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In Vivo Anergized CD4\(^+\) T Cells Have Defective Expression and Function of the Activating Protein-1 Transcription Factor

Annette Sundstedt* and Mikael Dohlsten†

The transcription factor activating protein-1 (AP-1) contributes significantly to the regulation of IL-2 gene expression during T cell activation and has been suggested to play a unique role in T cell anergy in vitro. In this study we have used the superantigen staphylococcal enterotoxin A to investigate the regulation of AP-1 in T cell anergy in vivo. Repeated injections of staphylococcal enterotoxin A induce a state of anergy in CD4\(^+\) T cells, characterized by reduced expression of IL-2 at mRNA and protein levels. The perturbed IL-2 response in anergic T cells correlated with reduced DNA binding activity of the transcription factors AP-1 and Fos/Jun-containing NF-AT. Using AP-1-luciferase reporter transgenic mice, we now demonstrate the lack of AP-1-dependent transcription. AP-1 activity is controlled by synthesis of its subunits Fos and Jun and by posttranslational phosphorylations. Analysis of Fos and Jun protein levels revealed no major differences in the expression of Jun proteins, but a marked decrease in c-Fos in anergic T cells. Experiments in transgenic mice overexpressing c-Fos (H2-c-fos) showed reconstituted AP-1 DNA binding. In contrast, the AP-1-driven transcription and IL-2 production remained suppressed. The Jun N-terminal kinase is known to play a critical role in regulating AP-1 trans-activation. Analyses of Jun N-terminal kinase demonstrated normal protein amounts, but reduced enzymatic activity, in anergic compared with activated CD4\(^+\) T cells. This suggests that in vivo anergized T cells have defects in the AP-1 pathway due to both reduced protein expression and perturbed posttranslational modifications. The Journal of Immunology, 1998, 161: 5930–5936.

Lymphocyte activation and IL-2 production require at least two signals, generated by the interaction of Ag/MHC complexes with the TCR (signal 1) and costimulation provided by the APC through the CD28 receptor on the T cells (signal 2) (1). The consequence of engagement of TCR in the absence of costimulation is not only poor induction of the growth factor IL-2, but the responding T cell is subsequently pushed into a state of anergy, characterized as long-lasting, Ag-specific unresponsiveness (2). Anergy was originally described by Schwartz and coworkers using an in vitro model with T cell clones that were rendered anergic by Ag presentation in the absence of appropriate costimulatory signals (3, 4). Studies of these Ag-specific T cell clones have shown that the deficient IL-2 production, which is a costimulatory signals (3, 4). Studies of these Ag-specific T cell clones have shown that the deficient IL-2 production, which is a hallmark of anergy, correlates with reduced levels of the activating protein-1 (AP-1)\(^2\) transcription factor (5).

Production of IL-2 in T cells is strongly regulated at the transcriptional level. Activation of the IL-2 promoter requires cooperative interactions of several transcription factors, including AP-1, NF-κB, NF-AT, and NF-IL-2 (6–9). AP-1 proteins seem to play a central role in IL-2 regulation by binding to the functionally important AP-1 site in the IL-2 promoter (10) as well as participating in the formation of transcriptionally active NF-AT and NF-IL-2 (8, 9). The AP-1 transcription factor is a complex between different members of the Fos (c-Fos, FosB, Fra-1, and Fra-2) and Jun (c-Jun, JunB, and JunD) families of proteins (11). These proteins contain a leucine zipper that permits dimerization with other members of the Fos/Jun family (12). Homodimers of Jun, but not of Fos, bind to 12-O-tetradecanoylphorbol-13-acetate-responsive elements (TREs). However, the DNA binding affinity of heterodimers is significantly higher (13).

AP-1 is regulated at the level of both jun and fos gene transcription and by posttranslational modifications of their gene products. Synthesis of c-Fos and the trans-activating capacity of c-Jun and c-Fos have been shown to be regulated by the mitogen-activated protein kinases (MAPK) extracellular signal-regulated kinases (ERK), Jun N-terminal kinases (JNK), and Fos-regulating kinase (FRK), respectively. ERK activation depends on coupling of the TCR-CD3 complex to p21\(^{ras}\) (14), with subsequent activation of the Raf-MEK kinase cascade (15, 16). JNK activation also occurs through p21\(^{ras}\), but requires an additional signal emerging from the CD28 costimulatory receptor (17). This involves activation of small GTP binding proteins such as Rac1 and Cdc42 (18, 19) that induce the kinase cascade MEKK-SEK-JNK (20).

Kang et al. previously showed that AP-1-dependent DNA binding and trans-activation at the IL-2 gene are defective in anergic Th1 cells (5). Recent studies have demonstrated that the TCR-inducible activities of ERK and JNK also are reduced in vitro anergized T cell clones (21), which correlated with reduced expression of c-Fos, FosB, and JunB proteins (22). However, the physiologic relevance of these findings is unclear, since only limited information is available on in vivo induced anergic T cells. We have previously shown that repeated injections of staphylococcal enterotoxin A (SEA) to mice transduce a state of long-lasting IL-2 hyporesponsiveness in the VB\(^3\)CD4\(^+\) T cell compartment (23) associated with reduced DNA binding of AP-1 and Fos/Jun-containing NF-AT (24). In this study we demonstrate lack of AP-1-dependent transcription in anergic CD4\(^+\) T cells. The defective AP-1 transcriptional activity seems to involve both reduced c-Fos

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2 Abbreviations used in this paper: AP-1, activating protein-1; TRE, 12-O-tetradecanoylphorbol-13-acetate-responsive element; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; FRK, Fos-regulating kinase; MEK, MAPK/ERK kinase; SEK, Stress-activated protein kinase; SEK, staphylococcal enterotoxin A; GST, glutathione S-transferase; EMSA, electrophoretic mobility shift assay.
protein levels and lack of JNK-mediated posttranslational modifi-
cations of AP-1 protein. We suggest that the perturbation of the
JNK pathway may be a critical factor in anergic CD4+ T cells
in vivo.

Materials and Methods

Animals and treatment

Transgenic C57BL/6 mice expressing a rearranged TCR-Vβ3 gene under
the influence of an inserted Ig heavy chain enhancer (25) were provided by
Dr. M. Davis (Stanford, CA). AP-1-luciferase reporter transgenic mice
(AP-1-luc) expressing the firefly luciferase gene driven by four human
collagenase TREs (7) were provided by Dr. R. Flavell (Howard Hughes
Medical Institute, Yale University, New Haven, CT). c-cfos transgenic mice
(H2-c-fos) carrying the mouse c-fos gene under the control of the H-2Kb
promoter (26) were provided by Dr. T. Tokuhisa (Chiba University
School of Medicine, Chiba, Japan). The AP-1-luc and the H2-c-fos mice
were crossed with TCR-β3 transgenic mice and used for experiments.

Recombinant SEA was expressed in Escherichia coli and purified to ho-
mogeneity as described previously (27). Various doses of SEA in PBS with
1% normal syngeneic serum or PBS alone were injected i.v. at 4-day in-
tervals. The TCR-β3 transgenic mice expressed 90–95% of TCR-β3 in
the CD3+ T cell population before and after SEA treatment.

Reagents

mAbs directed to murine CD3, CD4, CD220, CD19, and TCR-β3 were
purchased from PharMingen (San Diego, CA). Recombinant murine IL-2
and mAbs to murine IL-2 (JES6-1A12 and JES6-5H4) were obtained from
PharMingen. PMA and ionomycin were purchased from ICN Pharmaceu-
ticals (Costa Mesa, CA). A GST-c-Jun,-9g-expressing plasmid was pro-
vided by Dr. M. Karin (University of California-San Diego). Production
and purification of GST-c-Jun protein were performed as previously
described (28).

Cell separation

Spleens were prepared from mice injected i.v. with SEA or PBS at different
times before analysis. Purified CD4+ T cells (>95% CD4+ as determined
by FACS analysis) and B220− B cells (>95% CD19+) were obtained by
positive selection using magnetic beads coated with anti-CD4 mAb or anti-
B220 mAb (Miltenyi Biotec, Bergisch Gladbach, Germany) according to
the manufacturer’s instructions.

Luciferase activity analysis

Purified CD4+ T cells were incubated at 1 × 10^6 cells/well (24 wells/plate)
in the presence or the absence of various stimuli. Purified B220− B cells
were used as APCs at 1 × 10^5 cells/well. 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 5 min, and total
supernatant was analyzed using the luciferase reagent (Promega) and
measured in a luminometer (MicroLumat LB 96 P, Berthold, Nashua, NH).
Background measurement was subtracted from each sample, and experi-
mental values are expressed as recorded light units of luciferase activity.

IL-2 protein levels

Blood samples were drawn at various time points after i.v. injections of
SEA or PBS and were tested for IL-2 content by a specific ELISA using
mAbs JES6-1A12 and JES6-5H4 according to instructions from the manufac-
turer. The same method was used for IL-2 measurements in culture
supernatants.

Preparation of nuclear extracts

Nuclear extracts were made according to the method described by Schre-
iber et al. (29). Purified CD4+ T cells (5–10 × 10^6) were used for extrac-
tion, and the nuclear proteins were finally dissolved in 20 mM HEPES (pH
7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF,
0.5 μg/ml leupeptin, and 0.5 μg/ml antipain. The protein concentration of
all samples was measured by the Bio-Rad protein assay kit (Bio-Rad,
Hercules, CA), and the extracts were stored in aliquots at −70°C until used.

Western blot analysis

Before analysis by Western blotting, protein extracts were separated on
10% SDS-PAGE and electroblotted onto nitrocellulose membranes (0.2
μM; Bio-Rad). The membranes were probed with specific rabbit antiserum.

The immune complexes were detected by a 1/3000 dilution of goat anti-
rabbit Ig-horseradish peroxidase conjugate (Bio-Rad) and were visualized
using enhanced chemiluminescence detection (Amersham, Little Chalfont,
U.K.). For analyses of Jun and Fos expression, nuclear protein extracts
and the following rabbit polyclonal Abs were used: anti-c-Fos (4), anti-JunB
(N-17), and anti-JunD (329) purchased from Santa Cruz Biotechnology
(Santa Cruz, CA). A mouse anti-c-Jun mAb and a goat anti-mouse Ig-
horseradish peroxidase conjugate for detection were obtained from Trans-
duction Laboratories (Lexington, KY). To examine JNK protein abun-
dance, cellular extracts and JNK-1 (C-17) and JNK-2 (FL) rabbit polyclonal
Abs (Santa Cruz Biotechnology) were used.

Electrophoretic mobility shift assay (EMSA)

The AP-1 consensus oligonucleotide used contained the following se-
quence: 5′-CTAGTGATGAGTGACCGGATC-3′. The probe was end la-
egocted with [γ-32P]ATP using T4 polynucleotide kinase (Promega), accord-
ing to instructions from the manufacturer and was purified on 5% polyacryl-
amide gels in 1× TBE (89 mM Tris, 89 mM boric acid, and 2
mM EDTA). Binding reactions were performed with the same amount of
protein in each reaction (0.5–1 μg) in 10 mM Tris-HCl (pH 7.5), 50 mM
NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, and 2 μg of poly(dI-dC) (Phar-
macia, Piscataway, NJ). The reactions were incubated at 37°C for 30
min with 15,000 cpm of double-stranded [32P]-labeled oligonucleotides.
The samples were electrophoresed on 5% polyacrylamide gels in 1× TBE.
The gels were dried under vacuum and exposed to autoradiography at −70°C.

Preparation of cellular extracts

Total cellular extracts for immunocomplex protein kinase assays and West-
ern blots were made from 5–10 × 10^6 purified CD4+ T cells according to
the method of Hibi et al. (28). In individual experiments, the same number
of cells from each group was used for extraction. The cells were lysed in
600 μl of cold lysis buffer consisting of 20 mM Tris-HCl (pH 7.7), 250 mM
NaCl, 3 mM EDTA, 3 mM EGTA, and 0.5% Nonidet P-40; to which
protease and phosphatase inhibitors had been freshly added. After a 30-min
incubation on ice, the extracts were spun for 10 min in an Eppendorf
centrifuge at 4°C to pellet cellular debris. The supernatants were removed
and stored at −70°C.

Immunocomplex protein kinase assay

Immunocomplex kinase assays were performed as described by Hibi et al.
(28). Clarified cellular extracts were incubated with protein A/G-agarose
beads (Santa Cruz) precoated with polyclonal Ab to JNK-1 (C-17; Santa
Cruz) for 2 h at 4°C with gentle rotation. Immune complexes were washed
and then assayed for enzyme activity as described, using GST-c-Jun,-9g as
a substrate. The beads were pelleted and resuspended in 30 μl of kinase
buffer (20 mM HEPES (pH 7.6), 2 mM DTT, 10 mM MgCl2, 1 mM EDTA,
and 1 mM EGTA, to which phosphatase inhibitors had been freshly added)
containing 1 μg of GST-c-Jun, 1 μM ATP, and 1 μCi of [γ-32P]ATP.
Incubations were conducted for 20 min at 30°C, then reactions were
stopped by addition of 10 μl of 4× SDS sample buffer. Phosphorylation
of the substrates was analyzed by 10% SDS-PAGE followed by autoradiog-
raphy of the dried gel. [32P]ATP incorporation was determined by cutting
the gel and measuring radioactivity in a beta counter.

Results

Reduced AP-1 transcriptional activity in CD4+ T cells

energized by superantigen in vivo

We have previously shown that repeated injections of SEA to
TCR-β3 transgenic mice induce a state of IL-2 hyporesponsiveness
in the CD4+ compartment that correlates with reduced DNA
binding of the AP-1 transcription factor (24). To confirm that AP-1
is also perturbed at the transcriptional level, we used transgenic
mice expressing a luciferase reporter gene under the control of
AP-1 binding sites (AP-1-luc) (7). Double-transgenic TCR-
β3×AP-1-luc mice were treated with one injection of SEA
to induce activation and with repeated injections to induce anergy.
CD4+ transgenic spleen T cells were purified, stimulated in vitro
with SEA-coated syngeneic B220− B cells, and analyzed for AP-1
transcriptional activity. Activated CD4+ T cells (1× SEA) exhib-
ted significant AP-1 transcriptional activity in response to SEA
(Fig. 1A), while no activity could be detected in the anergic CD4+
in anergic T cells was due to reduced expression of Jun and Fos proteins, we analyzed nuclear Fos and Jun protein content by Western blotting. We have previously shown that large amounts of AP-1 DNA binding were induced in CD4+ T cells 1 h after a single SEA injection (24). The AP-1 complexes detected by EMSA contained mainly heterodimers between c-Fos and JunB or JunD (24). Using Western blot analysis of the nuclear extracts, we found that JunD was constitutively expressed, and the amount did not change following activation either by a single (1× SEA) or repeated (3× SEA) injections (Fig. 2A). In contrast, c-Fos, JunB, and c-Jun were expressed only after stimulation (Fig. 2, B–D). Interestingly, the induction of c-Fos was defective in anergic CD4+ T cells (Fig. 2D). On the contrary, JunB protein was induced in both groups to a similar extent (Fig. 2B). c-Jun was expressed at low levels, which were further reduced in the anergic cells (Fig. 2C). Taken together, these results suggested that the reduced c-Fos expression may explain the defective AP-1 DNA binding and trans-activation in the anergic CD4+ T cells.

Restored AP-1 DNA-binding in anergic CD4+ T cells from H2-c-fos transgenic mice

In an attempt to restore the possible c-Fos defect in the anergic T cells, we used transgenic mice that express c-fos under an MHC class I promoter (H2-K^b; H2-c-fos) (26). These mice were crossed with the TCR-Vβ3 transgenic mice and used for anergy experiments. Constitutive expression of c-fos was confirmed by RT-PCR (data not shown). Double-transgenic mice were injected one or three times with SEA. Nuclear extracts from purified CD4+ T cells were analyzed by EMSA for AP-1 DNA binding. Interestingly, CD4+ T cells from H2-c-fos × TCR-Vβ3 transgenic mice anergized by SEA (3× SEA) contained significantly more AP-1 than anergic CD4+ T cells from control mice (Fig. 3A). The levels of AP-1 in H2-c-fos transgenic mice anergized by SEA corresponded to the levels observed in CD4+ T cells from control mice activated by a single injection of SEA (1× SEA), suggesting that c-Fos indeed was a limiting factor for obtaining AP-1 band shifts. Supershift analysis of the obtained AP-1-binding protein complex in H2-c-fos transgenic mice demonstrated the same constitution as that previously described for SEA-treated TCR-Vβ3 transgenic mice (24), consisting mainly of heterodimers between c-Fos and JunB or JunD (data not shown). In contrast, analysis of IL-2 production in serum from double-transgenic mice revealed a perturbed IL-2 response similar to that in control mice (Fig. 3B). This suggests that there is an additional defect in the AP-1 pathway, possibly due to a perturbation in the posttranslational modifications of Fos and Jun.

Lack of AP-1 transcriptional activity in c-Fos-overexpressing mice

To exclude the possibility that AP-1 transcriptional activity was restored in the H2-c-fos mice and that the absence of IL-2 production instead represented additional defects in other transcription factors, we crossed the H2-c-fos mice with the AP-1-luc mice on TCR-Vβ3 background. Triple-transgenic mice were activated (1× SEA) or anergized (3× SEA) by SEA in vivo. Purified CD4+ T cells were stimulated with SEA-coated syngeneic B220+ B cells and PM2 in vitro and analyzed for AP-1 transcriptional activity. Activated H2-c-fos CD4+ T cells (1× SEA) exhibited significant AP-1 transcription in response to SEA and PM2 (Fig. 4A), while the anergic H2-c-fos CD4+ T cells failed to respond (Fig. 4A). Addition of ionomycin restored the response in the anergic cells (Fig. 4A), as shown above (Fig. 1A). Similarly, the AP-1 transcriptional activity correlated with the ability to synthesize IL-2 (Fig. 2B).
FIGURE 2. Reduced expression of c-Fos in anergic CD4+ T cells. TCR-Vβ3 transgenic mice were injected i.v. one or three times with 10 μg of SEA or PBS (untreated control) at 4-day intervals. Spleens were removed 1 h after the last SEA injection, and purified CD4+ T cells were prepared. Two micrograms of nuclear extracts were loaded on a 10% SDS-PAGE gel; transferred to a nitrocellulose filter; probed with anti-JunD antiserum (A), anti-JunB antiserum (B), anti-c-Jun Ab (C), and anti-c-Fos antisem (D); and detected by chemiluminescence. The specific proteins and m.w. (MW) standards are indicated in the figure. One of three similar experiments is shown.

4B). Thus, these results suggest that the AP-1 complex induced by overexpression of c-Fos is transcriptionally inactive.

Reduced JNK activity in anergic CD4+ T cells

Synthesis of Fos and Jun proteins is not sufficient to induce AP-1 activity. In addition, these proteins need to be phosphorylated to become transcriptionally active (30). Protein kinase activities that phosphorylate and activate Jun (JNK) and Fos (FRK) have been described (28, 31). However, the c-Fos kinase FRK is poorly understood. To investigate whether JNK activity was reduced in the anergic CD4+ T cells, we used a specific immunocomplex protein kinase assay. TCR-Vβ3 transgenic mice were injected one or three times with SEA to induce activation or anergy, respectively. Analysis of JNK activity in purified CD4+ T cells revealed that a significant response was rapidly induced in activated CD4+ T cells (1× SEA), with peak activity after 0.5 h in vivo (Fig. 5A). In contrast, the anergic cells exhibited significantly lower basal JNK activity before the last SEA injection and a minor activation-induced increase (Fig. 5A). Analysis of JNK protein levels by Western blotting showed that these proteins were constitutively expressed (Fig. 5B) in conformity with earlier studies (21). Thus, the defective AP-1 activity in anergic CD4+ T cells may be the result of deficient JNK activation.

Discussion

The AP-1 transcription factor is intimately involved in the regulation of IL-2 gene transcription (6–10) and may be a unique target in T cell clonal anergy (5, 22). We have previously shown that CD4+ T cells anergized by superantigen in vivo contained reduced AP-1 and NF-AT/AP-1 DNA binding (24). In this study we have further characterized the in vivo expression and function of AP-1 in the anergic CD4+ T cells. Using AP-1-luciferase reporter transgenic mice, we conclude that the AP-1 transcriptional activity in SEA-energized CD4+ T cells was repressed and correlated strongly with the inability to synthesize IL-2. Earlier studies in these mice by Rincón and Flavell (7) demonstrated a clear dissociation between the signals required for AP-1 DNA binding and transcriptional activity in primary T cells in vitro. While protein kinase C activation was sufficient to induce DNA binding activity, an additional calcium increase was required to induce AP-1-driven transcription and IL-2 production (7).

AP-1 protein activity is regulated both at the level of gene transcription and by posttranslational modifications, which are largely controlled by protein kinases of the MAPK family (30). c-fos transcription is controlled by ERK-dependent phosphorylation of the ternary complex factor Elk-1 that regulates the c-fos promoter (32). Activation of JNK results in the phosphorylation of c-Jun and JunD, which augments their transcriptional activities (28, 33). In addition, JNK phosphorylates ATF-2, which together with c-Jun bind to a nonconventional TRE site in the c-jun promoter and induce c-jun transcription (34). It was recently demonstrated that c-Fos, FosB, and JunB proteins are reduced in Th1 clones anergized by anti-CD3 treatment in vitro (22), which correlated with impaired activities of the MAPK family members ERK and JNK (21). In this study, we observed that in vivo anergized CD4+ T cells expressed less c-Fos protein than activated CD4+ T cells. In contrast, both JunB and JunD proteins were expressed at similar levels in activated and anergic CD4+ T cells. Thus, a potential explanation for defective AP-1 trans-activation in anergic cells in vivo would be poor activation of MAPK and subsequent suboptimal Fos protein expression. However, overexpression of c-Fos protein in H2-c-fos transgenic mice restored AP-1 DNA binding, but failed to induce IL-2 production and proper AP-1-dependent transcription. Thus, in addition to the observed defect in synthesis of the Fos/Jun family of proteins observed here and by others (22), anergic T cells lack posttranslational modifications of AP-1 proteins required for AP-1-dependent trans-activation.

Protein kinase activities that phosphorylate and activate Jun (JNK) and Fos (FRK) have been described. Whereas the c-Fos kinase FRK is poorly understood, the c-Jun kinase JNK has been studied in detail. The JNK protein kinases bind Jun proteins at a docking site between amino acids 30 and 60 (30) and subsequently phosphorylate the NH2-terminal activation domain of c-Jun, causing increased transcriptional activity (28, 33). JunD is also a substrate for JNK, but contains a weaker docking site for JNK than c-Jun (33). Interestingly, full activation of JNK1 and JNK2 has
been reported to require two signals in T cells, i.e., ligation of both TCR and CD28 (17). Studies of T cell cultures in vitro have further shown that the activity of JNK correlates to the ability of T cells to synthesize IL-2 (17). In contrast, MAPK ERK1 and ERK2 were activated by TCR stimulation alone and were not affected by CD28 (17). Hence, integration of signals that lead to full T cell activation may occur at the levels of JNK activation. In this study rapid induction of JNK activity in CD4$^+$ T cells was observed after SEA priming in vivo. In contrast, we observed inhibition of JNK activation in SEA-anergized T cells. This could explain the absence of AP-1 transcriptional activity in the c-Fos-overexpressing cells, although prominent AP-1 DNA binding was recorded. Supershift analysis of the induced AP-1 complexes showed that both the activated and anergic T cells contained mainly heterodimers between c-Fos and JunB or JunD (24) (data not shown). We did, however, detect c-Jun protein by Western blotting and found reduced protein levels in the anergic cells (Fig. 2). Indeed, a dominant negative mutant form of c-Jun was shown to inhibit NF-AT-driven transcription and to prevent IL-2 gene expression (36).

Inhibition of JNK activation may also explain the observed defective expression of c-Fos protein in the anergic T cells. In addition to its ability to activate Jun synthesis and transcriptional activity, it was recently demonstrated that JNK activates the c-fos promoter through phosphorylation of TCF/Elk-1 (37). Furthermore, expression of the protein kinase MEKK-1 in the JNK cascade induced c-fos transcription and Elk-1 transcriptional activity in the absence of considerable ERK activation (37). Interestingly, induction of MEKK-1 activity also requires two signals in T cells (38), suggesting similar activation requirements for JNK and
MEKK-1 proteins. However, the roles of ERK and JNK in the induction of Fos and Jun proteins and in the control of AP-1 transcription remain to be completely established in T cells. The absence of AP-1 transcriptional activity in anergized CD4+ T cells could in addition include a failure to phosphorylate c-Fos. However, little information is available about FRK (31). FRK is a protein kinase of the MAPK family that is stimulated via the p21ras pathway and phosphorylates c-Fos at Thr232 (31). The role of FRK in regulation of AP-1 activity in T cells is not known. Interestingly, when analyzing the induced c-Fos protein using higher resolution electrophoresis, we observed the presence of a slower migrating band in the activated CD4+ T cells (data not shown). Whether this represents a phosphorylated form of c-Fos remains to be determined.

In a recent study by Li et al., it was demonstrated that the block in ERK and JNK activation upon restimulation of anergic T cells in vitro could be completely overcome by stimulation with phorbol ester (21). They further showed that triggering of anergic cells resulted in dephosphorylation and translocation of the calcium/calcineurin-dependent transcription factor NF-AT (21). Taken together, these results suggested that the block in anergic T cells was not due to a global defect in TCR/CD3 signal transduction, but occurred specifically at the level of p21ras. In T cells, PMA activates protein kinase C, which, in turn, activates p21ras by inhibiting Ras GTPase-activating protein (39). A defect in Ras activation was directly demonstrated by Fields et al., who showed that conversion of GDP-p21ras to the activated GTP-bound form was prevented in the anergized cells (39). In this study we were unable to restore AP-1 transcription by PMA alone. A rise in intracellular calcium by ionomycin treatment was necessary to induce AP-1 activity in the anergized T cells. Thus, in addition to the defect in Ras activation observed in vitro (21, 40), the in vivo anergized CD4+ T cells seem to mobilize insufficient levels of calcium to trigger AP-1 transcription.

Rincón and Flavell (7) demonstrated the occurrence of DNA binding, but transcriptionally inactive AP-1 in primary T cells in response to PMA. Furthermore, they reported that blocking of the calcium-dependent pathway by cyclosporin A, which inhibits the protein phosphatase calcineurin (41), had minor effect on AP-1 DNA binding, but completely inhibited the transcriptional activity (7). In a recent study on B cells by Goodnow and co-workers, it was demonstrated that the amplitude and duration of calcium signals contribute to selective activation of NF-AT, NF-κB, and JNK (42). NF-κB and JNK required a large transient Ca2+ rise for activation, whereas NF-AT was activated by a low sustained Ca2+ plateau (42). Furthermore, stimulation of B cells tolerantized by self Ag was shown to trigger low calcium increases and activated NF-AT and ERK but not NF-κB and JNK (43). The need for activation of the calcium- and calcineurin-dependent pathway by ionomycin to restore AP-1 transcription may imply that suboptimal calcium mobilization contributes to the insufficient response in vivo anergized CD4+ T cells. Indeed, studies on T cell clones have implicated that low amplitude Ca2+ responses are involved in the induction of T cell anergy by altered peptide ligands (44).

In conclusion, we have demonstrated that in vivo anergized CD4+ T cells contain multiple defects in the AP-1 pathway, which strongly correlated to the inability of these cells to induce JNK kinase activity and to synthesize IL-2. The requirements for both PMA and ionomycin to restore AP-1 and IL-2 responses led us to propose that a block in p21ras activation and suboptimal increase in intracellular Ca2+ may be involved in the induction of anergy in vivo. Identification of the upstream targets responsible for regulation of calcium amplitude and uncoupling of the JNK pathway in anergic T cells will be a major focus for future studies.

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References
DEFECTIVE EXPRESSION OF AP-1 IN ANERGIC T CELLS


