phase and reached a maximum between 3.5 and 4 hours. Because the peak did not increase between 4 and 5 hours after metaphase, and because the height of the ELFH peak after initiation in cultured CHOC 400 cells (G_1-S) was not greater than those of the peaks at 4 and 5 hours, we conclude that the maximum attainable specificity was achieved by 4 hours after metaphase. The relative preference of *Xenopus* egg cytosol for initiation within the DHFR origin locus in nuclei prepared at different times after metaphase is plotted, together with nuclear membrane assembly and the onset of S phase, in Fig. 3B. The first cells entered S phase 7 hours after metaphase and 50% of the cells were engaged in DNA synthesis by 9 to 10 hours after metaphase, consistent with flow cytometric analysis that revealed an average G_1 phase of 10 to 11 hours. These data are also consistent with an increase in the percentage of cells that pass through a discrete regulatory point in G_1 between 3 and 4 hours after metaphase; we have termed this point the origin decision point (ODP) for the DHFR locus.

The data in Fig. 2 demonstrate that the DHFR locus ODP occurs 2 to 3 hours after replication licensing, the mechanism that converts eukaryotic nuclei from a postreplicative (G_2) to a prereplicative (G_1) state (*8*, *11*). Intact mammalian nuclei that have not undergone the licensing step will not replicate in *Xenopus* egg extracts (*11*, *12*). Pre-ODP nuclei have fully assembled nuclear envelopes (Fig. 1) and initiate replication at the same number of sites as post-ODP nuclei (Fig. 2, A and B). The DHFR locus ODP occurs close to the previously identified restriction point (R point), the time after which entry into S phase is independent of growth factors or moderate concentrations of protein synthesis inhibitors (*13*). Thus, it will be of interest to determine whether a similar ODP serves to establish origins of replication at other mammalian chromosomal loci and whether there is a mechanistic link between the commitment to cellular proliferation (R point) and the commitment to initiate replication at specific chromosomal loci (ODP).

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15. Total DNA synthesis was determined by acid precipitation after incubation in extracts containing [α-^{32}P]deoxyadenosine 5′-triphosphate (25 μCi/ml). 6-DMAP was added to 3 mM. The percentage of labeled nuclei was determined by indirect immunofluorescence after incubation in extracts containing 250 μM bromodeoxyuridine triphosphate. Similar results were obtained in four separate experiments, although the length of the lag period (10 to 30 min) and the total amount of DNA replicated varied with the batch of extract.

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Blocked Signal Transduction to the ERK and JNK Protein Kinases in Anergic CD4$^+$ T Cells

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T cells activated by antigen receptor stimulation in the absence of accessory cell-derived costimulatory signals lose the capacity to synthesize the growth factor interleukin-2 (IL-2), a state called clonal anergy. An analysis of CD3- and CD28-induced signal transduction revealed reduced ERK and JNK enzyme activities in murine anergic T cells. The amounts of ERK and JNK proteins were unchanged, and the kinases could be fully activated in the presence of phorbol 12-myristate 13-acetate. Dephosphorylation of the calcineurin substrate NFATp (preexisting nuclear factor of activated T cells) also remained inducible. These results suggest that a specific block in the activation of ERK and JNK contributes to defective IL-2 production in clonal anergy.

Bretscher and Cohn (*1*) first suggested that antigen-receptor stimulation unaccompanied by critical costimulatory signals could cause the inactivation of mature lymphocytes. Jenkins and Schwartz (*2*) subsequently demonstrated such a functional unresponsiveness in murine CD4$^+$ T cell clones exposed to peptide antigen (Ag) presented by chemically modified accessory cells (ACs) lacking costimulatory molecules such as those of the B7 family of CD28 ligands (*3*, *4*). They determined that these T cells were induced into a state of clonal anergy, in which they become incapable of producing IL-2 and proliferating upon reexposure to Ag and the proper costimulatory ligands (*2*, *5*). The biochemical basis for this IL-2 production defect in anergic T cells remains controversial (*6*); however, a study by Kang *et al.* (*7*) has indicated that IL-2 synthesis is blocked at the level of gene transcription because of poor AP-1 complex assembly and function.

We initiated a study of AP-1 signal transduction using murine CD4$^+$ helper T cell clones (*8*–*10*) in an attempt to identify the biochemical basis for this defect. Overnight treatment of T cells with immobilized monoclonal antibody (mAb) to CD3, in order to simulate Ag-receptor [T cell receptor (TCR)–CD3] occupancy in the absence of costimulatory ligands and to induce clonal anergy (*11*), resulted in a substantial defect in the capacity of the T cells to produce IL-2 (Fig. 1A). Nevertheless, the anergic T cells were not globally deficient in their ability to transduce signals to the nucleus, because restimulation with CD3 plus CD28 mAbs could still induce the rapid disappearance of the 120-kD form of the preexisting nuclear factor of activated T cells (NFATp) (*12*) from a Triton X-100–soluble cytosolic compartment (Fig. 1B) and result in the translocation of NFATp into the nucleus (*13*). To test signal transduction to AP-1–binding DNA enhancer elements, T cells were stably transfected with plasmid DNA containing consensus AP-1 enhancer sequences driving the ex-

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pression of a minimal IL-2 promoter fused to a bacterial *lacZ* gene (AP-1ZH cells) (*14–16*). β-Galactosidase (β-Gal) activity was induced in normal cells in response to CD3 stimulation, and incubation with the combination of mAbs to CD3 and CD28 was the most potent stimulus (Fig. 1C). In contrast, anergic AP-1ZH cells expressed no reporter gene activity in response to either mAb to CD3 alone or the combination of CD3 plus CD28 mAbs (Fig. 1C). Thus, a specific block in AP-1–dependent transactivation was apparent in anergic T cells.

The proline-directed serine-threonine kinases ERK (extracellular signal–regulated protein kinases) and JNK (c-Jun NH_2-terminal kinases) are mitogen-activated protein kinases (MAPKs) thought to play a key role in the transmission of signals from the outside of the T cell to AP-1–binding DNA sequences inside the nucleus. ERK activation probably relies on the coupling of the TCR-CD3 complex to p21ras (*17*), with subsequent activation of the RAF→MEK kinase cascade immediately upstream of ERK (*18*). JNK activation also depends on p21ras, as well as on signals mediated by calcineurin and signals coming from the CD28 costimulatory receptor (*19, 20*), and it results from the activation of small monomeric guanosine triphosphate (GTP)–binding proteins (such as Rac1 or Cdc42) that can induce the kinase cascade PAK→MEKK→SEK→JNK (*21*). Activated ERK and JNK phosphorylate the nuclear protein Elk-1, and this regulates the synthesis of c-Fos (*22*). JNK also phosphorylates c-Jun within its NH_2-terminus, resulting in enhanced transactivation by AP-1 protein complexes that contain c-Jun (*19, 20*). Therefore, a defect in the function of either ERK or JNK could be responsible for the poor AP-1–dependent transactivation observed in anergic T cells.

When we used an immune complex kinase assay, stimulation of normal T cells with the protein kinase C (PKC)–activating phorbol ester phorbol 12-myristate 13-acetate (PMA) resulted in maximal ERK activity (Fig. 2A). CD3 stimulation, either with or without the addition of mAb to CD28, also induced ERK activity that peaked after 5 min of stimulation and decayed by 60 min (Fig. 2, A and B). On the other hand, mAb to CD28 alone only modestly activated ERK, and in most experiments CD28 costimulation had only a small positive effect on CD3-induced ERK activity. Finally, ionomycin did not stimulate ERK (Fig. 2A), and ERK activation was insensitive to the addition of the calcineurin-specific inhibitor cyclosporin A (CSA) [Fig. 2A and (*23*)]. Thus, ERK activation in normal $CD4^+$ T cells is most dependent on those molecules that can influence p21ras activity (for example, TCR-CD3 and PKC) (*24*).

Anergic T cells had reduced ERK activity after CD3 stimulation, as compared with

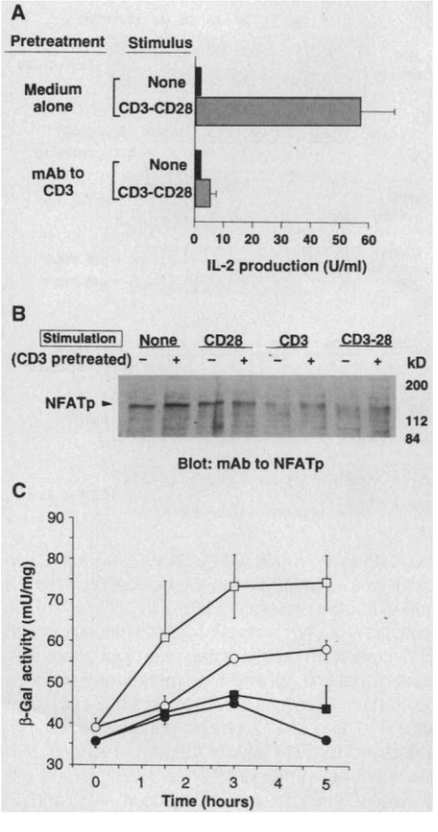

Fig. 1. Clonal anergy blocks transactivation by AP-1 elements and leads to defective IL-2 secretion, but signal transduction to NFATp remains inducible. In each experiment shown, T cells were pretreated overnight either with medium alone or with immobilized mAb 145-2C11 to CD3 to induce clonal anergy as previously described (*11*). The cells were subsequently rested for 5 days before restimulation with mAb to CD3 and the CD28 mAb 37.51 (*4*) either alone or in combination. (**A**) A.E7 T cells restimulated for 40 hours either with or without immobilized mAbs (10^5 cells per sample, in duplicate) with IL-2 production determined by bioassay (*10*). Data are ± SEM. Similar results were obtained with the 16B.2 cell line (*10*). (**B**) A.E7 T cells examined for the presence of cytosolic NFATp protein by protein immunoblot analysis after mAb stimulation (*13, 34*). T cells were incubated for 30 min on ice with primary antibodies as indicated. Free antibody was removed, and then all cells were incubated for 5 min at 37°C with goat anti-hamster IgG to cross-link the primary antibodies. The 120-kD phosphorylated NFATp species is marked by the arrowhead. This experiment is representative of three. (**C**) AP-1ZH transfected T cells (normal control cells, open symbols; cells pretreated with mAb to CD3, closed symbols) (*14–16*) were incubated either with immobilized mAb to CD3 alone (circles) or with the combination of mAb to CD3 and mAb to CD28 (squares) for various periods of time before the assay of reporter gene β-Gal activity (*36*). Two independent experiments were performed, and data for each time point are expressed as mean β-Gal activity ± SEM.

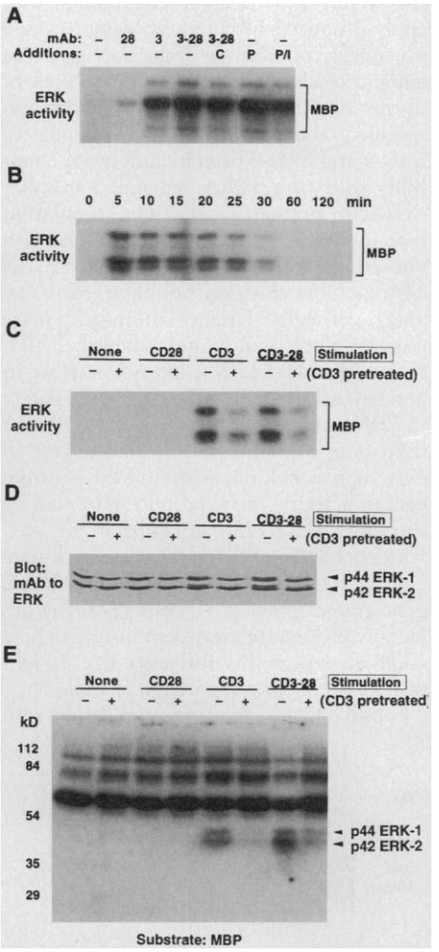

Fig. 2. ERK activation is defective in anergic T cells. (**A**) A.E7 T cells were incubated for 15 min at 37°C with no addition (−), with immobilized mAb to CD28 alone (28), with immobilized mAb to CD3 (3), with the combination (3-28), with PMA (50 ng/ml) (P), or with the combination of PMA plus 1.5 μM ionomycin (P-I). A single group of cells was pretreated with CSA (500 ng/ml) (C) for 15 min before the initiation of CD3 and CD28 signaling, and remained in CSA for the entire experiment. At the end of the 15-min incubation period, cells were assayed for ERK activity (*33*). An autoradiogram of the phosphorylated kinase substrate MBP is shown for each sample. (**B**) Time course of ERK activation in A.E7 T cells stimulated with the combination of immobilized mAb to CD3 and mAb to CD28. (**C** through **E**) Normal and anergic T cells (overnight pretreatment with immobilized mAb to CD3) were rested for 6 days and then stimulated as indicated for 15 min. Cell pellets were then (C) assayed for ERK activity in an immune complex kinase assay (*33*), (D) tested for ERK-1 and ERK-2 protein abundance by protein immunoblot (*37*), and (E) examined for ERK activity in an in-gel kinase assay with gel-immobilized MBP as substrate (*33*). Similar results were obtained with the 16B.2 T cell clone (*23*). Each experiment shown is representative of at least two, and the experiment shown in (C) was repeated six times with identical results.

that of normal control cells (Fig. 2C). Furthermore, a kinetic experiment revealed that a shift in the peak response within the anergic population could not account for this result (*23*). A protein immunoblot analysis of total ERK protein demonstrated no effect of anergy induction on the amount of ERK-1 or ERK-2 protein present in the cells (Fig. 2D). This experiment also revealed that a substantial amount of ERK-1 and ERK-2 protein underwent a mobility shift to a higher apparent molecular weight in normal T cells after stimulation with mAb to CD3, which is consistent with phosphorylation by an upstream kinase. This mobility shift was not observed in the anergic T cells. Finally, an in-gel kinase assay of whole-cell lysates established that both ERK-1 and ERK-2 did not activate in anergic cells (Fig. 2E). Therefore, the defect in ERK function most likely resulted either from poor phosphorylation and activation by an upstream kinase such as MEK, or from excess activity of a protein phosphatase such as PAC-1 (*25*). Given that the activation of RAF and its downstream kinase cascade is essential for the induction of IL-2 gene transcription in T cells (*17*), the defect in ERK activation demonstrated here could be expected to influence the amount of IL-2 production by anergic T cells.

Unlike ERK activation, stimulation with PMA alone resulted in only modest JNK activity in normal T cells, and ionomycin alone had no effect (Fig. 3, A and B). The combination of ionomycin and PMA did activate JNK, and this response was blocked by CSA (Fig. 3B). This calcium-ion responsiveness and sensitivity to CSA was consistent with an ability of calcineurin to influence JNK activity. Monoclonal antibody to CD3 induced only weak JNK activity, and mAb to CD28 alone had no effect; however, mAb to CD3 plus mAb to CD28 induced strong kinase activity that was also sensitive to inhibition by CSA (Fig. 3A). PMA plus mAb to CD28 proved to be a strong stimulus for JNK activation, and this response was entirely insensitive to the effects of CSA (Fig. 3A). JNK activation in these cells was less rapid than ERK activation, peaking at 15 min and falling to near background by 60 min (Fig. 3C). Therefore, extracellular signals that mimic Ag presentation by AC, namely mAb to CD3 plus mAb to CD28, costimulated JNK activity in normal $CD4^+$ helper T cells in a synergistic fashion, much as CD3 and CD28 signals costimulated to induce AP-1–dependent transactivation and the production of IL-2.

Like ERK activation, the JNK activity within anergic T cells was found to be only weakly induced upon incubation with the combination of mAb to CD3 and mAb to CD28 (Fig 3D, upper panel). The suboptimal activation of JNK seen with CD3 stimulation alone also was reduced in anergic cells. A time-course experiment was performed, and no shift in the kinetics of JNK activation could account for this poor response in anergic T cells (*23*). Finally, this impaired JNK activity appeared not to be the result of a defect in JNK expression, as the amounts of JNK-1 and JNK-2 proteins in anergic cells were equivalent to those of normal T cells (Fig. 3D, lower panel).

The findings of defective ERK and JNK function after the development of clonal anergy suggested two possible mechanisms for the impaired IL-2 inducibility of these cells: Anergy may specifically inhibit the activation of all MAPKs by their respective MAPK kinases, or alternatively, anergy may interfere with a common upstream signal transduction pathway. The defect in ERK activation could not easily be explained by a CD28 signal transduction block, as CD28 costimulation was unnecessary for ERK function. Furthermore, the defect observed in both JNK activation and AP-1–dependent transactivation after stimulation with mAb to CD3 was profound, regardless of whether CD28 was costimulated. Thus, CD28 proximal signal transduction was not necessarily defective in anergic T cells. To further examine upstream signaling pathways, T cells were induced into clonal anergy and then stimulated in the presence of PMA, in an attempt to bypass a defective proximal signal transduction pathway. ERK activity was found to be normal even in anergic cells when they were stimulated in the presence of PMA (Fig. 3E, upper panel). Likewise, JNK activation was essentially normal in anergic cells after stimulation with either PMA alone or with a combination of PMA plus ionomycin or PMA plus mAb to CD28 (Fig. 3E, lower panel). Therefore, strong stimulation of a PKC-dependent pathway in anergic T cells appeared to bypass an upstream defect that otherwise led to poor MAPK activation.

PKC-activating phorbol esters activate p21ras in normal T cells by inhibiting the GTPase activating protein rasGAP (*24*). The activation of ERK and JNK in response to PMA in anergic cells suggests then that p21ras protein is present, and signaling cascades downstream of p21ras are intact. In contrast, a direct inhibition of MAPK activities seems unlikely, because even suboptimal JNK activation in response to PMA alone was unaffected in anergic T cells. Finally, no specific abnormality in signal transduction initiated by the CD28 molecule was detected. Thus, the defect in signal transduction to the MAPK may prove to be at the level of specific coupling between the TCR-CD3 complex and p21ras. Nonetheless, this coupling defect must be specific,

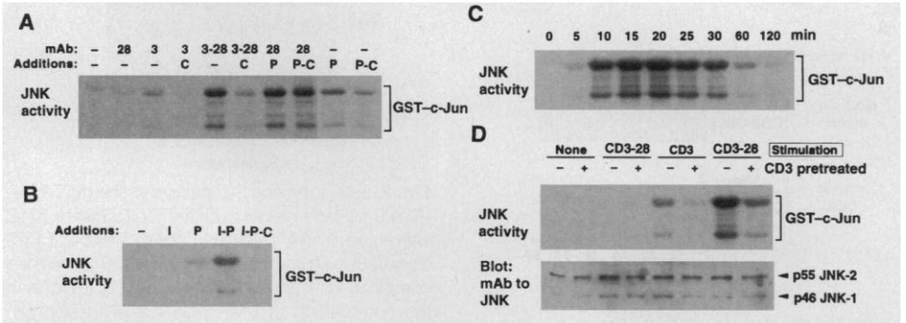

Fig. 3. JNK activation is defective in anergic T cells. (**A** and **B**) JNK activity assay after stimulation of A.E7 T cells without exposure to antibody (–), with immobilized mAb to CD28 (28), with immobilized mAb to CD3 (3), with the combination of the mAb to CD3 and mAb to CD28 (3-28). In some samples, T cells were first preincubated for 15 min with CSA (500 ng/ml) (**C**). In other samples, PMA (50 ng/ml) (P) or 1.5 μM ionomycin (I) was included during the incubation. After 15 min of stimulation at 37°C, cells were lysed and whole-cell extracts were assayed for JNK activity with the GST–c-Jun fusion protein as substrate (*38*). (**C**) Time course of JNK activation after stimulation with immobilized mAb to CD3 and mAb to CD28. (**D** and **E**) T cells were preincubated overnight either with medium alone or with immobilized mAb to CD3 to induce clonal anergy, and then rested for 6 days. At that point, the normal or pretreated T cells were restimulated for 15 min as indicated. In (D), T cells were analyzed for JNK activity (upper panel) (*38*), as well as JNK-1 and JNK-2 protein abundance (bottom panel) by protein immunoblot (*37*). In (E), T cells were assayed for ERK activity (upper panel) and JNK activity (lower panel) after a 15-min restimulation. The substrate proteins MBP and GST–c-Jun are indicated. Similar results were also seen with the 16B.2 clone (*23*). Each experiment shown in (D) and (E) is representative of at least four others.

given the ability of CD3 mAb to activate calcineurin and induce the dephosphorylation of NFATp.

Although the relation between the MAPK and AP-1 has yet to be fully established in T cells, these data predict that defective MAPK activation limits both the expression and function of Fos and Jun proteins at the *IL-2* enhancer. Poor inducibility of c-Fos, FosB, and JunB protein has, in fact, been observed in T cells after the induction of clonal anergy (*13*). As is also consistent with this hypothesis, anergic T cells demonstrated significantly less difficulty in proliferating when PMA was present to bypass the defect in MAPK activation. Whereas proliferation in response to Ag was inhibited to only 2.4 ± 0.9% of control amounts after the induction of clonal anergy, proliferation by anergic T cells in the presence of PMA plus ionomycin reached an average of 42.4 ± 4.2% of control amounts ($n = 3$, $P = 0.007$) (*26*). Finally, a Northern (RNA) blot analysis has indicated that this greatly improved proliferation of anergic T cells stimulated with PMA plus ionomycin is the result of a substantial accumulation of *IL-2* mRNA (*27*). Therefore, these experiments now provide support for the hypothesis that *IL-2* gene expression and proliferation are defective in anergic T cells because of a selective block in signal transduction to the MAPK that leads to impaired AP-1–dependent transactivation.

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14. Plasmid pNFATZH contains a multimer of the human NFAT enhancer element flanked by Xho I restriction sites that has been inserted into a minimal IL-2 promoter (−319 to +47 base pairs of *IL-2* gene DNA containing an internal deletion and replacement of base pairs −296 to −72 by Xho I linker DNA) and fused to a bacterial *lacZ* gene (*15*). This plasmid also contains a hygromycin resistance gene under the control of the herpes simplex virus *tk* promoter. Plasmid pSXNeo/AP-1 contains five tandem repeats of the sheep metallothionein *MT-II* gene AP-1 site (TGACTCA) ligated within an identical minimal IL-2 promoter at the internal Xho I site. To construct the pAP-1ZH plasmid, an Xmn I–Hind III fragment of pSXNeo/AP-1 containing the AP-1 multimer within the minimal IL-2 promoter was gel purified. The pNFATZH plasmid was similarly digested with Xmn I and Hind III to remove enhancer sequences, and then purified vector DNA was ligated to the AP-1 multimer-containing fragment. The pAP-1ZH construct thus has a bacterial *lacZ* gene fused to a 5× AP-1 multimer-containing minimal IL-2 promoter, as well as a hygromycin resistance gene. The pZH enhancerless plasmid was prepared by simple digestion of pNFATZH with Xho I and ligation of the empty vector. The construction of both plasmids was confirmed by restriction analysis.
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33. An immune complex kinase assay was performed to detect the activity of ERK molecules in the cell lysates of stimulated T cells. Cell pellets (2×10^6) were extracted on ice with whole-cell extract buffer according to the method of Hibi *et al.* (*19*). Using protein A–Sepharose beads precoated with the polyclonal rabbit antibody A249 to ERK-2, we immunoprecipitated ERK protein from the lysates for 1 hour at 4°C with gentle rocking. Immune complexes were washed and then assayed for enzyme activity as described (*19*), with the use of myelin basic protein (MBP) (Upstate Biotechnology) as a substrate. Incubations were carried out for 15 min at 37°C, then reactions were stopped by addition of 20 μl of 3× SDS sample buffer. Control experiments determined that incorporation of ^{32}P during the first 15 min of the kinase assay was dependent both on time and the amount of active kinase present. Phosphorylation of the MBP substrate was analyzed by SDS-PAGE (polyacrylamide gel electrophoresis) in a 16% gel, followed by autoradiography of the dried gel. Phosphorylated MBP often ran in the SDS gel as several bands, presumably as a result of protein degradation in the MBP preparation. In addition, we performed an in-gel kinase assay on whole-cell extract proteins to determine the individual activities of ERK-1 and ERK-2, using MBP as an immobilized substrate in a 10% polyacrylamide gel, according to the method of Chao *et al.* (*29*).
34. To examine cytosolic NFATp expression after stimulation, 2×10^6 normal or anergic A.E7 or 16B.2 T cells were pelleted and washed once with ice-cold phosphate-buffered saline containing protease and phosphatase inhibitors, and the cell pellet was then extracted with Triton X-100, as previously described (*30*). Lysates were recovered from the insoluble nuclear fraction and analyzed by protein immunoblot (8.5% SDS-PAGE with transfer to nitrocellulose) with a 1:1000 dilution of R59 rabbit antiserum to NFATp, goat antiserum to rabbit immunoglobulin G (IgG)–horseradish peroxidase (Bio-Rad), and ECL (Amersham) chemiluminescence reagents.
35. S. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988).
36. β-Gal activity was determined in vitro with the use of the substrate o-nitrophenyl β-D-galactopyranoside (Sigma) according to standard procedures (*31*). T cells (5×10^6) were harvested from six-well plates after stimulation, washed once, and then suspended (1.7×10^8 cells per milliliter) in 250 mM tris-HCl (pH 7.5). Freeze-thaw lysis of cells was followed by centrifugation and collection of the lysate, and protein concentration was determined by the BCA protein assay (Pierce). Duplicate enzyme reactions were carried out, followed by measurement of optical absorbance at 405 nm with a microplate reader (BIO-TEK Instruments). Units of activity were calculated based on a standard curve with purified enzyme (Sigma), and were corrected for the protein concentration of the extract. Values obtained in this assay for unstimulated AP-1ZH T cells are essentially identical to those obtained with either resting or activated ZH cells or untransfected T cells (*32*) and result from background optical absorbance in the assay.
37. A protein immunoblot analysis of cellular proteins was also used to examine ERK and JNK protein abundance. Approximately 20 μg of whole-cell extract (*33*) was mixed 1:1 with 2× SDS sample buffer, and then separated by 10% SDS-PAGE. Proteins were transferred to nitrocellulose, and blotted as described (*34*) with either polyclonal antibody 691 to ERK-1 and ERK-2 at a 1:1000 dilution or with polyclonal antibody to JNK-1 (1 μg/ml) (Santa Cruz Biotechnology).
38. JNK activity was measured with a solid-phase kinase assay essentially as previously described by Hibi *et al.* (*19*). Whole-cell extracts (2×10^6 cell equivalents) were prepared and then incubated with a glutathione S-transferase (GST)–c-Jun fusion protein bound to GSH agarose beads. This fusion protein was constructed from a polymerase chain reaction (PCR)–amplified product encoding amino acids 1 to 232 of murine c-*jun* complementary DNA. The PCR prod-

uct was inserted into the *Escherichia coli* expression vector pGEX-2T and then expressed in *E. coli* strain HB101 as described by Smith and Johnson (*35*). Whole-cell extracts were incubated with the fusion protein at 4°C to immobilize JNK proteins, then washed beads were analyzed for kinase activity by incubation for 15 min at 37°C in 30 μl of kinase reaction buffer (*33*). Again, control experiments determined that the incorporation of ^{32}P into the fusion protein during the first 15 min was linear with respect to both time and kinase concentration. The immobilized proteins were washed three times with kinase wash buffer, eluted in 1× SDS sample buffer, and then analyzed on a 10% SDS–polyacrylamide gel, followed by autoradiography to detect the presence of a phosphorylated GST–c-Jun substrate. The phosphorylated fusion protein electrophoresed through an SDS gel as several bands because of protein degradation.

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Blocked Ras Activation in Anergic CD4$^+$ T Cells

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T cell anergy is a state of functional unresponsiveness characterized by the inability to produce interleukin-2 (IL-2) upon T cell receptor stimulation. The mitogen-activated protein kinases ERK-1 and ERK-2 and the guanosine triphosphate–binding protein p21^{Ras} were found to remain unactivated upon stimulation of anergic murine T helper cell 1 clones. The inability to activate the Ras pathway did not result from a defect in association among Shc, Grb-2, and murine Son of Sevenless, nor from a defect in their tyrosine phosphorylation. This block in Ras activation may lead to defective transactivation at activator protein 1 sites in anergic cells and may enable T cells to shut down IL-2 production selectively during anergy.

Stimulation of T lymphocytes through the T cell receptor (TCR) in the absence of a second costimulatory signal results in a long-term, functionally nonresponsive state termed anergy (*1*). T cell anergy is characterized by the inability to produce the autocrine growth factor IL-2. Tyrosine phosphorylation patterns are altered after TCR stimulation of anergic T cells, implicating an early signal transduction defect (*2*, *3*). Transactivation at the activating protein 1 (AP-1) site or sites in the 5′ IL-2 gene enhancer is also defective in anergic cells (*4*). Activation of p21^{Ras} normally couples TCR stimulation to transactivation by AP-1 and other transcription factors that regulate IL-2 gene transcription. Because activation of Ras requires early tyrosine phosphorylation events, a targeted block of Ras activation in anergy might reconcile the previous observations of alterations in TCR-proximal tyrosine phosphorylation and the downstream block in transcriptional activity in anergic cells. To test the hypothesis that Ras function is altered in anergy, we examined Ras activation in the murine T helper cell 1 (T_H1) clone pGL10 during TCR stimulation (*5*).

Anergy was induced in this clone by stimulation with antibody to CD3 [monoclonal antibody (mAb) 145-2C11], which was immobilized on tissue culture dishes (Fig. 1, A and B) (*3*). When restimulated with antigen (ovalbumin) plus antigen-presenting cells (APCs), the anergized clone did not proliferate (Fig. 1A) or produce IL-2 (Fig. 1B) (*6*). The cells were specifically defective in their ability to produce IL-2 and proliferate, as they secreted amounts of interferon γ that were comparable to amounts secreted by control cells upon restimulation (Fig. 1B). The unresponsiveness was not attributable to alterations in expression of cell surface proteins associated with T cell activation (TCRαβ, CD3, CD45, and CD4), as these proteins were comparably expressed on control and anergic cells (*7*). These data are consistent with data in other published reports (*1*, *3*, *8*). Anergized pGL10 cells were capable of proliferating in response to exogenous IL-2 (Fig. 1A), as reported by other laboratories (*1*).

Anergic T_H1 cells have altered TCR-induced protein tyrosine phosphorylation, including a substantial reduction in the phosphorylation of two proteins of apparent mass of 42 and 44 kD (*3*). We suspected that these proteins might be the mitogen-activated protein (MAP) kinases ERK-1 and ERK-2, so we did immunoblot analysis of ERK immunoprecipitates (IPs) (*9*). Control and anergic pGL10 cells were stimulated by antibody-mediated, soluble cross-linking of CD3 together with the coreceptor, CD4 (TCR + CD4) (*10*). Such stimulation of control, but not anergic, pGL10 cells induced tyrosine phosphorylation of both ERK-1 and ERK-2 (Fig. 1C). In addition, such stimulation induced a shift in the electrophoretic mobilities of these proteins only in control cells (Fig. 1C). Tyrosine phosphorylation and mobility shift of ERK (attributable to threonine phosphorylation) have been associated with activation of this enzyme (*11*). Indeed, another group has demonstrated that induction of ERK activity is impaired in anergic T cells, which corroborates these findings (*12*). To exclude the possibility that differences in kinetics of the response account for our observations, longer times of activation were examined. Even after stimulation for 45 min, these events were not observed in anergic cells (*7*). Growth for 1 to 2 weeks in IL-2 has been shown to enable anergized cells to regain the ability to proliferate and produce IL-2 in response to TCR stimulation (*13*). This treatment of anergized pGL10 enables the cells to regain the ability to activate ERK as well (*7*). The block in ERK activation seen in pGL10 was also detected when another T_H1 clone, pGL2, was similarly tested (*7*).

Because of the apparent dependence of MAP kinase activation upon Ras activation (*14*), the inability to activate MAP kinase in anergic clones suggested a defect in Ras activation. The capacity of anergic clones to activate Ras by TCR + CD4 stimulation was therefore tested (*15*). Stimulation of control pGL10 cells resulted in accumulation of Ras in the guanosine triphosphate (GTP)–bound, or active, form (Fig. 2, A and B). This increase in GTP binding was maximal at 5 min and returned to resting levels by 30 min (Fig. 2B). In contrast, TCR + CD4 stimulation of anergized clones resulted in no accumulation of Ras in the GTP-bound form (Fig. 2, A and B). Stimulation with IL-2, although capable of activating Ras in control cells (Fig. 2A), did not activate Ras in anergic cells (Fig. 2A), despite the ability of these cells to proliferate normally to this growth factor (Fig. 1A). Thus, although IL-2 can induce proliferation of anergic cells, it may act through a Ras-independent mechanism. This suggests that a common component used by both the T cell antigen receptor and IL-2 receptor systems for Ras activation may be a target of anergy.

At least two possible explanations can account for our observations concerning Ras. (i) Ras itself is affected in anergy, in such a way that its ability either to bind GTP or to be regulated by guanine nucleo-

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