Anergic T Cells are Defective in Both Jun NH$_2$-terminal Kinase and Mitogen-activated Protein Kinase Signaling Pathways

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Summary

T helper type 1 cells (Th1) become anergic when stimulated through the antigen receptor in the absence of costimulation. They do not produce IL-2 or proliferate in response to subsequent stimulation. Previous studies have indicated that anergic T cells are defective in the transactivational activity of the transcription factor, AP-1, which is required for optimal IL-2 transcription. Using two murine Th1 cell clones, we demonstrate that anergic Th1 cells have defects in both jun NH$_2$-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) activities. These kinases have been shown to be important for the upregulation of AP-1 activity. Furthermore, our data show that ERK and JNK activities are restored when anergy is induced in the presence of the protein synthesis inhibitor cycloheximide, or when anergic T cells are allowed to proliferate in response to exogenous IL-2. These treatments have previously been shown to prevent or reverse the anergic state. Our results suggest that defects in both JNK and ERK may result in the decreased AP-1 activity and the reduced IL-2 transcription observed in anergic T cells.

In 1987, Jenkins and Schwartz showed experimentally that IL-2 and interferon-γ producing T cells (Th1 cells) require two signals from antigen-presenting cells (APC)[1] in order to produce these cytokines (1, reviewed in reference 2). The first signal is provided by the TCR following occupancy by antigenic peptides bound to class II major histocompatibility complex (MHC) molecules. The second or "costimulatory" signal is known to be provided by B7-1 (3, 4, 5) and B7-2 (6, 7) molecules on the surface of APC. TCR occupancy in the absence of costimulation results in suboptimal activation of T cells, with no IL-2 production and no resultant proliferation (1). In addition, such cells are also unresponsive (anergic) to subsequent stimulation with normal APC plus antigen (1).

In response to antigen plus APC, anergized T cells have been shown to undergo early events characteristic of T cell activation such as inositol phosphate production (8), IL-2 receptor (IL-2R) upregulation and increases in levels of intracellular calcium (9). Surface expression of TCR, CD4 (9), and CD28 (D.R. DeSilva and M.K. Jenkins, unpub-

lished data) are also not measurably altered. The major defect in anergic T cells seems to be a lack of accumulation of IL-2 mRNA since anergic cells proliferate in response to exogenously added IL-2 (9). In addition, T cell clones stimulated with antigen plus APC become anergized when treated with neutralizing antibodies to IL-2 (10).

The series of events culminating in IL-2 production by activated Th1 cells is complex (11, 12, 13). The IL-2 promoter contains an enhancer region extending 300 nucleotides 5' of the initiation site of transcription (14, 15). The transcription factors necessary for positive regulation of IL-2 gene expression include the NF-AT/AP-1 complex, AP-1 itself, NFκB and octamer-binding proteins. All of these factors are either newly synthesized or posttranslationally modified subsequent to TCR occupancy. Alteration of any one of these binding sites results in a 60–90% decrease in the inducibility of the IL-2 gene. These factors alone, however, do not result in maximal production of IL-2. A second stimulus, mediated by CD28 in complex with its ligand B7, activates yet another transcription factor, the CD28 receptor complex (CD28RC). Binding of CD28RC together with the transcription factors produced upon TCR occupancy results in maximal IL-2 production in Th1 cells (16, 17, 18).

Recent data have indicated that AP-1 transactivation is defective in anergic T cells (19) that correlates with a lack

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[1] *Abbreviations used in this paper:* APC, antigen-presenting cells; CHX, cycloheximide; ERK, extracellular signal-regulated kinase; HA, hemagglutinin; HAU, HA units; JNK, c-jun NH$_2$-terminal kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MHC, major histocompatibility complex; Th1, IL-2 and interferon-γ-producing T cells.

of IL-2 transcription by these cells. AP-1 is a heterodimer composed of Fos and Jun family members. During T cell activation, Fos and Jun levels increase and the Jun proteins become phosphorylated, increasing their ability to transactivate (20). Two major kinase pathways have been implicated in the upregulation of AP-1 activity during the T cell response to antigen. The first is the mitogen-activated protein kinase (MAPK) pathway that is downstream of p21ras (21). Ras, when it is in the GTP-bound state, localizes the serine/threonine kinase Raf to the membrane where it is phosphorylated and activated. Raf can then phosphorylate and stimulate the dual-specific kinase MEK, which activates extracellular signal-regulated kinase (ERK) by direct phosphorylation on threonine and tyrosine residues. ERK phosphorylates and increases the activity of a number of proteins including elk-1, a transcription factor involved in the upregulation of the c-fos gene. The second pathway involved in AP-1 upregulation is the c-jun NH_2-terminal kinase (JNK) pathway. Although the upstream components of this pathway are not as clearly defined, it is thought that the serine/threonine kinase MEKK1 phosphorylates and stimulates the dual specificity kinase MKK4/SEK1 that phosphorylates the MAPK family member, JNK (20, 22). Activated JNK can then phosphorylate c-jun on two NH_2-terminal serine residues (Ser63 and Ser73) critical for its transactivational activity.

The lack of AP-1 transactivation observed in anergic cells could therefore be due to impaired signaling in the pathways leading to fos and jun upregulation and activation. In this report, we examined the levels of ERK2 and JNK activation in control vs. anergic T cells after stimulation through the TCR. We demonstrate that both ERK and JNK activities are reduced in anergic cells. These data suggest that defects in the MAPK and JNK pathways may be responsible for the decreased AP-1 activity and the reduced IL-2 transcription observed in anergic T cells.

Materials and Methods

Mice

B10.BR/SgSn mice were purchased from the Jackson Laboratory (Bar Harbor, ME). BALB/c mice were purchased from Charles River (Raleigh, NC). Single-cell suspensions of whole spleen obtained from these mice were used as APC for maintenance of Th1 clones and in proliferation assays.

T Cell Clones

A.E7. This is a B10.A-derived CD4⁺8⁻, CD28⁺ murine Th1 clone (23) that expresses a Vβ3/Vα11 TCR. A.E7 cells produce IL-2, IL-3, and IFN-γ upon stimulation with the COOH-terminal cyanogen bromide fragment (residues 81–104) of pigeon cytochrome *c* or a synthetic peptide based on the moth cytochrome *c* sequence (called DASP) (24) bound to I-E^k molecules on the surface of APC. The DASP peptide was synthesized using the Merrifield method at the University of Minnesota Microchemical facility.

Th17. This murine Th1 clone was derived from BALB/c mice immunized s.c. with the influenza virus A/PR/8/34 (PR8) as described (25). It is CD4⁺8⁻, CD28⁺ and produces IL-2 and

IFN-γ upon stimulation with the hemagglutinin (HA) molecule of influenza virus PR8. The viral antigen was in the form of allantoic fluid from virus-infected embryonated hen eggs. Virus titers were determined by agglutination of chicken erythrocytes and are expressed as hemagglutinating units (HAU) (25).

Both A.E7 and Th17 cells were maintained in 24-well plates on a "rest-stimulation" protocol (23) using mitomycin-c treated APC, antigen and recombinant human IL-2 (Boehringer Mannheim, Indianapolis, IN). Cells used in experiments were rested at least 5 d after addition of IL-2 and were separated from debris and dead APC by histopaque 1077 (Sigma Chem. Co., St. Louis, MO) density gradient centrifugation.

Anergy Induction

During the preculture, A.E7 or Th17 T cells (1–3 × 10⁵/well or 2–10 × 10⁶/plate) were incubated in 24-well plates or 10-cm Falcon 3003 petri plates coated with 10 μg/ml purified anti-CD3 mAb (clone 145-2C11; 26) as previously described (27). In some experiments, 0.1 μg/ml cycloheximide was added to cells to prevent induction of anergy (28). T cells were removed from the immobilized anti-CD3 after 1–2 days and transferred into fresh wells where they were allowed to rest in medium alone for 2 d.

To reverse the anergic state, anergic T cells from some experiments were removed from immobilized anti-CD3 after 1–2 d, washed and cultured for 7 d in the presence of 30 U/ml recombinant human IL-2. After culture in IL-2, cells were washed and rested for 2–5 d in medium alone before stimulation.

After the rest period, Th1 cells were treated with anti-CD3 plus anti-CD28 (2 μg/ml each) cross-linked with 2.0 μg/ml goat anti-hamster IgG (Cappel Research Products, Durham, NC) or PMA plus ionomycin (100 ng/ml and 1.0 μM, respectively) for activation before performing various assays for detecting differences between control resting T cells and anergic T cells.

Cellular Extracts

Total cellular extracts were made from A.E7 or Th17 T cells (3–15 × 10⁶ cells/treatment) which were unstimulated, or stimulated for 15 min at 37°C with either anti-CD3 plus anti-CD28 antibodies or PMA plus ionomycin. These extracts were used in Western blots, ERK KINASE assays, and JNK assays. After the 15 min incubation, T cells were pelleted and washed twice with cold PBS. The cells were then lysed in 100 μl of cold lysis buffer consisting of 10 mM Tris (pH 7.2), 150 mM NaCl, 1.0% Triton X-100, 1.0% sodium deoxycholate, and 0.1% SDS to which protease inhibitors and phosphatase inhibitors had been freshly added. After a 10-min incubation on ice, the extracts were spun for 15 min at 14,000 rpm in Eppendorf tubes at 4°C to pellet cellular debris. The supernatant was removed and protein concentrations determined using the Bio Rad protein assay (Bio Rad Labs., Hercules, CA).

Western Blots

Cellular extracts from A.E7 or Th17 T cells (10–20 μg protein/lane) were run on a 10% SDS–polyacrylamide gel following denaturation by boiling for 5 min in SDS loading buffer. Following electrophoresis, proteins were transferred onto Hybond-ECL nitrocellulose membranes (Amersham Life Science Inc., Arlington Heights, IL) and probed with the primary antibody (Raf-1; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by a peroxidase-conjugated secondary antibody, and detected using ECL Western blotting detection reagents (Amersham Life Sciences, Buckinghamshire, England). Blots were exposed to autoradiographic film (Du Pont & Co., Wilmington, DE) for 1–2 min for detection.

JNK Assay

Cellular extracts from A.E7 or Th17 T cells (25–50 μg protein diluted 1:10 in water for a final concentration of 0.1% Triton X-100) were incubated for 3 h at 4°C with 10 ng GST-c-jun (29) bound to agarose beads. During this incubation, JNK in the extracts binds c-jun. The beads were then washed three times in cold lysis buffer consisting of Tris-buffered saline containing 0.1% Triton X-100 and protease inhibitors. The beads were pelleted and resuspended in 25 μl of kinase buffer containing 20 mM Hepes (pH 7.0), 5 mM 2-ME, 10 mM $MgCl_2$, 0.1 mg/ml BSA, 10 μM unlabeled ATP and 10 μCi [^{33}P]γATP. The reaction mix was incubated at 37°C for 20 min, and washed three times in cold lysis buffer. The beads were resuspended in SDS loading buffer and boiled for 5 min to extract bound proteins. The beads were pelleted and the proteins run on a 10% SDS–polyacrylamide gel. The gel was dried and exposed to a PhosphorImager screen overnight after which quantitation was performed using the Image Quant. program (Molecular Dynamics, Sunnyvale, CA).

Kinase Assays for ERK Activity

Cellular extracts from A.E7 or Th17 T cells (100–300 μg protein) were brought up to 0.5 ml with PBS containing protease inhibitors and rocked for 1 h at 4°C with 1.0 μg of ERK2 mAb (Santa Cruz Biotechnology Inc.). A 50-μl slurry of protein A–agarose was then added to each tube and the tubes incubated for 1 h at 4°C with rocking. The tubes were centrifuged at 8,000 rpm for 5 min at 4°C and washed three times with PBS containing protease inhibitors. The pellets were then washed once with kinase buffer (see JNK assay), and resuspended in 25 μl of kinase buffer containing 10 μCi [^{33}P]γATP and 2.5 μg myelin basic protein (MBP; Upstate Biotechnology Inc., Lake Placid, NY). The tubes were then incubated for 20 min at 30°C. Samples were resuspended in SDS loading buffer and boiled for 5 min to extract bound proteins. The

tubes were centrifuged and the supernatent was run on a 14% SDS–polyacrylamide gel. The gel was dried and exposed to a PhosphorImager screen overnight after which quantitation was performed using the Image Quant. program (Molecular Dynamics).

Results

JNK Activity Is Abrogated in Anergic T Cells. Anergic Th1 cells have been reported to show decreased AP-1 binding compared to control T cells 3 h after stimulation, a time point preceding peak transcription of IL-2 (19). This could be due to defects in the signal transduction cascades leading to the upregulation and/or posttranslational modification of the AP-1 subunits, fos and jun. We therefore decided to examine anergic T cells for their ability to phosphorylate c-jun since it has been shown that transactivation by jun is dependent on its phosphorylation state (20). T cells were anergized by pretreatment with immobilized anti-CD3 in the absence of APC. The cells were rested for 2–3 d and then stimulated with anti-CD3 plus anti-CD28 or with PMA plus ionomycin. Cells anergized in this way failed to proliferate upon restimulation with antigen and failed to produce IL-2 although they proliferated in response to exogenous IL-2 (data not shown).

Cell lysates from anergic A.E7 T cells were compared with extracts from control T cells for their ability to phosphorylate the NH_2 terminus of recombinant human c-jun protein. As shown in Fig. 1, unstimulated T cells (both control and anergic) showed a basal, low level of JNK ac-
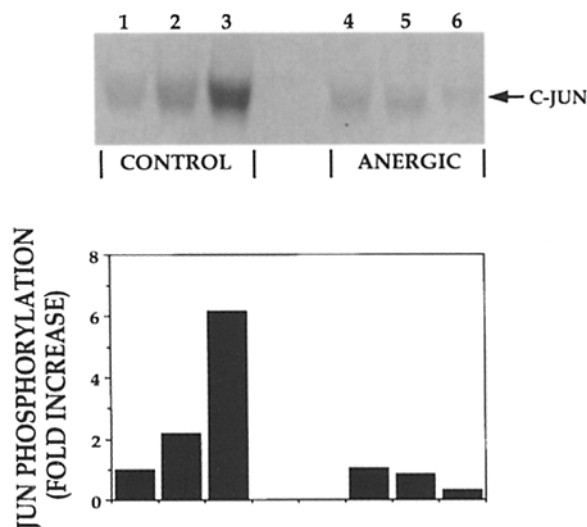

Figure 1. JNK activity is defective in anergic T cells. The ability of JNK from cellular extracts of control or anergic A.E7 T cells to phosphorylate a recombinant c-jun protein was tested. The band represents [^{33}P]γATP incorporated into phosphorylated c-jun. Lanes *1–3* represent control cells and lanes *4–6* represent anergic cells. Cells were either unstimulated (lanes *1* and *4*), stimulated with cross-linked anti-CD3 plus anti-CD28 (lanes *2* and *5*) or stimulated with PMA plus ionomycin (lanes *3* and *6*). Quantitation of the increase in kinase activity was performed using the Image Quant. program.
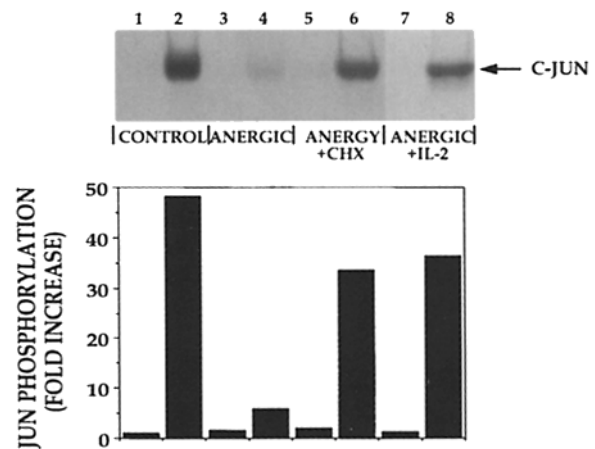

Figure 2. JNK activity is restored when T cells are made anergic in the presence of CHX or when anergic T cells are allowed to undergo proliferation. The ability of JNK from cellular extracts of control or anergic Th17 T cells to phosphorylate a recombinant c-jun protein was tested. The band represents [^{33}P]γATP incorporated into phosphorylated c-jun. Lanes *1* and *2* represent control cells; lanes *3* and *4* represent anergic cells; lanes *5* and *6* represent cells anergized in the presence of CHX and lanes 7 and *8* represent anergic cells allowed to undergo proliferation in the presence of exogenously added IL-2 (these cells were a subset of the anergic cells shown in lanes *3* and *4*). Odd numbers represent lanes in which cells were unstimulated, and even numbers represent lanes in which cells were stimulated with PMA plus ionomycin. Quantitation of the increase in kinase activity was performed using the Image Quant. program.

tivity. JNK activity in control T cells increased following anti-CD3 plus anti-CD28 stimulation (lane 2) and was dramatically increased during PMA plus ionomycin treatment (lane 3). In contrast, JNK activity did not increase in anergic T cells stimulated with either anti-CD3 plus anti-CD28 (compare lane 5 with lane 2) or PMA plus ionomycin (compare lane 6 with lane 3). Identical results have been obtained using another Th1 clone, Th17 (Fig. 2).
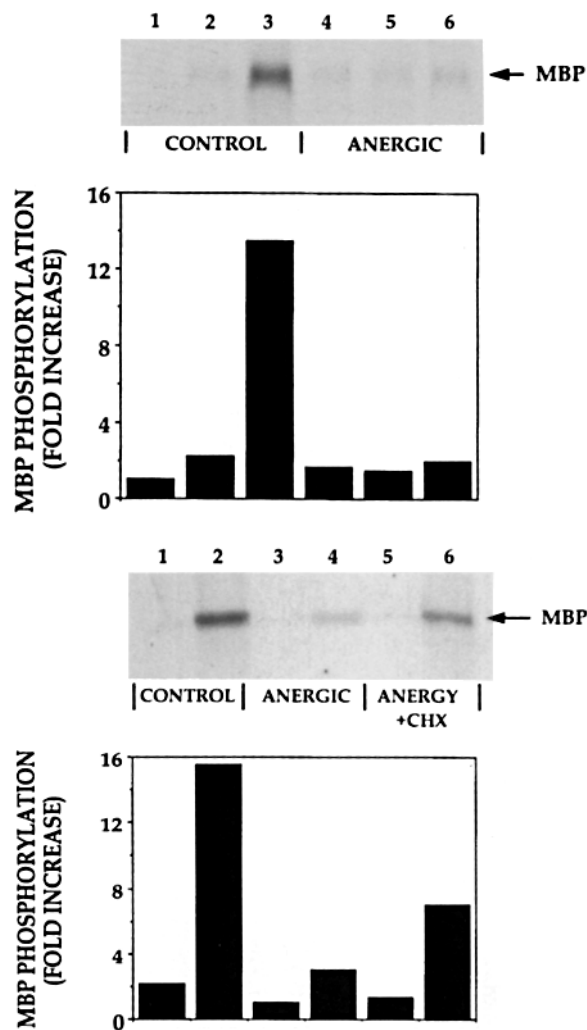

Figure 3. ERK KINASE activity is defective in anergic T cells and is restored when anergy is induced in the presence of CHX. (A) The ability of ERK from cellular extracts of control or anergic A.E7 T cells to phosphorylate MBP was tested. The band represents [^{33}P]γATP incorporated into phosphorylated MBP. Lanes 1-3 represent control cells and lanes 4-6 represent anergic cells. Cells were either unstimulated (lanes 1 and 4), stimulated with cross-linked anti-CD3 plus anti-CD28 (lanes 2 and 5) or stimulated with PMA plus ionomycin (lanes 3 and 6). Quantitation was performed using the Image Quant. program. (B) The ability of ERK from cellular extracts of control or anergic Th17 T cells to phosphorylate MBP was tested. The band represents [^{33}P]γATP incorporated into phosphorylated MBP. Lanes 1 and 2 represent control cells; lanes 3 and 4 represent anergic cells; and lanes 5 and 6 represent cells anergized in the presence of CHX. Odd numbers represent lanes in which cells were unstimulated, and even numbers represent lanes in which cells were stimulated with PMA plus ionomycin. Quantitation of the increase in kinase activity was performed using the Image Quant. program.

Activation of JNK was restored by including the protein synthesis inhibitor cycloheximide (CHX) during the induction of anergy (Fig. 2, compare lane 6 with lane 4), or when anergic Th1 cells were allowed to undergo proliferation in response to exogenous IL-2 (Fig. 2, compare lane 8 with lane 4). Therefore, our data indicate that JNK activity is restored by treatments that are known to prevent (CHX) or reverse (IL-2) the anergic phenotype, suggesting that defective JNK is responsible for maintaining the anergic phenotype. The lack of JNK activity observed in anergic cells was not due to a shift in the kinetics of the response. A time course experiment indicated that JNK activity in control resting T cells peaked at 20 min and was at background levels by 60 min post-stimulation. JNK activity remained at background levels at all time points tested in anergic T cells (data not shown).

ERK Activity Is Defective in Anergic T Cells. To study the other major signal transduction pathway downstream of TCR ligation and preceding AP-1 binding at the IL-2 enhancer, we examined ERK2 activity in anergic or control T cells. As shown in Fig. 3 *A*, ERK activity increased in control T cells stimulated with either anti-CD3 plus anti-CD28 or PMA plus ionomycin as measured by phosphorylation of MBP. In contrast, no increase in ERK activity was observed after stimulation of anergic cells (Fig. 3 *A*, compare lane 6 with lane 3). Similar to the results observed for JNK activity, the presence of cycloheximide during anergy induction prevented the loss of ERK activity observed in anergic T cells (Fig. 3 *B*, compare lane 6 to lane 4). Further, proliferation of anergic T cells in the presence of IL-2 restored the ability of ERK to become activated upon restimulation (data not shown).

Phosphorylation of Raf Is Not Defective in Anergic T Cells. To determine if kinase function was generally suppressed in anergic cells, we examined the phosphorylation state of Raf-1, an upstream kinase in the MAPK pathway. The unphosphorylated and phosphorylated forms of Raf can be distinguished because phosphorylation of Raf results in its decreased mobility on SDS-PAGE. As shown in Fig. 4, the phosphorylated form of Raf was seen in both control and anergic T cells after stimulation with PMA plus ionomycin. Thus, anergy does not prevent the increased phosphorylation of Raf associated with T cell activation. The kinase activity of Raf-1 in anergic cells, however, was lower than that seen in control T cells. Control cells increased their kinase activity two- to threefold during stimulation with PMA and ionomycin, and this increase is absent in anergic T cells (data not shown).

Discussion

The primary deficiency in anergic Th1 cells appears to be their inability to produce IL-2. Anergic T cells possess comparable levels of both TCR (9) and CD28 (D.R. DeSilva and M.K. Jenkins, unpublished data) on their surface compared to control resting cells. However, several factors downstream of TCR and CD28 occupancy have been implicated in causing an IL-2 production deficiency in anergic T cells. Studies have indicated decreased p56[lck] levels and increased p59[fyn] levels (30), altered tyrosine phosphorylation
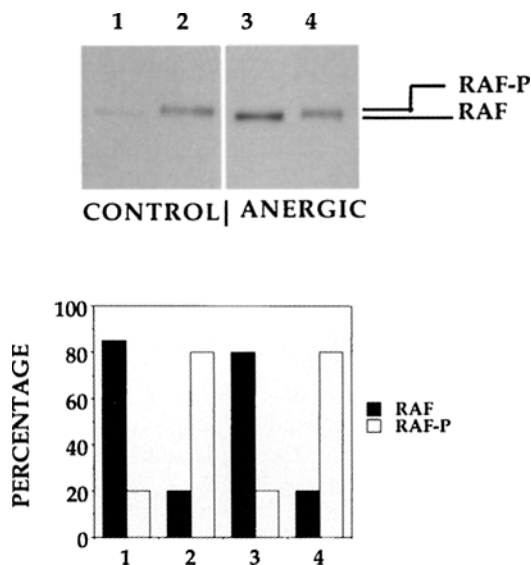
Figure 4. Raf phosphorylation is not defective in anergic T cells. Western blots of control or anergic T cells which were unstimulated or stimulated with PMA plus ionomycin were probed with an antibody to Raf-1, followed by a peroxidase-conjugated goat anti–rabbit antibody as the second step. A slower migrating band represents the phosphorylated form of Raf, and the faster migrating band is the unphosphorylated form of Raf. Lanes *1* and *2* represent control cells and lanes *3* and *4* represent anergic cells. Cells were either unstimulated (lanes *1* and *3*) or stimulated with PMA plus ionomycin (lanes *2* and *4*). Quantitation of the percentage of Raf-1 in the unphosphorylated and phosphorylated states was performed using the Image Quant. program.

(31), and decreased AP-1 transactivation (19) in anergic T cells. Our data have indicated that anergic Th1 cells also have reduced levels of JNK and ERK activity during restimulation. This finding suggests that both fos and jun subunits of the transcription factor AP-1 will be affected in anergic T cells. Jun activity may be reduced due to its lack of phosphorylation by JNK and Fos levels may be decreased due to the lack of ERK phosphorylation of Elk-1, a known regulator of Fos transcription. Together, these effects may result in the decreased levels of AP-1 activity in anergic cells reported by other researchers (19, D.R. DeSilva and M.K. Jenkins, unpublished data).

Ras and Raf-1 activities are known to be required for the stimulation of IL-2 promoter activity in T cells (32, 33). A recent report has also indicated that stimulating the ERK pathway by expressing active MEK-1 enhanced IL-2 promoter and NF-AT-driven transcription in a manner identical to expressing active Raf-1, whereas inhibiting ERK activity by expressing interfering mutants of MEK-1 or by expressing the ERK-specific phosphatase, MKP-1 decreased IL-2 promoter activity (34). These authors conclude that MEK-1 and ERKs function in conveying stimulatory signals from Raf-1 to the IL-2 gene. This data is compelling evidence that ERK is a required kinase in IL-2 gene transcription, and that a defect in its kinase ability could lead to the paralysis in IL-2 production seen in anergic T cells.

The defects in JNK and ERK activity were overcome by the presence of cycloheximide during anergy induction or by allowing anergic T cells to undergo several rounds of cell division in response to exogenous IL-2. Previous studies have shown that anergy could not be induced in the presence of cycloheximide (28), and that anergic Th1 cells reverse their anergic phenotype following proliferation (10, 35). Kang et al. (19) also observed that anergic Th1 cells that proliferated in response to exogenously added IL-2 no longer showed decreased AP-1 binding. In combination, these results suggest that components of AP-1 may be regulated by a factor that is newly synthesized in cells receiving TCR signals in the absence of cell division and that this factor is lost if proliferation occurs. Our results suggest that this factor acts to suppress JNK and ERK activation. This may occur by (*a*) preventing the synthesis or activation of JNK and ERK or (*b*) enhancing the degradation or inactivation of JNK and ERK in anergic T cells.

Although we have demonstrated that Raf is phosphorylated normally in anergic cells, the kinase activity of Raf-1 in anergic cells was lower than that seen in control T cells. Control cells increased their kinase activity two- to threefold during stimulation with PMA and ionomycin, and this increase is absent in anergic T cells (data not shown). Previous studies have demonstrated that the activity of Raf does not correlate with its level of phosphorylation (36). The finding that Raf activity is also decreased in anergic cells suggests that there is a defect in the activation of an upstream component common to both JNK and ERK pathways, such as Ras (29, 37). Recent data from Frank Fitch's laboratory has indicated that Ras activation is blocked in anergic Th1 clones (38). Downstream of Ras, these authors found that anergic Th1 cells were defective in TCR-mediated MAP-2 kinase activation. These results are consistent with our data. However, these authors found no alterations in the inductive tyrosine phosphorylation of the guanine nucleotide-releasing factor, Vav, or in that of Shc. Also, association of Grb-2 with Shc after TCR stimulation was seen to be comparable in control and anergic cells. They also found no significant differences in levels of rasGAP in anergic cells. These data suggest that the early signaling events are intact in anergic cells. In addition to a defect in Ras, there may also be simultaneous effects on upstream components unique to each pathway, such as Rac and/or Rho family members in the case of JNK (39, 40) or on the individual MEK family members. Finally, it is also possible that anergic T cells have higher levels of phosphatases which result in rapid inactivation of several kinases within these cells.

T cell clonal anergy may be an important mechanism for peripheral tolerance. In vivo models for clonal anergy exist, although the true incidence of clonal anergy in self tolerance is not yet known. There are many examples for anergic T cells being present in the periphery (41, 42, 43, 44). Blackman et al. (45) have shown that tolerance to the superantigen Mls-1ᵃ in mice transgenic for a Vβ8.1 TCR is maintained by both clonal deletion and anergy. It is plausible, therefore, that anergic T cells may possess defects in more than one key signaling event leading to IL-2 production, since loss of the anergic phenotype could result in autoimmunity and tissue destruction, serious consequences to the individual.

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