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Raf-1 is required for T cell IL2 production

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Engagement of the T cell receptor/CD3 complex activates the serine/threonine kinase, Raf-1, but the physiologic consequences of its activation have not been determined. The effects of Raf-1 on interleukin 2 (IL2) production in T cells were examined using activated and inhibitory forms of Raf-1. A truncated active form of Raf-1 was expressed constitutively from the metallothionein promoter in a malignant T cell line, Jurkat. Treatment of the cells with zinc and cadmium greatly increased active Raf-1 expression. This increase in Raf-1 expression allowed antibodies to CD3 and to CD28 to stimulate IL2 production in the absence of phorbol myristate acetate (PMA) and enhanced IL2 production stimulated by these antibodies in the presence of PMA. The action of active Raf-1 was to increase IL2 gene transcription as it enhanced transcription of a reporter gene linked to IL2 promoter. Finally, the dominant negative form of Raf-1 inhibited transcription directed by the IL2 promoter that was induced by the mitogen phytohemagglutinin (PHA) and PMA. We conclude that Raf-1 activity is necessary for IL2 gene transcription and secretion. These data indicate a role for Raf-1 in the immune response.

Key words: Raf-1/IL2 production/IL2 enhancer driven transcription/T cells/kinase

Introduction

T cell activation is triggered by the recognition of antigen in the context of an antigen presenting cell (Allen and Unanue, 1987; Geppert and Lipsky, 1989). The signals generated by this interaction stimulate lymphokine production and proliferation leading to expansion of antigen-specific lymphocytes and the initiation of an immune response. One functional consequence of T cell activation is the production of interleukin 2 [IL2 (Smith, 1984)]. IL2 is an autocrine growth factor that is a critical stimulus for the growth and differentiation of both B and T cells (Smith, 1984; Jelinek *et al.*, 1986).

Model systems have been developed to study the biochemical events triggered by antigen recognition and leading to IL2 production. One such system exploits monoclonal antibodies (mAbs) to the T cell receptor/CD3 complex to mimic antigen recognition. Another model system employs the mitogenic plant lectin, phytohemagglutinin (PHA). Both PHA and mAbs to CD3 trigger T cell IL2 production in the presence of accessory cells or phorbol esters (Hara and Fu, 1985; Lipsky *et al.*, 1976) that activate protein kinase C (PKC). The requirement for phorbol esters can be partially replaced by immobilizing the PHA or anti-CD3 mAbs to a solid substrate (Geppert and Lipsky, 1987; Vine *et al.*, 1988). Calcium ionophores can also co-stimulate T cell IL2 production with phorbol esters (Isakov and Altman, 1985; Truneh *et al.*, 1984). Thus, the biochemical signals leading to IL2 production can be separated into calcium dependent and PKC dependent events.

Recent studies suggest that T cell IL2 production is dependent upon the activity of the proto-oncogene, *ras* (Rayter *et al.*, 1992). The Ras protein is thought to transmit PKC dependent signals that are necessary for the induction of IL2 gene transcription. In nonlymphoid cells, the product of the proto-oncogene *raf*-1 has been implicated in mediating the effects of Ras on gene activation (Bruder *et al.*, 1992; Troppmair *et al.*, 1992; Wood *et al.*, 1992). The current studies explore the role of Raf-1 in T cell IL2 production. Raf-1 is a 72 kDa serine/threonine kinase that is made up of an N-terminal regulatory domain and a C-terminal kinase domain (Heidecker *et al.*, 1990; Bruder *et al.*, 1992). Expression of the kinase domain without the regulatory domain results in a constitutively active kinase with regard to transformation of NIH-3T3 cells and the induction of transcription factor AP-1 driven promoter activity (Heidecker *et al.*, 1990; Kolch *et al.*, 1991; Bruder *et al.*, 1992). Expression of the regulatory domain without the kinase domain results in a protein that inhibits endogenous Raf-1 activity (dominant inhibitory effect) (Bruder *et al.*, 1992). The current studies examined the effect of active and dominant inhibitory forms of Raf-1 on IL2 gene transcription and secretion.

T cell IL2 production is driven primarily by a T cell-specific enhancer region extending ~300 bp upstream of the transcriptional start site for the IL2 gene (Durand *et al.*, 1987; Shaw *et al.*, 1988; Crabtree, 1989; Fraser *et al.*, 1991). This region contains several antigen specific and PKC dependent regulatory response elements. A reporter construct containing the minimal IL2 promoter driving the chloramphenicol acetyl transferase (CAT) gene was used to determine whether Raf-1 regulated IL2 gene transcription. The data demonstrate that active Raf-1 promotes IL2 gene transcription and secretion in Jurkat cells whereas dominant negative Raf-1 suppresses IL2 gene transcription. These studies implicate Raf-1 as an important constituent of the signaling cascade that is activated by antigen recognition and functions with other signaling cascades to induce IL2 gene transcription.

Results

The current studies employ three Raf-1 constructs. One (Raf-BXB) carries a large in-frame deletion of amino acids 26-302. This deletion renders Raf-1 constitutively active

with regard to transformation of NIH-3T3 cells and the induction of AP-1 driven promoter activity (Heidecker *et al.*, 1990; Kolch *et al.*, 1991; Bruder *et al.*, 1992). A second construct (Raf-C4B) encodes the 257 N-terminal amino acids of Raf-1, but lacks the C-terminal catalytic domain. Expression of this construct inhibits Raf-1 activity with regard to transformation and AP-1 driven promoter activity (Bruder *et al.*, 1992). This has been termed the dominant negative effect. Finally, we employed Raf-C4pm17B, which is identical to Raf-C4B except for the substitution of a serine residue for Cys168. This substitution eliminates the ability of Raf-C4B to regulate Raf-1 function (Bruder *et al.*, 1992). Raf-C4B and Raf-C4pm17B were tagged with the C-terminus of B-Raf, a closely related family member of Raf-1, to facilitate their detection.

Raf-BXB is expressed in transfected Jurkat cells

Figure 1 demonstrates that active Raf-1 (Raf-BXB) was expressed constitutively in transfected Jurkat cells, but exposure to a mixture of zinc and cadmium (Zn/Cd) greatly increased expression. Raf-BXB expression was significantly greater than endogenous Raf-1 even in the absence of Zn/Cd. As expected, Raf-C4pm17B, which lacks the C-terminus of Raf-1, was not recognized by the antiserum used in the immunoblot in Figure 1. Immunoblots with an antiserum that recognizes a B-Raf tag on Raf-C4pm17B did not detect Raf-C4pm17B expression (data not shown). These cells were hygromycin resistant and, therefore, served as controls for the transfection and subsequent culture in hygromycin. The response of this cell line (depicted below) was similar to the response of other cell lines transfected with vector alone (data not shown) and to the response of untransfected Jurkat cells as described in the literature (Manger *et al.*, 1985; Wiskocil *et al.*, 1985; Geppert *et al.*, 1989).

Active Raf-1 promotes anti-CD3 induced Jurkat cell IL2 production

The experiments depicted in Figure 2 illustrate the effect of active Raf-1 expression on anti-CD3 induced IL2 production. Soluble anti-CD3 alone does not normally stimulate Jurkat cell IL2 production (Geppert and Lipsky, 1987). As expected, anti-CD3 mAbs alone stimulated minimal IL2 production from Jurkat cells transfected with Raf-C4pm17B regardless of exposure to Zn/Cd and Jurkat cells expressing low levels of active Raf-1 (Figure 2). In contrast, Jurkat cells in which active Raf-1 expression had been enhanced by exposure to Zn/Cd produced IL2 when stimulated with soluble anti-CD3 mAbs. The amount of IL2 produced was similar to that generated by control Jurkat-C4pm17B cells stimulated with immobilized antibodies to CD3, but less than that of control cells stimulated by anti-CD3 and phorbol myristate acetate (PMA). Active Raf-1 expression also enhanced IL2 production stimulated by anti-CD3 and PMA.

Active Raf-1 promotes anti-CD28 induced IL2 production

We also examined the effect of active Raf-1 expression on IL2 production triggered via the CD28 pathway of activation. MAbs directed against CD28 on T cells trigger IL2 production through a pathway that is believed to be distinct from that triggered through the T cell antigen receptor (Geppert *et al.*, 1989; Ledbetter *et al.*, 1990; Fraser *et al.*, 1991). However, both pathways require activation of PKC (Hara *et al.*, 1985; Hara and Fu, 1985). Anti-CD28 mAbs alone stimulated IL2 production from Jurkat cells expressing optimal levels of active Raf-1 (Figure 3), but not from Jurkat cells expressing low levels of active Raf-1 or control Jurkat cells. Active Raf-1 expression also enhanced IL2 production stimulated by anti-CD28 and PMA (1 ng/ml) in Figure 3.

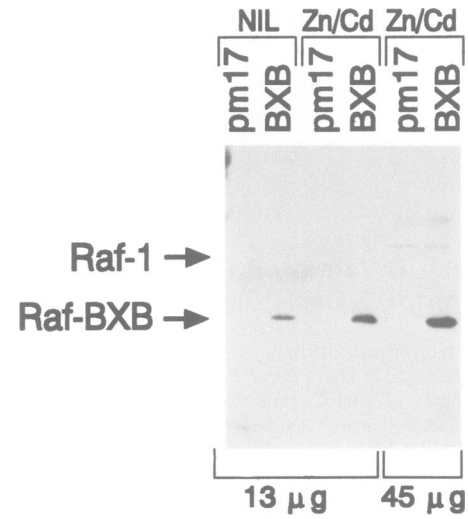

Fig. 1. Raf-BXB is expressed in a Zn/Cd inducible manner. A cDNA encoding Raf-BXB or Raf-C4pm17B was ligated into a metallothionein promoter driven episomal replicon expression vector containing the hygromycin resistance gene (pMEP, Invitrogen). The cDNAs were introduced into a malignant T cell line, Jurkat, by electroporation and selected with hygromycin. The Raf-BXB and Raf-C4pm17B transfected Jurkat cell lines were incubated with and without $ZnSO_4$ (75 μM) and $CdSO_4$ (0.75 μM) for 48 h and analyzed for BXB expression by immunoblotting.

Active Raf-1 does not co-stimulate IL2 production with ionomycin

To determine whether active Raf-1 could completely replace the requirement for PKC in T cells, we examined the capacity of Raf-1 to co-stimulate with ionomycin (Figure 4). Induction of active Raf-1 expression modestly enhanced IL2 production stimulated by ionomycin and PMA when 1 ng/ml of PMA was employed, but did not enhance responses when higher concentrations of PMA were employed. Ionomycin did not co-stimulate IL2 production with active Raf-1 regardless of the concentration of active Raf-1 (Figure 4).

Active Raf-1 enhances IL2 promoter driven transcription

To determine whether the effect of active Raf-1 was to enhance IL2 gene transcription, we examined the effect of Raf-BXB expression on IL2 promoter driven transcription of the CAT gene. Preliminary experiments (not shown) demonstrated that neither PHA nor anti-CD3 alone triggered an increase in CAT activity, but either stimulus induced CAT activity in the presence of PMA.

The experiments in Figure 5 compared CAT activity stimulated by anti-CD3 and PMA or PHA and PMA in Jurkat cells transfected with active Raf-1 with that present in Jurkat cells transfected with Raf-C4pm17B or the vector alone. Transfection of Raf-BXB enhanced CAT activity stimulated

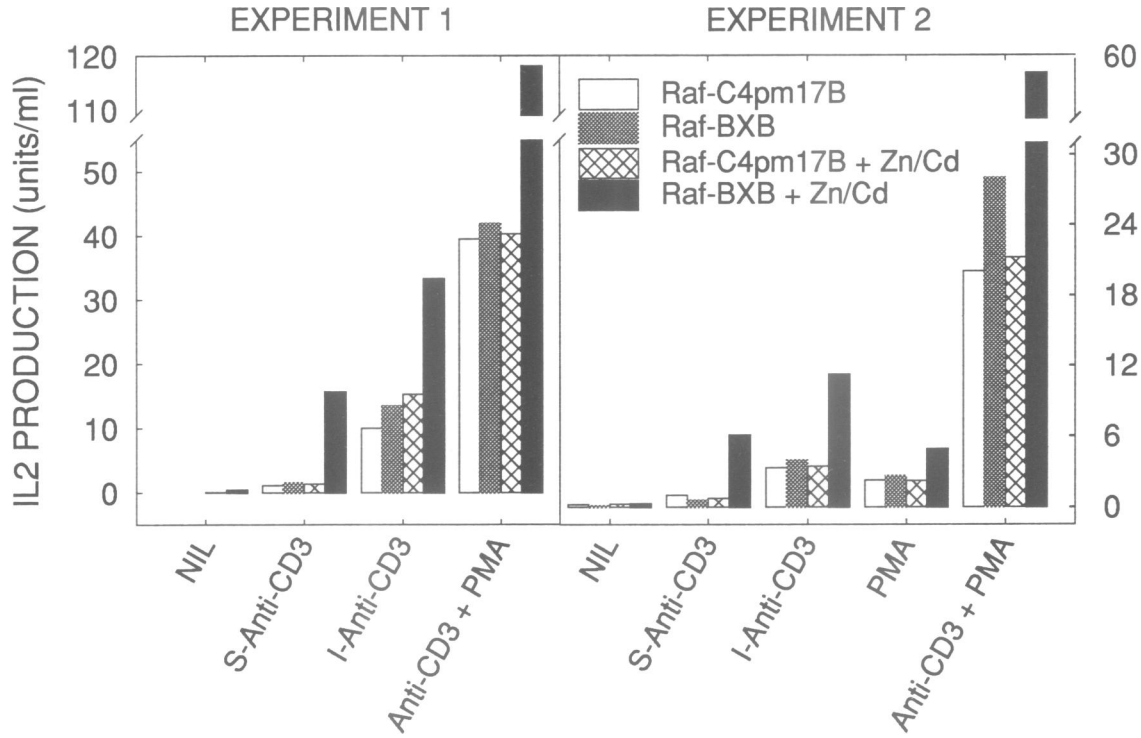

Fig. 2. Active Raf-BXB promotes anti-CD3 mAb induced IL2 production. Raf-BXB and Raf-C4pm17B transfected with Jurkat cell lines were incubated with or without Zn/Cd for 48 h, washed, and then cultured (1×10^5 cells/well) with 1 μg/ml soluble or 500 ng/well immobilized anti-CD3, with or without PMA (1 ng/ml) for 24 h. The supernatants were then harvested and IL2 production assessed. One unit of IL2 was defined as the amount of IL2 that caused half maximal CTLL cell proliferation.

by either PHA and PMA or anti-CD3 and PMA compared with transfection with vector alone or Raf-C4pm17B.

Dominant inhibitory Raf-1 inhibits IL2 promoter driven transcription

The studies described earlier suggest that Raf-1 activity promotes T cell IL2 production. To determine whether Raf-1 was required for T cell IL2 production, we examined the effect of a dominant negative form of Raf-1 (Raf-C4B) on IL2 promoter driven transcription induced by PHA and PMA. As can be seen in Figure 6, CAT activity induced by PHA and PMA was inhibited by Raf-C4B. Raf-C4B did not affect basal CAT activity.

Dominant inhibitory Raf-1 does not block transcription from the CRE promoter induced by cholera toxin

To examine whether the inhibitory effect of Raf-C4B was specific for the IL2 promoter, we examined the effect of Raf-C4B on CAT transcription driven by a cAMP regulated enhancer element (CRE) reporter construct. In control experiments, CRE regulated CAT expression was induced by cholera toxin, which has been found to stimulate an increase in cAMP in Jurkat cells (Minakuchi *et al.*, 1990), but not by PHA and PMA (Figure 7). Transfection of Raf-C4B did not inhibit cholera toxin stimulated CRE driven transcription of CAT. In additional experiments (not shown), we found that Raf-C4B did not inhibit transcription from the CMV promoter in Jurkat cells.

Discussion

The current studies examined the role of Raf-1 in T cell IL2 production. The data demonstrate that active Raf-1 expression promotes T cell IL2 production stimulated by anti-CD3 or anti-CD28 and IL2 promoter driven transcription induced by anti-CD3 or PHA and PMA. In addition, the data demonstrate that expression of a dominant negative form of Raf-1 specifically inhibits IL2 production stimulated by PHA and PMA. The data indicate that Raf-1 plays a critical role in T cell IL2 production.

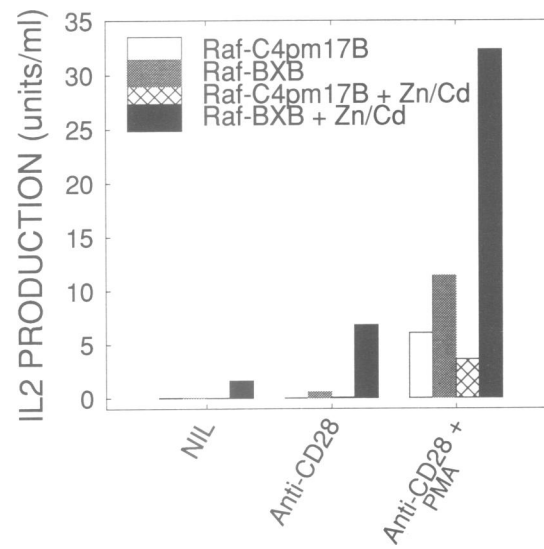

Fig. 3. Active Raf-BXB promotes anti-CD28 mAb induced IL2 production. Cells prepared as in Figure 2 were stimulated with soluble anti-CD28 with or without PMA (1 ng/ml) and IL2 production was assessed.

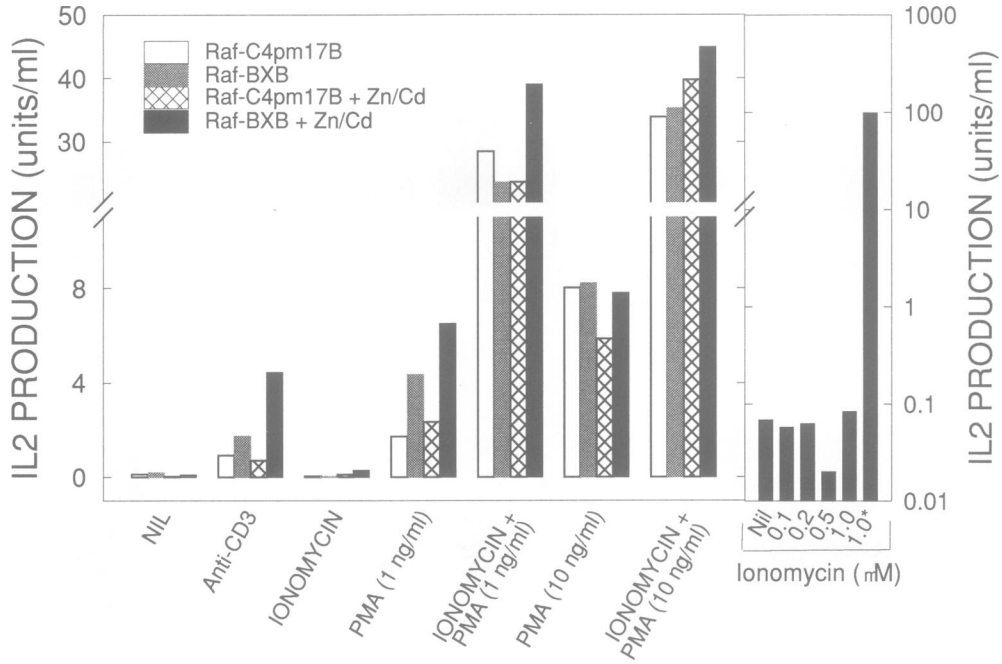

Fig. 4. Active Raf-1 does not co-stimulate T cell IL2 production with ionomycin. Raf-BXB and Raf-C4pm17B transfected Jurkat cell lines were incubated with or without Zn/Cd for 48 h, washed and cultured (1×10^5 cells/well) with ionomycin in the presence or absence of PMA as indicated for 24 h. The asterisk indicates the addition of 10 ng/ml of PMA. The supernatants were then harvested and IL2 production assessed.

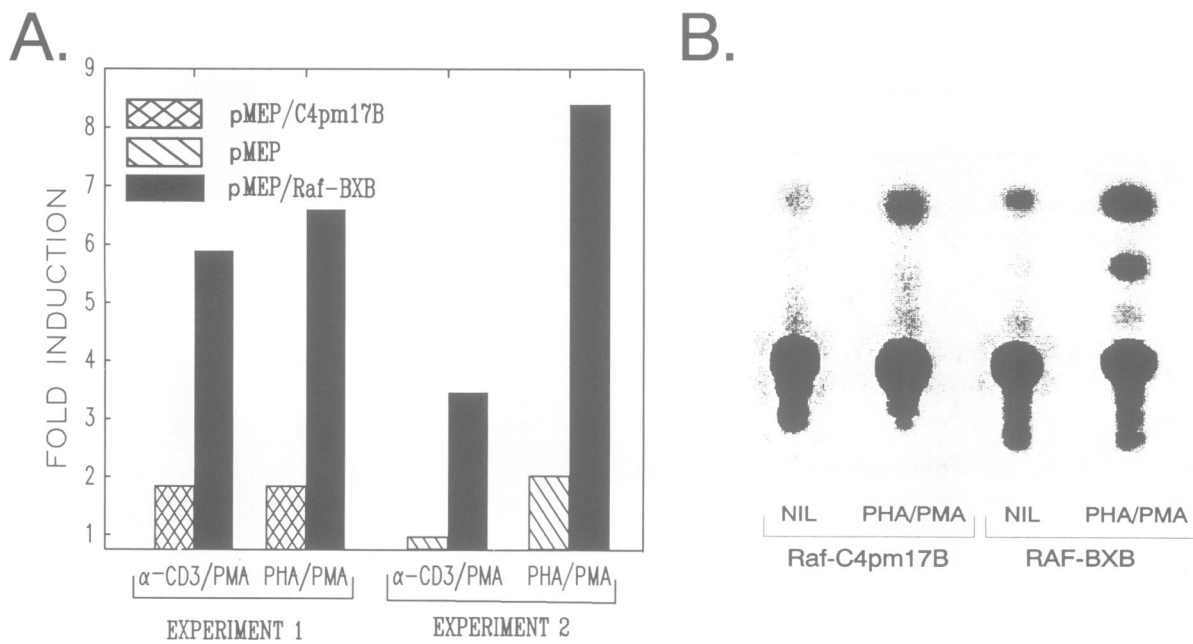

Fig. 5. PHA and PMA induced IL2 promoter driven transcription is enhanced by active Raf-1. Jurkat cells (2×10^7) were co-transfected with the pMEP vector or the pMEP vector containing the Raf-BXB construct (40 μg) together with the IL2 promoter−CAT construct (40 μg) and cultured in the presence of Zn/Cd for 24 h at 37°C. The cells were then divided into two groups and cultured in medium alone or in medium containing PHA (2 μg/ml) and PMA (20 ng/ml). CAT activity was assessed 18 h after stimulation. The data are presented as (**A**) the ratio of the percent conversion induced by stimulation over the percent conversion of unstimulated cells (fold induction), or (**B**) an image generated using the AMBIS radioanalytical imaging system which correlates density with emissions.

In the absence of Zn/Cd, Raf-BXB expression was several fold greater than Raf-1 expression in control Jurkat cells. Despite this, constitutive Raf-BXB expression was insufficient to promote IL2 production. There are several potential explanations for this finding. First, other isoforms of Raf-1 may exist in Jurkat cells. Thus, constitutive Raf-BXB expression may not be greater than the expression of other isoforms of Raf. Alternatively, the C-terminus of Raf-BXB may be required for the kinase to interact with cytoskeletal or signaling proteins which may facilitate its interaction with substrate proteins. In this regard, previous studies have demonstrated that Raf-1 associates with tyrosine kinases (App *et al.*, 1991; Maslinski *et al.*, 1992; Prasad and Rudd, 1992). If the C-terminus is required for this interaction or other as

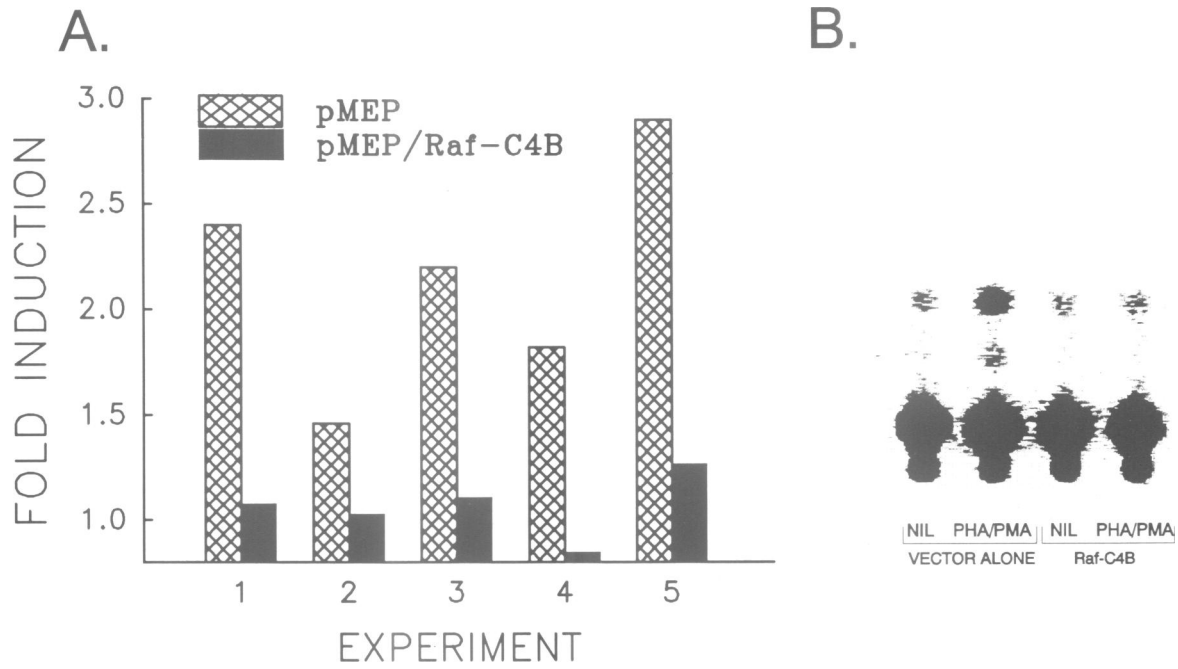

Fig. 6. PHA and PMA induced IL2 promoter driven transcription is inhibited by dominant negative Raf-1. Jurkat cells (2×10^7) were co-transfected with pMEP vector or the pMEP vector containing the Raf-CB4 construct (40 μg) together with the IL2 promoter−CAT construct (40 μg) and cultured in the presence of Zn/Cd for 24 h at 37°C. The cells were then divided into two groups and cultured in medium alone or in medium containing PHA (2 μg/ml) and PMA (20 ng/ml). CAT activity was assessed 18 h after stimulation. The data are presented as (**A**) the ratio of the percent conversion induced by PHA and PMA over the percent conversion of unstimulated cells (fold induction), or (**B**) an image generated using the AMBIS radioanalytical imaging system which correlates density with emissions.

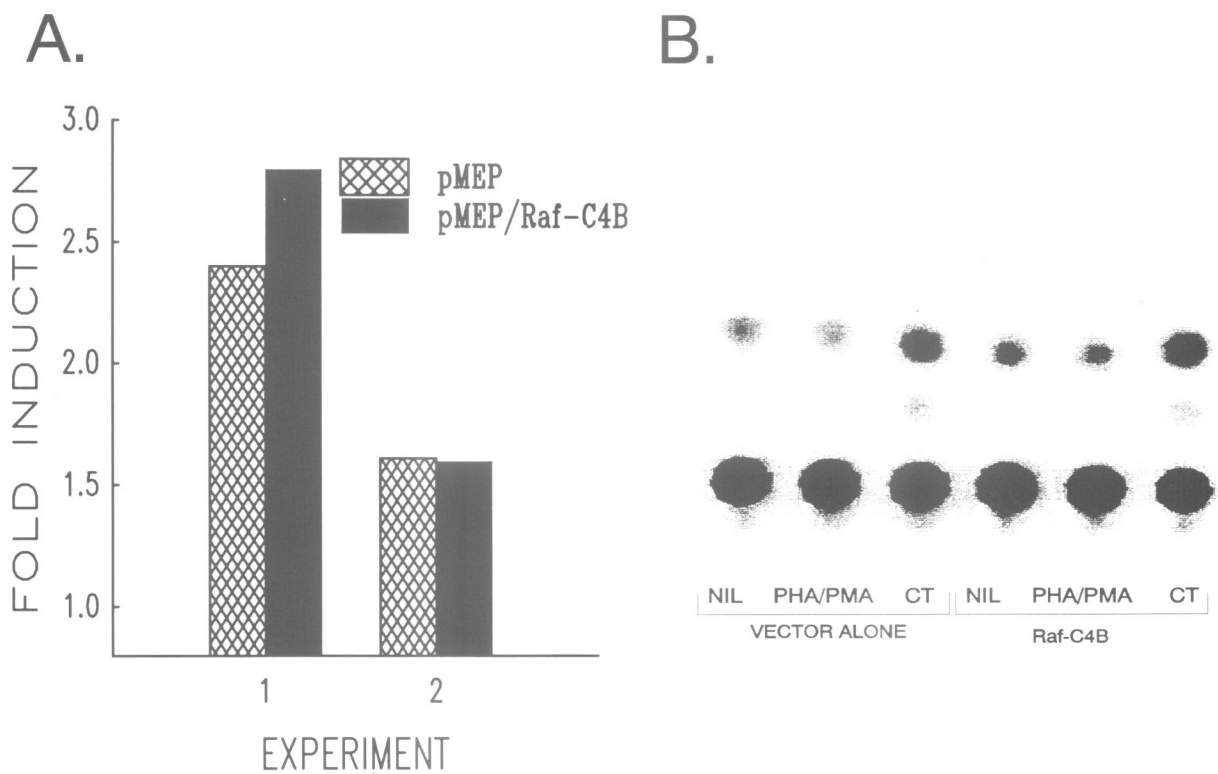

Fig. 7. Dominant negative Raf-1 does not inhibit CRE driven transcription. Jurkat cells (2×10^7) were co-transfected with pMEP vector or the pMEP vector containing the Raf-CB4 construct (40 μg) together with the CRE−CAT construct (40 μg) and cultured in the presence of Zn/Cd for 24 h at 37°C. The cells were then divided into two groups and cultured in medium alone or in medium containing either PHA (2 μg/ml) and PMA (20 ng/ml) or cholera toxin (CT; 100 ng/ml). CAT activity was assessed 18 h after stimulation. The data are presented as (**A**) the ratio of the percent conversion induced by PHA and PMA over the percent conversion of unstimulated cells (fold induction), or (**B**) an image generated using the AMBIS radioanalytical imaging system which correlates density with emissions.

yet undescribed interactions, then high levels of Raf-BXB expression may be required to trigger the physiologic events normally triggered by active Raf-1. Regardless of the mechanism, the data clearly demonstrate that an increase in Raf-1 activity correlates with an increase in IL2 production.

The relationship between Raf-1 activity and IL2 production was confirmed by additional studies demonstrating that transient expression of active Raf-1 enhanced IL2 promoter driven transcription. This finding demonstrates that Raf-1 enhances IL2 production through a direct effect on IL2 transcription, suggesting that Raf-1 activity is likely to influence the transcriptional activity of one or more of the nuclear binding factors regulating IL2 production. The IL2 promoter is known to contain a number of discrete elements (Durand *et al.*, 1987, 1988; Shaw *et al.*, 1988; Crabtree, 1989; Verweij *et al.*, 1990). These elements include the c-Fos/c-Jun heterodimer (AP-1), NFAT (nuclear factor of activated T cells) which contains AP-1 (Jain *et al.*, 1992), NF-κB, and Oct-1. Since Raf-1 is known to regulate AP-1 activity in other cell types (Bruder *et al.*, 1992), it is likely that the effect of Raf-1 on IL2 production noted in the current studies is mediated through an effect on AP-1.

Active Raf-1 expression was not sufficient to induce IL2 production alone or in the presence of ionomycin. This finding suggests that IL2 production is dependent on signals besides those delivered by ionomycin and Raf-1. Since the combination of ionomycin and PMA are sufficient to induce IL2 production (Truneh *et al.*, 1984; Isakov and Altman, 1985), PKC must initiate events required for IL2 production in addition to activation of Raf-1. Moreover, since anti-CD3 and anti-CD28 mAbs do co-stimulate IL2 production with Raf-1, the data suggest that they must provide a signal not provided by ionomycin. Both anti-CD3 and anti-CD28 mAbs are known to activate PKC. Thus, their ability to co-stimulate with Raf-1 may relate to their ability to activate PKC. Alternatively, they may activate as yet undescribed pathways that are PKC independent.

Active Raf-1 also promoted responses stimulated by low concentrations of PMA alone or in combination with anti-CD3, anti-CD28 or ionomycin. Active Raf-1 did not enhance IL2 production stimulated by higher concentrations of PMA, however. Therefore, although the signals generated by active Raf-1 can enhance phorbol ester induced T cell IL2 production, they do not mimic the co-stimulatory effects of ionomycin or anti-CD3, each of which is active at all concentrations of PMA.

It was not possible to generate stably transfected Jurkat cell lines that expressed dominant negative Raf-1. Therefore, to examine the effect of dominant negative Raf-1 on T cell IL2 production, we examined its effect on IL2 promoter driven transcription using a transient transfection system. The data clearly demonstrate that dominant negative Raf-1 expression inhibits IL2 promoter driven transcription induced by PHA and PMA. This effect was specific for the IL2 promoter, since dominant negative Raf-1 expression did not inhibit cholera toxin stimulated, CRE driven or unstimulated, CMV driven transcription. This finding strongly supports the hypothesis that Raf-1 is required for T cell IL2 production.

The effects of active and dominant negative Raf-1 on T cell IL2 promoter driven transcription are similar to the effects of active and dominant negative Ras reported previously (Baldari *et al.*, 1992; Rayter *et al.*, 1992). Active forms of both signaling molecules partially replace the requirement for PMA in anti-CD3 induced T cell activation (Baldari *et al.*, 1992; Rayter *et al.*, 1992). Active Ras co-stimulates IL2 production with ionomycin somewhat, but the response is very small compared with that of ionomycin and PMA. Dominant negative forms of both Ras and Raf-1 inhibit T cell IL2 production (Rayter *et al.*, 1992). The data are consistent with a model in which Raf-1 functions to transmit signals generated by Ras that are involved in IL2 production. These signals are probably triggered by PKC activity (Rayter *et al.*, 1992), but neither active Raf-1 nor Ras can completely mimic the effects of agents that activate PKC such as phorbol esters.

The conclusion that Raf-1 is required for T cell IL2 production expands the repertoire of functional activities regulated by Raf-1 activity. Previous studies have suggested that Raf-1 may function to transmit signals important in cell growth (Heidecker *et al.*, 1990; Kolch *et al.*, 1991; Troppmair *et al.*, 1992) and differentiation (Klinken *et al.*, 1988; Principato *et al.*, 1990). The current studies suggest that signals transmitted by Raf-1 may also control physiologic processes such as transcription of cytokine genes.

Materials and methods

Monoclonal antibodies and reagents

64.1, an IgG2a mAb directed at the TCR/CD3 molecular complex on mature T cells, and P1.17, an IgG2a control mAb, were purified over a column of Sepharose 4B coupled with staphylococcal protein A. Recombinant IL2 was kindly provided by Cetus. PMA (Sigma Chemical Co.) was dissolved in ethanol and added to the cultures at the indicated concentrations. Cholera toxin (Sigma Chemical Co.) was dissolved in water and used at a final concentration of 100 ng/ml. PHA was obtained from Wellcome Reagents Division, Burroughs Wellcome Co.

DNA constructs

Raf-BXB, Raf-C4B and Raf-C4pm17B have been described previously (Bruder *et al.*, 1992). Each vector was obtained in a Rous sarcoma virus driven mammalian expression vector. This vector was digested with *Xho*I and *Xba*I and ligated into *Xho*I and *Xba*I digested Bluescript (Stratagene). The Bluescript plasmids were then digested with *Hin*dIII and *Not*I and ligated into a metallothionein driven expression vector, pMEP (Invitrogen). pMEP contains the hygromycin resistance gene driven by the thymidine kinase promoter. A construct containing the cAMP regulated enhancer element (CRE) driving CAT (Yamamoto *et al.*, 1988) was obtained from Dr Marc Montminy (Salk Institute, La Jolla, CA).

A construct containing the minimal IL2 enhancer/promoter element driving CAT was obtained from Dr Richard Gaynor (UTSWMC, Dallas, TX). The promoter/enhancer was created from human T cell cDNA by PCR using primers that were synthesized based on the published sequence of the IL2 promoter (Siebenlist *et al.*, 1986). The region amplified (−342 to +47) includes most of the T cell specific transcription factor binding sites within the IL2 enhancer/promoter (Durand *et al.*, 1987). The sequence of the amplified segment was identical to the sequence reported previously. The IL2 enhancer/promoter was then ligated into the expression construct pJGFCAT19 (Garcia *et al.*, 1989) upstream of the CAT gene. The only promoter elements present in the final construct were those from the IL2 gene.

Cells and medium

The human T cell leukemia line, Jurkat, was obtained from Dr Arthur Weiss (UCSF, San Francisco, CA) and maintained in RPMI 1640 medium (Whittaker) supplemented with 10% fetal bovine serum (FBS; Gibco Laboratories, Grand Island, NY), penicillin G (200 U/ml), gentamycin (10 μg/ml) and L-glutamine (0.3 mg/ml).

DNA transfection

DNA-mediated gene transfer into Jurkat cells was accomplished by electroporation. Cells were washed and resuspended in RPMI 1640 (Whittaker) at a concentration of 1×10^7/ml at room temperature. Afterwards 0.8 ml of the cell suspension was transferred into a 4 cm electroporation cuvette (Bio-Rad) and the relevant construct or constructs (20 μg for stable transfection and 40 μg of each construct for transient transfection) were added. Electroporation was accomplished with a Gene Pulser apparatus (Bio-Rad) with a capacitance of 960 μF and an

electrical field of 0.2 V. The electroporated cells were transferred into a 75 cm^2 tissue culture flask (Costar) containing 20 ml of culture medium prewarmed to 37°C.

Development of stable cell lines expressing Raf-1 constructs

Transfected Jurkat cells were cultured for 48 h in medium and then hygromycin (200 μg/ml) was added to select for cells containing the episome. The concentration of hygromycin was increased to 800 μg/ml over the next 3 weeks.

Cell culture techniques

Jurkat cell lines expressing Raf-BXB or Raf-C4pm17B (1×10^6/well) were incubated with 64.1 (1 μg/ml), 9.3 (anti-CD28, 1:1000 dilution of 9.3 ascites; Bristol Myers Squibb), or the indicated concentrations of ionomycin alone or in combination with PMA (1 or 10 ng/ml) or the cells were incubated with immobilized 64.1 (500 ng/well) alone. After 24 h in culture the supernatants were harvested and IL2 activity assessed. 64.1 was immobilized to polystyrene wells as described (Geppert and Lipsky, 1987).

Assay of IL2 production

Culture supernatants were tested for the presence of IL2 with the use of CTLL cells, as described by Geppert *et al.* (1989). IL2 dependent CTLL cells (4×10^3 cells/well) were incubated with varying dilutions of culture supernatant in 96-well microtiter plates. Recombinant IL2 was used as a standard for the assay. The cells were incubated for 16 h at 37°C, then pulsed with 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) and harvested 8 h later. [^{3}H]Thymidine incorporation was measured in a liquid scintillation counter. Units of IL2 were determined by comparing the responses with a standard curve of IL2. One unit of IL2 was defined as the amount of IL2 that induced half-maximal responses.

Immunoblotting

Cells were lysed in 1% NP40 in homogenization buffer (20 mM Tris pH 7.5, 20 mM *p*-nitrophenyl-phosphate, 1 mM EGTA, 2 mM $MgCl_2$, 50 mM NaF, 50 μM sodium vanadate, 3 μg/ml aprotinin, 1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide). The soluble protein was boiled in gel loading buffer containing β-mercaptoethanol. 13 or 45 μg of protein was loaded into lanes as indicated and analyzed by electrophoresis on 12% SDS–polyacrylamide gels, transferred to nitrocellulose (Bio-Rad, Richmond, CA) and immunoblotted with a rabbit polyclonal antiserum to the C-terminus of Raf-1 [SP63 (Heidecker *et al.*, 1990)]. Reactive proteins were visualized with goat anti-rabbit immunoglobulin (Cappel) conjugated to horseradish peroxidase and a chemiluminescent procedure according to the manufacturer's specifications (Amersham International, Amersham, UK).

Assessment of IL2 promoter or CRE driven transcription of the CAT gene

Jurkat cells were co-transfected with the IL2 promoter/CAT construct or the CRE/CAT construct together with control constructs or various Raf-1 constructs and cultured at 37°C in the presence of $ZnSO_4$ (75 μM) and $CdSO_4$ (0.75 μM) for 24 h to allow the cells to recover and to produce truncated Raf-1 proteins. To avoid errors caused by variations in transfection efficiency, comparisons of stimuli were carried out on aliquots from a single pool of transfected cells. Each aliquot was cultured with medium alone, cholera toxin (100 ng/ml), PHA (2 μg/ml) and PMA (20 ng/ml), or anti-CD3 (1 μg/ml) and PMA (20 ng/ml) for 20 h. CAT enzyme activity was assayed using [^{14}C]chloramphenicol as described by Gorman *et al.* (1982). Thin layer chromatograms were visualized and the radioactivity quantified using an automated β-detection and imaging device (AMBIS). The percent conversion was determined by dividing the c.p.m. present in acetylated chloramphenicol into that present in unacetylated chloramphenicol. To allow comparisons between transfections, the data were expressed as the increase in the percent conversion induced by various stimuli.

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