had little effect on the DNA binding of AP-1 induced by PMA alone, PMA plus ionomycin or anti-CD3 mAb in spleen cells (Figure 4A). In contrast, treatment with CsA blocked AP-1 transcriptional activity induced by both PMA plus ionomycin or anti-CD3 mAb (Figure 4B) in splenocytes from transgenic mice. These data together demonstrated that although PKC activation alone was sufficient to induce DNA binding of AP-1 in primary T-cells, a CsA sensitive calcium signal was also required to induce AP-1-mediated transcription.

Comparative analysis of the AP-1 binding complexes

One explanation for the dissociation between the activation requirements for DNA binding versus transactivation activity is the possibility that different members of the Jun or Fos family participate in the AP-1 complexes induced by PMA plus ionomycin or anti-CD3 mAb, but not in the complex induced by PMA alone, so that, for example, a non-functional form of AP-1 could result from PMA stimulation. To address this possibility, we carried out a comparative analysis of the composition of AP-1 DNA-binding complexes in splenocytes induced by different stimuli, using antisera to Fos or Jun family members to compete the binding to the TRE oligo. No significant differences in the percentage of inhibition by anti-c-Jun, -JunB or -JunD specific antisera were observed among the AP-1 DNA-binding complexes induced by PMA, anti-CD3 mAb or PMA plus ionomycin (Figure 5A and B). Using an antiserum that recognizes all the Jun family members so far described, we were able to eliminate the AP-1 complex almost completely, further demonstrating the specificity of the DNA-binding complex. Similarly, we did not detect significant differences in the composition of the Fos family members in the AP-1 complex induced by PMA compared with the complex induced by anti-CD3 mAb or PMA plus ionomycin (Figure 5C and D). We do not believe that the greater inhibition of the PMA-induced DNA binding by anti-Fra2 antiserum (Figure 5D) was significant since it was not observed in other experiments. In summary, these results suggest that it is

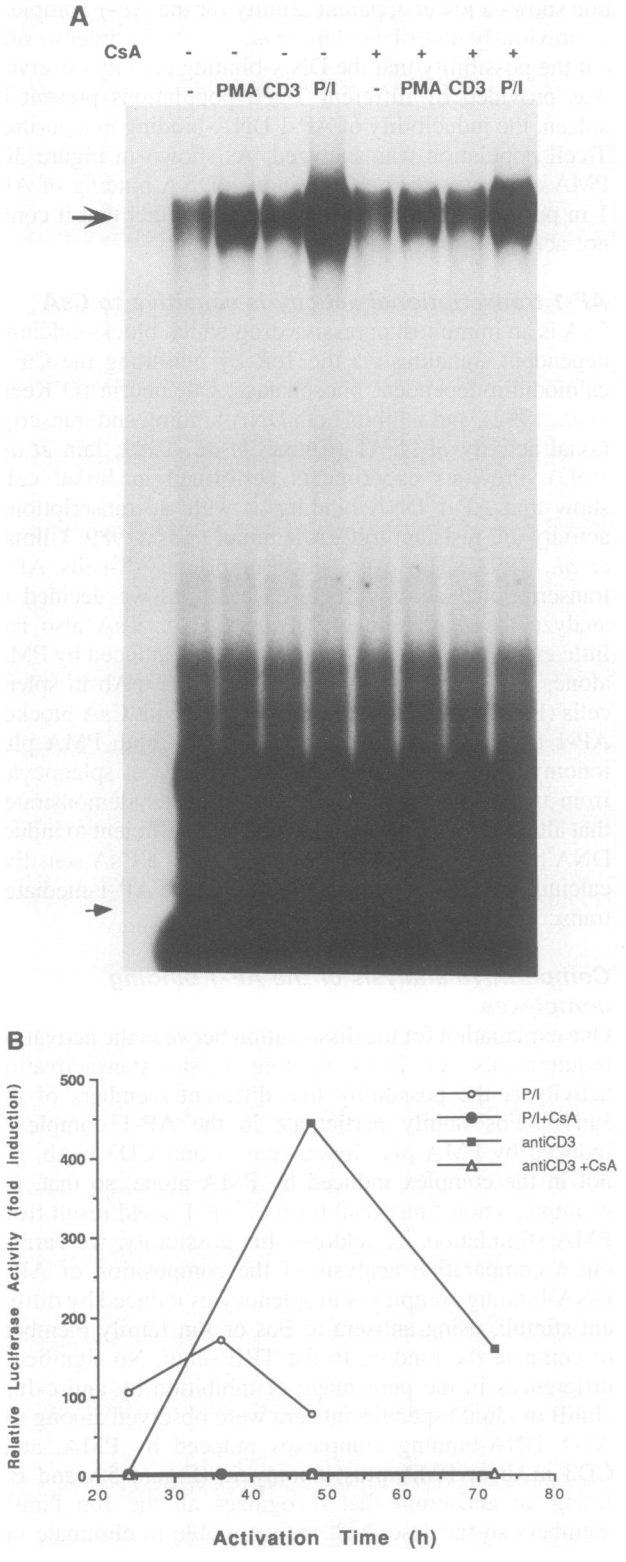

Fig. 4. Blocking of AP-1 transcriptional activity by CsA. (**A**) EMS assay of nuclear extracts from spleen cells unstimulated (−) or stimulated with PMA (5 ng/ml), PMA (5 ng/ml) plus ionomycin (250 ng/ml) (P/I) or anti-CD3 mAb (1 μg/ml) (CD3) in the presence or absence of CsA (50 ng/ml) for 4 h. For each assay, 3 μg of nuclear extracts were incubated with a ^{32}P end-labeled oligonucleotide containing the human collagenase TRE. (**B**) Spleen cells from transgenic mice were stimulated as described for EMS assay. Luciferase activity was analyzed at 24, 48 and 72 h after activation, as described. This is a representative experiment of four carried out.

unlikely that the composition of the AP-1 complex stimulated by PMA is responsible for its lack of transcriptional activity.

Tyrosine phosphorylation is required for AP-1 transcriptional activity

Specific phosphorylation events mediated by protein kinases and phosphatases have been shown to modulate AP-1 activity within the cell (Hunter and Karin, 1992). PMA treatment of cells induces rapid dephosphorylation of Jun at specific sites which lie very close to the DNA-binding domain, resulting in increased DNA-binding activity (Boyle *et al.*, 1991; Lin *et al.*, 1992). In addition, Jun is also phosphorylated at residues located in the transactivation domain and this phosphorylation is required to activate Jun (Binétruy *et al.*, 1991; Smeal *et al.*, 1991). Therefore, the dissociation between the activation requirements for DNA-binding activity versus transactivation activity might be also explained by the requirement for post-transcriptional modification of the AP-1 complex for functional activity. To address this possibility, we analyzed the effect of okadaic acid, a specific phosphatase inhibitor (Haystead *et al.*, 1989), and herbimycin, a specific tyrosine kinase inhibitor (Uehara *et al.*, 1989). When dephosphorylation was inhibited by treatment with okadaic acid, we observed a strong reduction of the AP-1 DNA binding induced either by PMA alone, anti-CD3 mAb or PMA plus ionomycin (Figure 6A) and, therefore, also a blocking of the AP-1-mediated transcription induced by these stimuli (Figure 6B). Since binding was inhibited after only a short-term incubation with okadaic acid, and since cells remained viable, we do not believe that this inhibition was the result of non-specific toxicity. Interestingly, herbimycin treatment did not inhibit AP-1 DNA binding induced by PMA alone or PMA plus ionomycin and only slightly reduced the binding induced by anti-CD3 (Figure 6A). However, herbimycin completely blocked AP-1 transcriptional activity induced by anti-CD3 mAb or PMA plus ionomycin (Figure 6C). These data together suggest that an additional modification mediated by a tyrosine kinase is required for transcriptional activity, but not for DNA-binding activity, of AP-1 in primary lymphocytes.

Role of co-stimulatory signal in AP-1 transcriptional activity in purified T-cells

In addition to the signaling delivered by the TcR, T-cell activation requires a second, antigen-independent, co-stimulatory signal. In the absence of such a signal, stimulation of T-cells through the TcR results in a failure to express the IL-2 gene, suggesting that co-stimulatory signals also participate in the regulation of IL-2 gene transcription (Jenkins and Schwartz, 1987; Mueller *et al.*, 1989). The stage in the signal transduction cascade at which co-stimulation acts is, however, unknown. In order to elucidate the role of co-stimulation in the transactivation function of AP-1, we activated primary T-cells either in the presence or the absence of co-stimulation. T-cells were highly purified from spleen or LN from transgenic mice by cell sorting, so that no APC were present in the preparation. The same results were obtained when anti-CD3 (Figure 7) or anti-TcRβ (data not shown) mAb were used in the T-cell sorting, making it unlikely that these

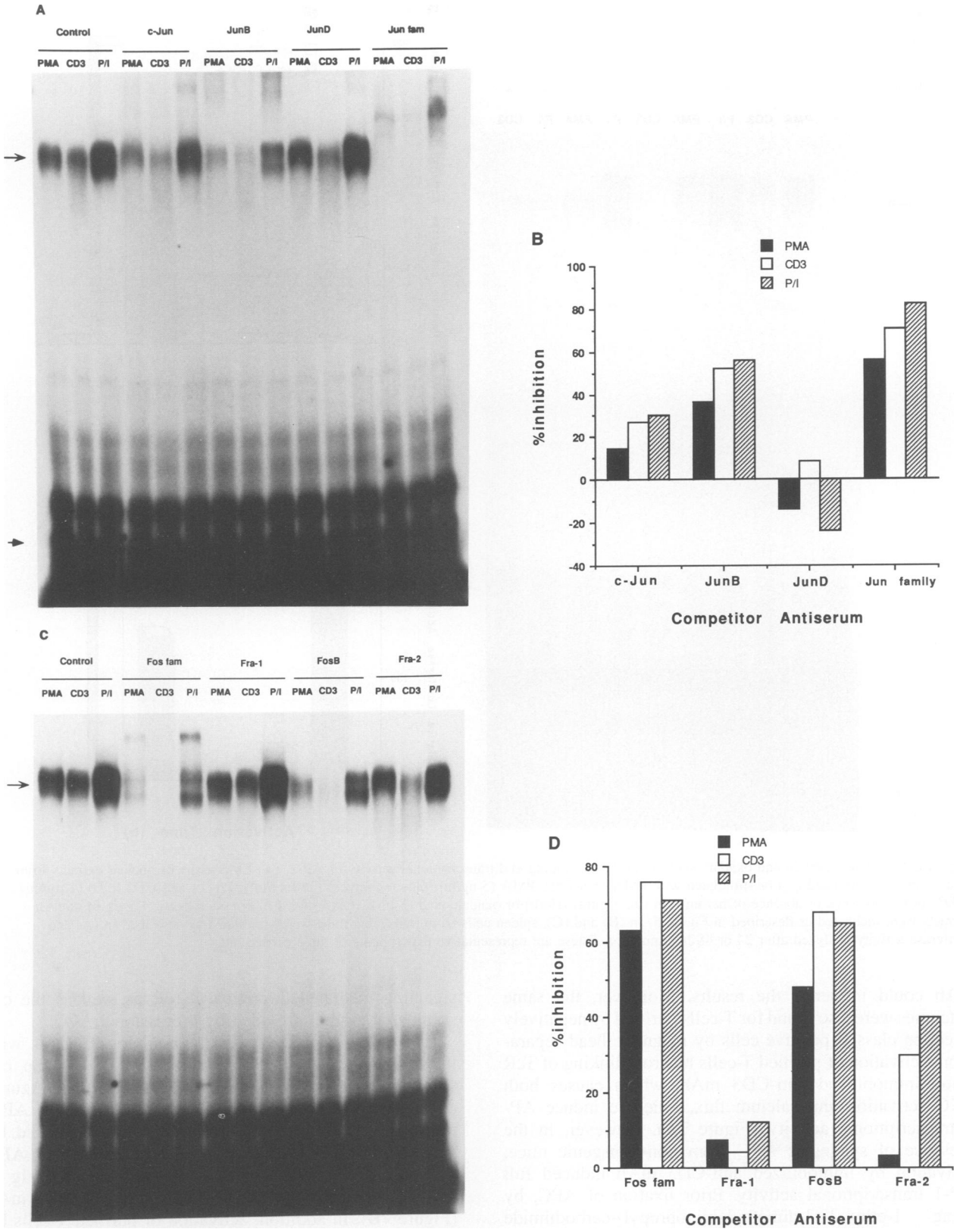

Fig. 5. Comparative analysis of AP-1 DNA-binding complexes. EMS analysis of nuclear extracts from spleen cells stimulated with PMA (5 ng/ml), PMA (5 ng/ml) plus ionomycin (250 ng/ml) (P/I) or anti-CD3 mAb (1 μg/ml) (CD3) for 5 h. For each assay, 2.5 μg of nuclear extracts were incubated with a ^{32}P end-labeled oligonucleotide containing the human collagenase TRE. Nuclear extracts were pre-incubated for 5 min on ice with 1 μl of each antiserum or 0.12 μg of purified mAb against M Fos peptide. The labeled probe was then added and incubated for an additional 15 min. Antisera against glutathione S-transferase (Control), c-Jun, JunB, JunD or all the Jun family members (Jun fam) were used in (**A**), and antisera against Fra-1, Fra-2 and Fos B or purified mAb against Fos peptide (Fos family) were used in (**C**). After drying, gels were exposed to PhosphorImager screens and the volume of the area for each complex was determined. (**B**) and (**D**) represent the percentage of reduction of this volume for each complex in the presence of a specific antiserum compared with control antiserum.

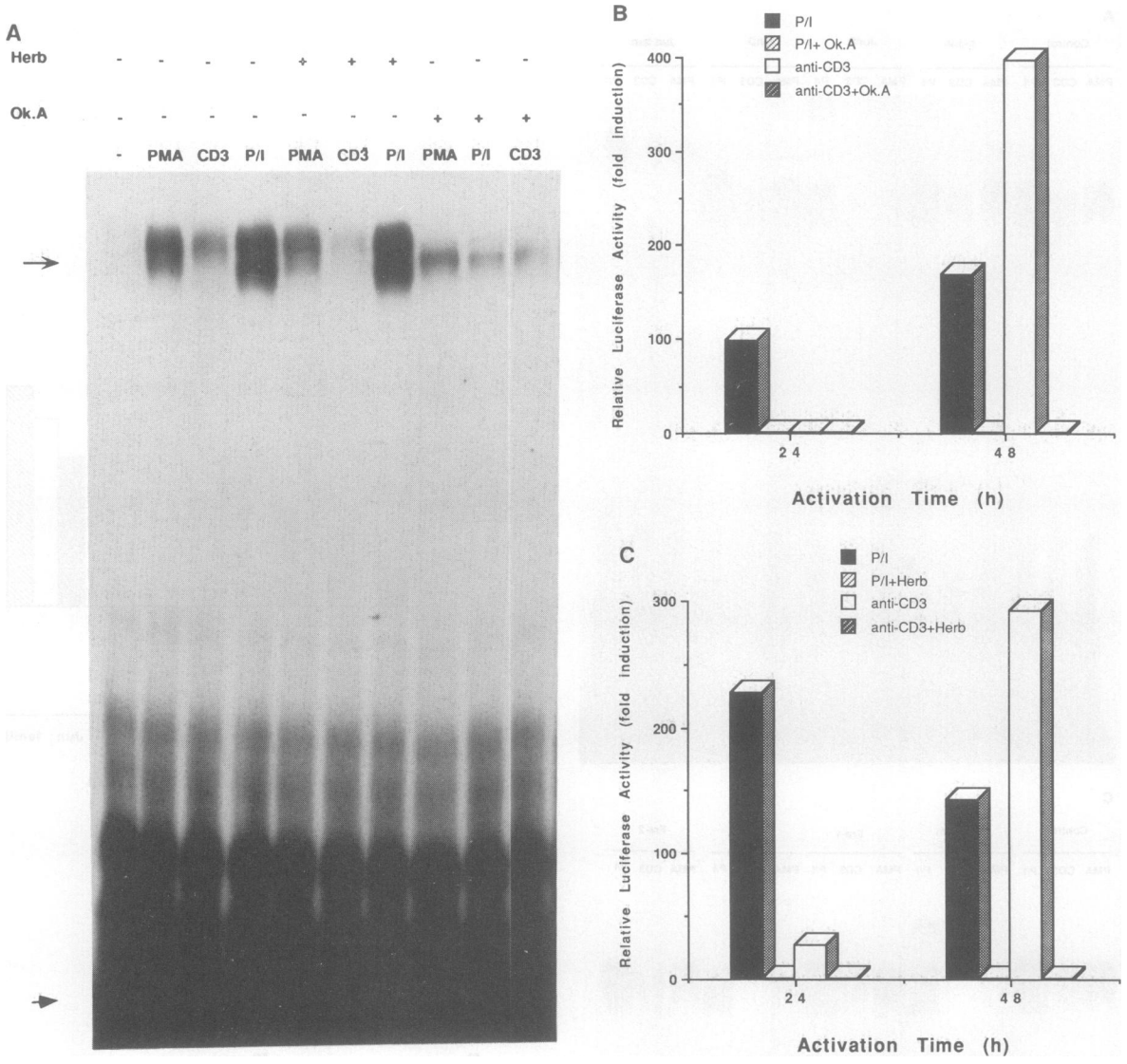

Fig. 6. Effect of herbimycin and okadaic acid on the DNA-binding and transcriptional activity of AP-1. (**A**) EMS assay of nuclear extracts from spleen cells unstimulated (−) or stimulated with PMA (5 ng/ml), PMA (5 ng/ml) plus ionomycin (250 ng/ml) (P/I) or anti-CD3 mAb (1 μg/ml) (CD3) in the presence or absence of herbimycin (0.5 μg/ml) (Herb) or okadaic acid (1 μM) (Ok.A) for 4 h. For each assay, 1.5 μg of nuclear extracts were incubated as described in Figure 5. In (**B**) and (**C**), spleen cells from transgenic mice were stimulated as described in (A) and luciferase activity analyzed after 24 or 48 h stimulation. These are representative experiments of three carried out.

mAb could influence the results. Moreover, the same outcomes were also found for T-cells purified by negatively selecting class II positive cells by magnetic bead separation. Activation of purified T-cells by cross-linking of TcR using immobilized anti-CD3 mAb, which causes both PKC activation and calcium flux, failed to induce AP-1 transcriptional activity (Figure 7A). However, in the presence of syngeneic APC from non-transgenic mice, activation by immobilized anti-CD3 mAb induced full AP-1 transcriptional activity. Prior fixation of APC, by using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (ECDI), which blocks the co-stimulatory signal (Jenkins and Schwartz, 1987), also prevented AP-1 activation. Similarly, soluble anti-CD3 mAb in the presence of normal APC, but not in the presence of ECDI-fixed APC, were able to induce AP-1-mediated transactivation (Figure 7A). These data show that in primary mouse T-cells the induction of transcriptional activity of AP-1 requires signaling mediated through the TcR as well as the co-stimulatory signal provided by professional APC.

Interaction of CD28 and its homolog CTLA4 with the ligands B7-1 or B7-2 expressed by APC can co-stimulate T-cells (Freeman *et al.*, 1989, 1993; Azuma *et al.*, 1993; Linsley and Ledbetter, 1993). The AP-1 transcriptional activity of purified T-cells stimulated by soluble anti-CD3 mAb in the presence of syngeneic APC was almost completely blocked by soluble CTLA4-Ig or anti-B7-mAb and was strongly reduced by anti-B7-1 mAb (Figure 7B). In addition, activation of purified T-cells by immobilized anti-CD3 mAb and soluble anti-CD28 in the absence of APC resulted in a strong induction of AP-1 transcriptional activity. We also analyzed the effect of co-stimulation on the DNA-binding activity of AP-1. As shown in Figure 7C, co-stimulation via anti-CD28 mAb did not significantly increase the AP-1 DNA binding stimulated by cross-inking of TcR by immobilized anti-

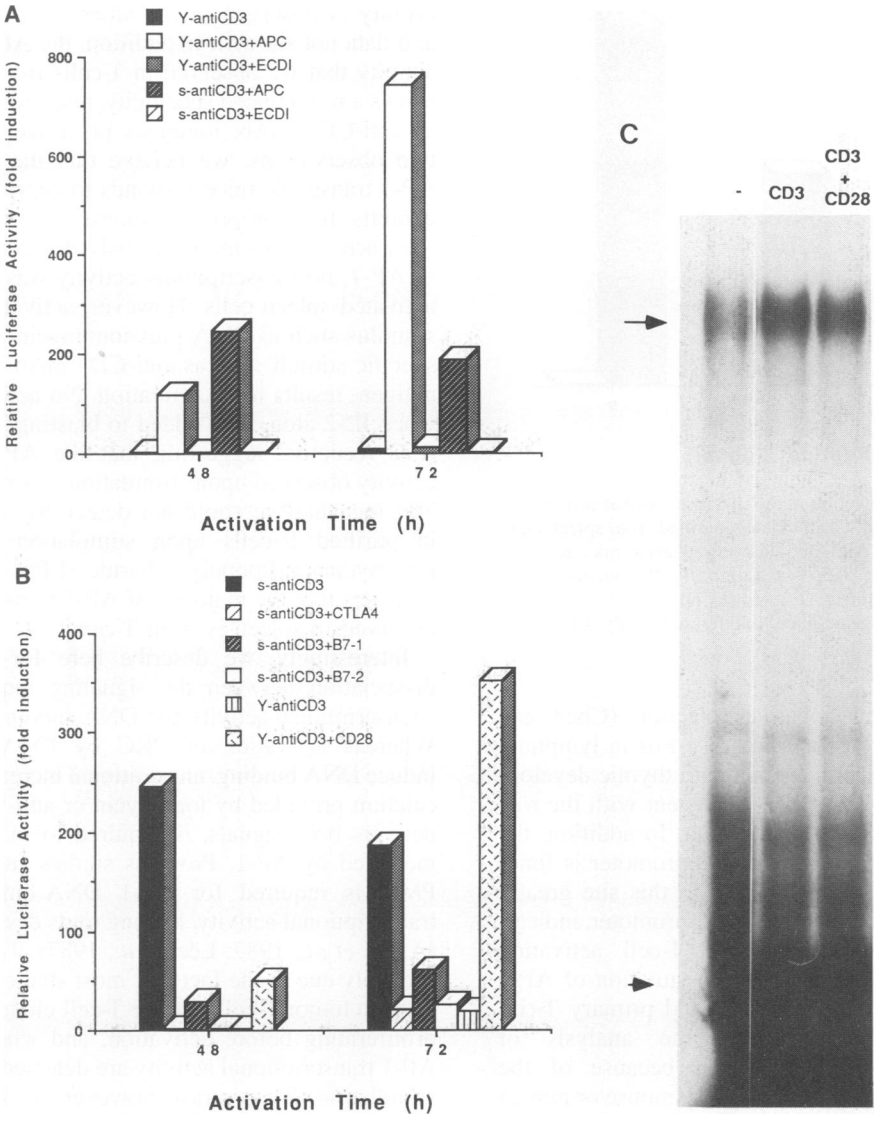

Fig. 7. A co-stimulatory signal is required for AP-1 transcriptional activity in purified resting T-cells. T lymphocytes purified from spleen and lymph nodes from transgenic mice were stimulated with (**A**) immobilized anti-CD3 mAb (125 µl/well at 5 µg/ml) (Y-antiCD3) or soluble anti-CD3 mAb (1 µg/ml) (s-antiCD3) in the presence or absence of mitomycin C-treated (APC) or ECDI-fixed (ECDI) spleen cells from non-transgenic B6 mice, or (**B**) soluble anti-CD3 mAb (1 µg/ml) plus APC in the presence or absence of soluble CTLA4-Ig (10 µg/ml) (CTLA4), anti-B7-1 mAb (10 µg/ml) (B7-1) or anti-B7-2 mAb (10 µg/ml) (B7-2), or immobilized anti-CD3 mAb without APC in the presence or absence of soluble anti-CD28 mAb (1 µg/ml) (CD28). Luciferase activity was analyzed after 48 or 72 h of activation. Similar results were obtained in two other individual experiments. (**C**) EMS assay of nuclear extracts from purified T-cells unstimulated (−) or stimulated with immobilized anti-CD3 (5 µg/ml) mAb alone (CD3) or in the presence of soluble anti-CD28 mAb (1 µg/ml) (CD28) (no APC were present during the culture) for 12 h. For each assay, 2 µg of nuclear extract were incubated with ^{32}P end-labeled oligonucleotide containing the human collagenase TRE.

CD3 mAb. These results suggest that the co-stimulatory signal is required for transcriptional activity, but not for DNA binding of AP-1, and that this signal is provided at least partially by CD28.

In order to demonstrate the requirement of co-stimulation for AP-1 transcriptional activity mediated by antigen-specific T-cell activation, we crossed the AP-1 transgenic mice with α/β TCR transgenic mice (Cyt *c* TcR) which express a receptor specific for pigeon cytochrome *c* (Cyt *c*) in association with I-E^k class II MHC molecules (Kaye *et al.*, 1989). We analyzed AP-1 transcriptional activity in CD4$^+$ T-cells from double transgenic mice upon stimulation with different doses of Cyt *c* peptide (amino acids 81–103) which is specifically recognized by the TcR expressed in these mice. Specific stimulation of CD4$^+$ T-cells with an increasing amount of peptide in the presence of syngeneic APC from non-transgenic mice resulted in significant AP-1 transcriptional activity (Figure 8). However, previous ECDI fixation of APC blocked the ability of peptide to induce AP-1 transcriptional activity. These results show that specific antigen stimulation of mouse primary T-cells is able to induce AP-1 transcriptional activity and that this response also requires the co-stimulatory signal provided by APC.

Discussion

It is likely that AP-1 proteins play a major role in T-lymphocyte development and activation. DNA binding of AP-1 can be induced in mature CD4$^+$CD8$^-$ TcR^{high} and immature CD4$^-$CD8$^-$TcR$^-$ thymocytes, but not in the transitional stages when the cells (CD4$^+$CD8$^+$ TcR^{low}) are

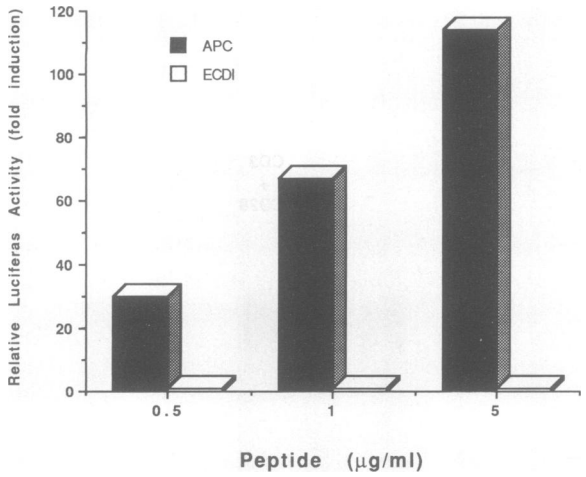

Fig. 8. Antigen-specific stimulation of AP-1 transcriptional activity requires co-stimulatory signal. $CD4^+$ T-cells purified from spleen and lymph nodes of Cyt *c* TcR×AP1−luc double transgenic mice were stimulated with Cyt *c* peptide (0.5, 1 or 5 μg/ml) in the presence of mytomycin C-treated (APC) or ECDI-fixed (ECDI) spleen cells from syngeneic B10.BR non-transgenic mice. Luciferase activity was analyzed 48 h after activation.

subject to positive and negative selection (Chen and Rothenberg, 1993). Overexpression of c-Fos in lymphoid tissues of transgenic mice interferes with thymic development (Ruther *et al.*, 1988), again consistent with the role of this protein in thymocyte maturation. In addition, the AP-1 proximal site present in the IL-2 promoter is functional *in vivo* and *in vitro*; mutations in this site greatly diminish or abolish induction of the IL-2 promoter, indicating the relevance of AP-1 during T-cell activation (Crabtree, 1989). Most studies of the regulation of AP-1 activity during thymic differentiation and primary T-cell activation have been based on the analysis of DNA−protein interactions. However, because of the extreme difficulty in transfecting primary mouse or human T-cells, the regulation of the AP-1 transcriptional activity in these cells remains unclear, and it is known that binding of a transcriptional factor to DNA does not always result in an induction of transcription. We have therefore developed an AP1−luciferase reporter transgenic mouse model that permits the analysis of the transcriptional activity of AP-1 in any murine cell types, including T-cells. In our studies, we make the assumption that luciferase activity measures the transcription rate of the AP-1-driven promoter. This approach and assumption have been validated in numerous studies of the transcriptional activity of particular transcription factors (Kliewer *et al.*, 1992; Boise *et al.*, 1993; Devary *et al.*, 1993; Fraser and Weiss, 1993; Saatcioglu *et al.*, 1993; Ullman *et al.*, 1993) as well as whole endogenous promoters (van Zonnuveld *et al*, 1988; Fraser *et al*, 1991; Ten *et al.*, 1992; Xu and Stavnezer, 1992). The same type of transgenic model has recently been applied for studying the regulation of NFAT and NFκB factors using different reporter genes (Verweij *et al.*, 1990; Lernbecher *et al.*, 1993). The human collagenase TRE, which we have used to analyze the AP-1 transcriptional activity, as well as the human metallotheonein TRE, have been extensively used in T-cell studies and a correlation with the proximal AP-1 site of the IL-2 promoter has been described, although a lower apparent affinity is observed in the latter case (Jain *et al.*, 1992, and data not shown). In addition, the AP-1 transcriptional activity that we observed in T-cells from transgenic mice shows a pronounced specificity, responding to stimuli such as anti-CD3 mAb, mitogens or antigen. Based on these two observations, we believe that the reporter in these AP-1 transgenic mice responds to appropriate signals and exhibits the appropriate response.

In accord with the previously described characteristics of AP-1, no transcriptional activity was observed in non-activated spleen cells. However, activation with a global stimulus such as PMA plus ionomycin, as well as T-cell-specific stimuli such as anti-CD3 mAb, ConA or specific antigen, results in its activation. No activity was detected when IL-2 alone was added to blasting T-cells expressing IL-2 receptor, suggesting that the AP-1 transcriptional activity observed upon stimulation was not IL-2 mediated. The fact that we could not detect any luciferase activity in purified B-cells upon stimulation with PMA plus ionomycin or lipopolysacharide (LPS, data not shown), indicates that the majority of AP-1 transcriptional activity observed in spleen is from T-cells.

Interestingly, we describe here for the first time a dissociation between the signaling requirement for the transcriptional activity and DNA-binding activity of AP-1 Whereas activation of PKC by PMA is sufficient to induce DNA binding, an additional increase of intracellular calcium provided by ionomycin or anti-CD3 mAb, which delivers both signals, is required to induce transcription mediated by AP-1. Previous studies suggested that only PMA is required for AP-1 DNA-binding as well as transcriptional activity, leading to its designation as a TRE (Angel *et al.*, 1987; Lee *et al.*, 1987). This discrepancy is probably due to the fact that most studies done previously were in tumor T-cell lines or T-cell clones that are already proliferating before activation, and where low levels of AP-1 transcriptional activity are detected without previous stimulation. By contrast, however, no luciferase activity was ever detected in unstimulated or PMA-treated primary lymphocytes from the transgenic mice.

It has been shown that CsA blocks NFAT DNA binding (Emmel *et al.*, 1989; Jain *et al.*, 1993) as well as Oct-1-associated protein (OAP) transcriptional activity (Ullman *et al.*, 1993), both factors containing Jun and Fos family members. Previous descriptions in cell lines indicated that both DNA-binding and transcriptional activity of AP-1 were resistant to CsA (Emmel *et al.*, 1989; Ullman *et al.*, 1993). However, we have found that in primary T-cells the calcium signal required for the AP-1 transcriptional activity is sensitive to CsA, even though it has no effect on DNA binding. The mechanism by which calcineurin might regulate AP-1 transcriptional activity remains unclear.

Two possible explanations might account for the dissociation between the signaling requirement for AP-1 transcriptional versus DNA-binding activity. First, even though PMA is able to induce DNA binding of AP-1, the complex induced might be non-functional due to an inappropiate subunit composition that differs from that of anti-CD3 or PMA plus ionomycin-induced complexes. Although c-Jun, JunB and JunD proteins bind to the AP-1 site, they exhibit different properties; for example, JunB has been reported to inhibit c-Jun-mediated transcription (Chiu

et al., 1989; Schutte *et al.*, 1989). Similarly, FosB2 interferes with the transactivation and transformation potential of c-Fos and FosB (Nakabeppu and Nathans, 1991; Yen *et al.*, 1991). However, we can eliminate this possibility since the comparative analysis done among all three different DNA-binding complexes does not show significant differences in the complexes induced with or without a Ca^{2+} signal that could account for the difference observed in transcriptional activity.

A second possibility is that an additional post-translational modification might be required for transcriptional activity. For example, the cyclic AMP-response element binding protein (CREB) can bind to the specific DNA element CRE, but it is able to mediate transcription only upon phosphorylation by a protein kinase A (Gonzalez *et al.*, 1991). Both Fos and Jun proteins are targets for different kinases and phosphatases that can regulate their activities. Casein kinase II has been reported to negatively regulate c-Jun DNA binding by phosphorylation at the C-terminal domain (Lin *et al.*, 1992). Mitogen-activated protein (MAP) kinases have also been involved in the phophorylation of Jun at the N-terminus after treatment with PMA (Pulverer *et al.*, 1991), suggesting that this pathway does not require two signals to be active. However, a new Jun kinase (JNK-1) has recently been identified (Hibi *et al.*, 1993; Dérijard *et al.*, 1994) which binds to a specific region within the c-Jun transactivation domain and phosphorylates serines 63 and 73. Interestingly, optimal activation of this kinase requires both PKC and calcium signals in Jurkat T-cell line as well as in spleen cells (Su *et al.*, 1994). The phosphorylation by JNK-1 in the transactivation domain can therefore explain the requirement of two signals for the AP-1 transcriptional activity, while only PKC activation is sufficient to induce DNA binding. Our data using herbimycin as a tyrosine kinase inhibitor show that inhibition of tyrosine phosphorylation abolishes AP-1 transcriptional activity. The blocking of AP-1 transcriptional activity induced by anti-CD3 mAb could be due partially to an inhibition of the earliest steps in the TcR-mediated signaling which involved different tyrosine kinases such as $p56^{lck}$, $p59^{fyn}$ or Zap70 (Perlmutter *et al.*, 1993). However, only a slight reduction in the DNA binding induced by anti-CD3 mAb was observed, indicating that the TcR signaling is not completely impaired in the presence of herbimycin. More importantly, the AP-1 transcriptional activity, but not DNA binding, stimulated by PMA plus ionomycin was also blocked by herbimycin, even though this stimulation bypasses the earliest tyrosine phosphorylation events proximal to the TcR. These results suggest that tyrosine kinases are involved in an intermediate step of the regulation of AP-1 transcriptional activity. In the same vein, JNK is phosphorylated at two residues, Thr and Tyr, located in the same peptide, which play a critical role in the regulation of JNK activity (Dérijard *et al.*, 1994). This Tyr residue might be the substrate for a herbimycin-sensitive tyrosine kinase involved in the regulation of AP-1.

In addition to the signaling delivered specifically by the TcR, IL-2 gene expression requires co-stimulatory signals provided by professional APC (Jenkins and Schwartz, 1987; Mueller *et al.*, 1989). However, it is not yet clear how this co-stimulatory signal participates in the transcription of the IL-2 gene. Go and Miller (1992) have reported that antigen stimulation of an IL-2 producer T-cell clone in the absence of co-stimulation results in a reduction of DNA-binding activity for NFAT, but a normal DNA-binding activity for AP-1. Here, we show that abolition of co-stimulation by prior fixation of APC or by blocking the interaction of CD28/CTLA4 with the counter-receptors B7-1/B7-2 blocks the AP-1 transcriptional activity induced by anti-CD3 mAb or specific antigen. Moreover, soluble anti-CD28 mAb is sufficient to provide the co-stimulatory signal which, in conjuction with TcR stimulation, will induce AP-1-mediated transcription with no significant modification of DNA-binding activity. These activation requirements correlate with those for IL-2 gene expression and suggest first that co-stimulation acts on at least AP-1 and, further, that the lack of AP-1 transcriptional activity in the absence of co-stimulation during T-cell activation might account for the failure to express the IL-2 gene. In addition, Kang *et al.* (1992) have previously described that the lack of IL-2 gene transcription upon restimulation of an anergic T-cell clone is associated with a reduction in both DNA-binding and transcriptional activity of AP-1, suggesting that the regulation of AP-1 activity plays a critical role not only during the induction of anergy in the absence of co-stimulation, but also during the restimulation of unresponsive T-cells.

How does CD28 mediate regulation of IL-2 gene expression? The initial studies indicated that CD28 mediates post-transcriptional regulation mechanisms by increasing the stability of several lymphokine mRNAs (Lindstein *et al.*, 1989). In addition, Fraser and co-workers described a CD28 response element (CD28RE) present in the IL-2 promoter which binds a specific protein induced by anti-CD28 (Fraser *et al.*, 1991; Fraser and Weiss, 1992). However, it has recently been reported that the CD28-responsive complex bound to CD28RE contains at least three members of the NFκB family (Verweij *et al.*, 1991; Ghosh *et al.*, 1993) and that distinct mitogenic T-cell activation signals converge on the induction of CD28RE binding activity (Civil *et al.*, 1992), suggesting the lack of specificity for the CD28-responsive nuclear factor. In this report, we demonstrate that CD28 is involved in the regulation of transcriptional activity of AP-1, although at present the mechanism is unknown. The biochemical nature of the signal mediated by CD28 remains unclear. Evidence has been presented for a calcium-mediated pathway, as well as a tyrosine phosphorylation pathway (reviewed in Linsley and Ledbetter, 1993), although a specific tyrosine kinase associated with CD28 has not yet been described. It is possible that a CD28-specific tyrosine kinase may participate in the post-translational modification of Jun and Fos and, therefore, in the regulation of the AP-1 transcriptional activity.

We propose a new model for the mechanism by which TcR- and CD28-mediated signals are integrated in the regulation of IL-2 gene transcription. Thus, PKC activation mediated by the interaction of an antigen in the context of MHC with a specific TcR is sufficient to induce binding of AP-1 to the IL-2 promoter. In addition, an intracellular Ca^{2+} increase also mediated by the TcR and another signal, probably tyrosine phosphorylation, provided by CD28, are required for post-translational modifications of the Jun or Fos family proteins. This post-translational modification results in the stimulation of transcriptional

activity of AP-1 and, likely, other transcriptional factors such as NFAT or OAP which also contain Jun and Fos proteins.

Materials and methods

Generation of transgenic mice

The 2.8 kb *Hpa*I digestion fragment isolated from the plasmid 2×AP1−luc was microinjected into fertilized C57BL/6×SJL F2 eggs and transgenic mice generated as described previously (Hogan *et al.*, 1986). Five positive founder lines were established. These founder lines were backcrossed onto C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) to obtain progeny for these studies. The construct contains the firefly luciferase gene (De Wet *et al.*, 1987) being driven by the rat prolactin minimal promoter (−36 to +37) under the control of two copies of the oligonucleotide containing two human collagenase TRE sites (TCGATTGAGTCAGGGTAACGATTGAGTCAGGAG) cloned into the *Xho*I site in an orientation indicated in Figure 1A.

Cell preparation

Spleen cells were treated with Gey's medium to remove red blood cells. T- and B-cell populations were purified by a double staining with directly fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (KT3; Tomonari, 1988) or anti-TcR (H57-557) mAbs and biotinylated anti-CD45R (B220; Caltag, San Francisco, CA) in combination with phycoerythrin−streptavidin (PE) (Pharmingen, San Diego, CA). The single FITC-positive (T-cells) and single PE-positive (B-cells) populations were separated and collected by using a Becton DickinsonFACStar^{PLUS} cell sorter. The average purity for each population was 99%. Identical results were obtained when anti-CD3 or anti-TcR mAbs were used for T-cell purification.

To obtain CD4$^+$ T-cells from Cyt *c* TcR×AP-1−luc double transgenic mice, red blood cell-depleted splenocytes were incubated with anti-IE, IA (m5/115) and anti-CD8 (TIB105) mAbs (Yale University Hybridoma Facility) for 30 min at 4°C, washed and incubated with goat anti-mouse and anti-rat IgG bound to magnetic beads (Collaborative Research) for 30 min at 4°C. They were then passaged over a magnet twice and 90% average purity was obtained.

To generate APCs, red blood cell-depleted splenocytes from non-transgenic mice [C57BL/6 or B10.BR (SGSNJ)] were treated with mitomycin C (50 μg/ml) (Sigma, St Louis, MO) for 45 min at 37°C and extensively washed after incubation. ECDI−APC were prepared by fixation of spleen cells (Jenkins and Schwartz, 1987) in 75 mM of ECDI (Calbiochem, La Jolla, CA) in phosphate-buffered saline (PBS) for 1 h on ice and extensively washed.

Nuclear extracts and mobility shift assay

'Mini' nuclear extracts were obtained from a small number of cells (4–6 x 10^6) by using a previously described procedure (Schreiber *et al.*, 1989; Tugores *et al.*, 1992). Binding reactions were carried out by using 2–3 μg of nuclear proteins and 3.5×10^4 c.p.m. of ^{32}P end-labeled double-stranded oligonucleotide as previously described (Schreiber *et al.*, 1989; Tugores *et al.*, 1992). The double-stranded oligonucleotides used in the mobility shift assay were the human collagenase TRE (GTCGACGTGAGTCAGCGCGC) (Angel *et al.*, 1987; Lee *et al.*, 1987) and the proximal AP-1 site from the IL-2 promoter (GTCGACAGAG-AGTCATCGCGC) (Serfling *et al.*, 1989).

Luciferase activity analysis

Cells from different sources were incubated at 5×10^5 cells/per well (48 wells/plate) in the presence or absence of different stimuli. After specific periods of time, cells from each independent well were harvested, washed twice in PBS and lysed in lysis buffer (Luciferase Assay, Promega, Madison, WI) for 30 min at room temperature. Lysate was spun down for 2 min and total supernatant was analyzed using the Luciferase Reagent (Promega) and measured in a luminometer (Lumat LB9501) for 10 s (two measurements of each independent sample were made). Background measurement was subtracted from each duplicate and experimental values are expressed relative to the activity found in extracts from unstimulated cells (equal to the background). The absolute values upon stimulation vary between 50 and 1000 relative luciferase units.

Reagents and antisera

The mAbs that we used for T-cell activation were anti-CD3 (145-2C11), anti-CD28 (37.51, Pharmingen), anti-B7-1/BB1 (Pharmingen) and anti-B7-2/GL1 (Pharmingen). Other reagents used in T-cell activation were CTLA4-Ig (kindly provided by P.Linsley), ConA (Boehring, Mannheim, Germany), SEA (Sigma), PMA (Sigma), ionomycin (Sigma), CsA (Sandoz Pharmaceutical, East Hanover, NJ), herbimycin A (Gibco BRL, Gaithersburg, MO), okadaic acid (Sigma) and synthetic Cyt *c* peptide kindly provided by K.Bottomly (Yale University). Antisera against c-Jun (2.2) and Fra-2 (13.2) were kindly provided by T.Curran (Hoffman-LaRoche Inc., Nutley, NJ). Antisera anti-JunB (725/4), -JunD (783/1), -Jun family (636/4) and -Fra-1 (RR21/3) were a gift from R.Bravo (Bristol-Mayer Squibb, Princeton, NJ). Purified Fos mAb against M peptide was kindly provided by A.Rao (Dana-Farber Cancer Institute, Boston, MA). Anti-FosB antiserum was provided by R.Winston (La Jolla, San Diego, CA).

Acknowledgements

We thank Dr A.Tugores, S.Guerder, B.Al-Ramadi, S.Ghosh and A.Bothwell for critical reading of the manuscript, T.Taylor for cell sorting technical assistance, C.Hughes and D.Buktus for generating the transgenic mice, and S.Hedrick for providing the Cyt *c* transgenic mice.This work was supported by National Institute of Health grant 1R1AI2902.

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Received on April 13, 1994; revised on June 8, 1994