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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tide exchange factors or guanosine triphosphatase (GTPase) activating factors is impaired. (ii) Upstream or immediate downstream regulators of Ras are affected in such a way that nucleotide exchange or regulation of GTPase activity is impaired. In order to distinguish between these possibilities, we stimulated cells with phorbol myristate acetate (PMA), which has been shown to activate Ras in T cells (*16*), plus the calcium ionophore ionomycin. PMA + ionomycin induced Ras activation in both control and anergic cells (Fig. 2A). In addition, stimulation of anergic cells with the combination of PMA and ionomycin causes the production of IL-2 and proliferation (*7*, *17*). Thus, Ras protein is capable of GTP binding and nucleotide exchange, and the defect causing the inability to activate Ras observed in anergic cells may reside in factors that regulate nucleotide exchange or intrinsic GTPase activity. We cannot exclude the possibility, however, that stimulation of these cells with PMA + ionomycin is able to overcome the effects of an alteration of Ras itself.

It appears that regulation of Ras activation is defective in anergic T cells. One potential mechanism implicated in TCR-mediated Ras activation is the stimulation-induced recruitment of the Ras–guanine nucleotide releasing factor (GRF) murine Son of Sevenless (mSOS) to the cell membrane, which involves the formation of a complex of mSOS with adaptor proteins Grb-2 and Shc; Shc is tyrosine-phosphorylated and associated with the zeta chain of CD3 upon TCR stimulation (*18*). To assess Shc function in anergic T cells, anti-phosphotyrosine and anti–Grb-2 immunoblotting of immunoprecipitates was performed. TCR + CD4 stimulation of both control and anergized pGL10 cells induced a rapid induction of tyrosine phosphorylation of Shc protein as well as its association with Grb-2 (Fig. 2C). The Grb-2–mSOS association in anergic cells was assessed by immunoblotting of anti-mSOS immunoprecipitates. In both control and anergic T cells, Grb-2 was detected in anti-mSOS immunoprecipitates, which indicates that these proteins were coprecipitated, and their association was unaltered in anergic cells (Fig. 2D) (*19*). The mSOS immunoblot of control cell immunoprecipitates revealed a shift in the electrophoretic mobility of mSOS after TCR stimulation, attributed to serine-threonine phosphorylation of mSOS by MAP kinase (*20*). In stimulated anergic clones, we detected no mobility shift in mSOS, which is consistent with the inability to activate MAP kinase in these cells. Ras activation in T cells can also be regulated by Vav (*21*) or GTPase activating protein (GAP), but Vav was normally phos-

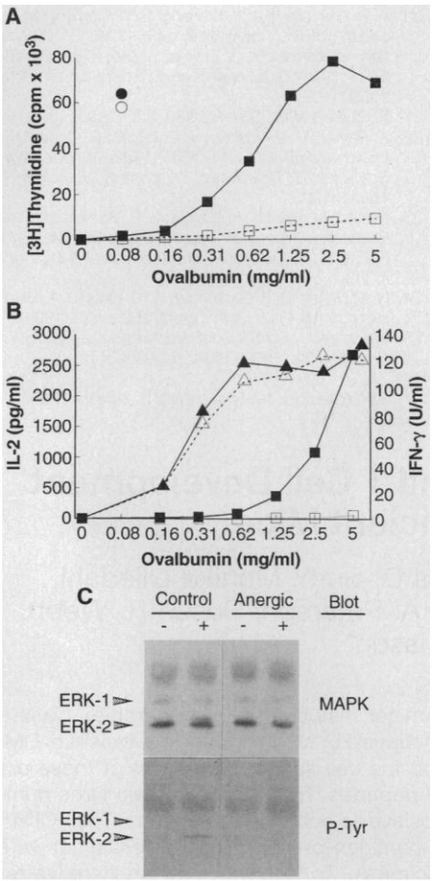

Fig. 1. (**A**) Proliferation of control (solid squares) and anergic (open squares) pGL10 cells in response to antigen (ovalbumin) plus APCs. Also shown is proliferation of control (solid circle) and anergic (open circle) pGL10 cells in response to recombinant human IL-2 (20 U/ml) (Cetus). Incorporation of [^{3}H]thymidine was measured at 48 hours (*24*). (**B**) IL-2 (squares) and interferon γ (IFN-γ) (triangles) production by control (solid symbols) and anergic (open symbols) pGL10 cells in response to antigen (ovalbumin) plus APCs. (**C**) Defective activation of ERK-1 and ERK-2 in control and anergic pGL10 cells. Unstimulated (4×10^7) (−) or TCR + CD4 stimulated (+) pGL10 cells were immunoprecipitated with mAb to MAP kinase (MAPK) (Zymed, San Francisco, California). The immunoprecipitates were split and resolved under reducing conditions by SDS–polyacrylamide gel electrophoresis (PAGE) on a 9% gel. Approximately 3.5×10^7 cell equivalents were probed with the mAb to phosphotyrosine, 4G10 (P-Tyr; bottom) (Upstate Biotechnology, Lake Placid, New York); and 5×10^6 cell equivalents were probed with mAb to MAP kinase mAb (MAPK; top) (Zymed).

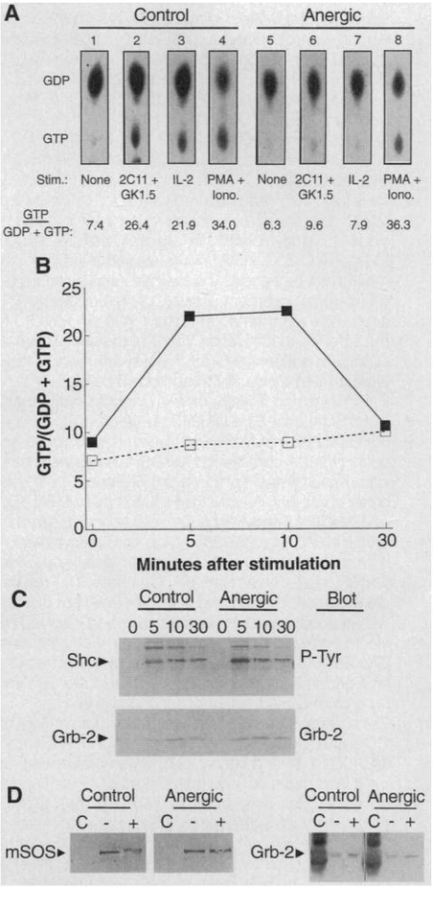

Fig. 2. (**A**) Defective Ras activation in anergic pGL10 cells. ^{32}P-Labeled control or anergic cells (1×10^7) were unstimulated (lanes 1 and 5) or were stimulated (stim.) by TCR + CD4 cross-linking (lanes 2 and 6), by recombinant human IL-2 (100 U/ml) (lanes 3 and 7), or by PMA (50 ng/ml) + 1 μM ionomycin (iono.) (lanes 4 and 8) for 5 min. Nucleotide binding was measured by direct scanning of beta radiation (AMBIS Systems). A stimulation index was calculated as GTP counts/(GDP + GTP) counts × 100 (numbers at bottom). (**B**) Time course of Ras activation in control (solid squares) and anergic (open squares) pGL10 after TCR + CD4 stimulation. ^{32}P-Labeled control or anergic cells (1×10^7) were unstimulated or were stimulated for different times by TCR + CD4 cross-linking. The GTP/(GDP + GTP) ratio was calculated as in (A). (**C**) Shc tyrosine phosphorylation and association with Grb-2 in control and anergic pGL10 cells. Control or anergic pGL10 cells (1×10^7) were stimulated by TCR + CD4 cross-linking for the times indicated at top (in minutes). Cell lysates were immunoprecipitated with antiserum to Shc (Upstate Biotechnology), and immunoprecipitates were resolved by SDS-PAGE (12% gel). The gel was transferred onto nitrocellulose and blotted with mAb 4G10 (P-Tyr) as well as antisera to Grb-2 (Upstate Biotechnology). (**D**) Association of mSOS with Grb-2 in control and anergic pGL10 cells. Control and anergic pGL10 cells were either unstimulated (−) or were stimulated by TCR + CD4 cross-linking for 5 min (+). Cells (2×10^7) were lysed and subjected to immunoprecipitation with either rabbit pre-immune serum (C) or antiserum to mSOS (Santa Cruz Biotechnology). The immunoprecipitates were resolved by SDS-PAGE (10% gel) and transferred to nitrocellulose. The presence of mSOS or Grb-2 was detected by immunoblotting (mAb to mSOS, Transduction Laboratories; antiserum to Grb-2, Santa Cruz Biotechnology).

phorylated in anergic pGL10 cells and we detected no differences in expression of GAP between normal and anergic cells (*7*).

Although many aspects of TCR signal transduction remain unaffected in anergic T cells, we show clearly that a defect in Ras activation persists in these cells, and activation of this pathway is known to be required for IL-2 gene transcription (*22*). We postulate that an early signaling defect results in the inability to activate the Ras pathway upon T cell stimulation. This block in Ras activation may result in an inability to engage the transcription factors Fos and Jun, which drive transcription at AP-1 sites, thereby preventing IL-2 gene transcription in anergic T cells. The ability to regulate Ras activation in this way may be used by the immune system to specifically prevent IL-2 production while allowing other T cell functions to proceed. Determination of the precise target or targets in the TCR signaling cascade responsible for this block in Ras activation should provide important insight into the mechanisms involved in the maintenance of T cell tolerance.

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6. Cells (5×10^4) were cultured with 1×10^6 irradiated (2000 rad) and T cell–depleted syngeneic splenocytes plus ovalbumin in 96-well culture plates (Costar) at 37°C. At 20 hours, supernatant was removed and IL-2 and IL-4 were measured by enzyme-linked immunosorbent assay (ELISA) (Endogen). Anergic cells were rested in culture medium for 2 to 6 days before restimulation. Control cells were stimulated with antigen-APC for 7 days, after which they were treated identically to anergized cells.
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9. For ERK determinations, cells were lysed in boiling tris buffer (10 mM) containing 1% SDS. Lysates were sonicated and diluted 1:10 in a 0.5% Triton X-100 lysis buffer containing 50 mM tris-HCl, 5.0 mM EDTA, 150 mM NaCl, 1.0 mM Na_3VO_4, leupeptin (10 μg/ml), aprotinin (10 μg/ml), 25 mM *p*-nitrophenyl *p*-guanidinebenzoate, 1.0 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mM NaF (pH 7.4) (buffer 1). Proteins were immunoprecipitated from post-nuclear lysates with antibody-coated agarose beads and resolved by electrophoresis and immunoblotting. For Shc and Grb-2–mSOS determinations, cells were lysed in buffer 1 and immunoprecipitation and resolution of proteins were carried out as described above.
10. Cells were stimulated by soluble cross-linking of CD3 and CD4 with the use of mAbs GK1.5 and 145-2C11 (1:1 mixture) (*23*). After precoating of cells with this mAb cocktail, 0.5 ml of cross-linking antisera goat antiserum to hamster [(20 μg/ml) (Cappel)] was added and cells were stimulated while being mixed on a rotator at 37°C. This antibody bound to both GK1.5 and 145-2C11 efficiently (*7*). At various times, cells were pelleted by centrifugation (10,000*g* for 5 s) for lysis.
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15. Assessment of guanosine diphosphate (GDP)–GTP binding to Ras was carried out as described (*16*). Ficoll-Hypaque purified T cells were labeled with [^{32}P]orthophosphate. Stimulated cells were lysed in buffer containing 50 mM Hepes (pH 7.4), 1.0% Triton X-100, 100 mM NaCl, 20 mM $MgCl_2$, bovine serum albumin (1 mg/ml), 0.1 mM GTP, 0.1 mM GDP, 1 mM adenosine triphosphate, 1 mM sodium phosphate (pH 7.4), 0.4 mM PMSF, aprotinin (10 μg/ml), soybean trypsin inhibitor (10 μg/ml), and 10 mM benzamidine. Ras was immunoprecipitated with the mAb Y13-259 (American Type Tissue Collection). Nucleotides were eluted from Ras, separated by thin-layer chromatography, and quantified by direct scanning for beta emissions (AMBIS Systems, San Diego, CA). Ras immunoblot analysis showed that similar amounts of protein were immunoprecipitated from both control and anergic cells (*7*). Data are expressed as the ratio of GTP bound to Ras to total guanine nucleotide bound to Ras.
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17. IL-2 production by antigen-stimulated control and anergic cells was 3969 pg/ml and 33 pg/ml, respectively; whereas control and anergic cells stimulated with PMA (100 ng/ml) + 1.0 μM ionomycin produced IL-2 amounts of 1561 pg/ml and 791 pg/ml, respectively. Similar results were obtained in three separate experiments. The data must be interpreted cautiously, because stimulation of T cells with these agents may not precisely mimic TCR stimulation. Although PMA + ionomycin equivalently activated Ras in control and anergic cells, there was an incomplete recovery of functional responses in the anergic clones, which suggests that other Ras-independent pathways may be affected in anergy.
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Antigen Presentation and T Cell Development in H2-M–Deficient Mice

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HLA-DM (DM) facilitates peptide loading of major histocompatibility complex class II molecules in human cell lines. Mice lacking functional H2-M, the mouse equivalent of DM, have normal amounts of class II molecules at the cell surface, but most of these are associated with invariant chain–derived CLIP peptides. These mice contain large numbers of $CD4^+$ T cells, which is indicative of positive selection in the thymus. Their $CD4^+$ cells were unresponsive to self H2-M–deficient antigen-presenting cells (APCs) but were hyperreactive to wild-type APCs. H2-M–deficient APCs failed to elicit proliferative responses from wild-type T cells.

Experiments in vitro suggest that DM facilitates the exchange of major histocompatibility complex (MHC) class II–associated invariant chain peptides (CLIP) for antigenic peptides (*1*). The general importance of DM for peptide loading is debatable, however, because mouse class II molecules have been reported to be less dependent on DM (*2*) than human class II molecules (*3*). To evaluate the function of H2-M in vivo, we generated mice lacking H2-M_α (Fig. 1, A and B) (*4*). H2-M αβ heterodimer formation is necessary for H2-M function, and in the absence of H2-M_α H2-M_β has a reduced half-life and does not leave the endoplasmic reticulum (*5*, *6*).

Splenocytes from H2-$M^{+/+}$ (wild-type) and H2-$M^{-/-}$ (H2-M–deficient) mice were analyzed for H2-M expression with the use of indirect immunofluorescence (*7*). In H2-$M^{+/+}$ mice, H2-M staining was located in vesicular structures (Fig. 1C), as described previously (*6*), whereas no H2-M staining was detected in cells from H2-$M^{-/-}$ mice (Fig. 1D). Costaining with H2-A^b mono-

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