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# Early Growth Response Gene-2, a Zinc-Finger Transcription Factor, Is Required for Full Induction of Clonal Anergy in CD4$^+$ T Cells[1]

**John E. Harris,[2]* Kenneth D. Bishop,[2]* Nancy E. Phillips,[†] John P. Mordes,[†] Dale L. Greiner,[†‡] Aldo A. Rossini,*[†‡] and Michael P. Czech[3]***

**Ag-specific immune tolerance results from the induction of cellular mechanisms that limit T cell responses to selective Ags. One of these mechanisms is characterized by attenuated proliferation and decreased IL-2 production in fully stimulated CD4$^+$ Th cells and is denoted T cell anergy. We report the identification of the early growth response gene (Egr-2; Krox-20), a zinc-finger transcription factor, as a key protein required for induction of anergy in cultured T cells. Gene array screening revealed high Egr-2 expression distinctly persists in anergized but not proliferating murine A.E7 T cells. In contrast, Egr-1, a related family member induced upon costimulation, displays little or no expression in the anergic state. IL-2-mediated abrogation of anergy causes rapid depletion of Egr-2 protein. Full stimulation of anergic A.E7 T cells fails to enhance IL-2 and Egr-1 expression, whereas Egr-2 expression is greatly increased. Silencing Egr-2 gene expression by small interfering RNA treatment of cultured A.E7 T cells before incubation with anti-CD3 alone prevents full induction of anergy. However, small interfering RNA-mediated depletion of Egr-2 5 days after anergy induction does not appear to abrogate hyporesponsiveness to stimulation. These data indicate that sustained Egr-2 expression is necessary to induce a full anergic state through the actions of genes regulated by this transcription factor.** *The Journal of Immunology,* **2004, 173: 7331–7338.**

Antigen stimulation of T lymphocytes through the TCR in the absence of costimulation through accessory receptors results in defective IL-2 production and a lack of proliferation. The consequence of this partial signaling is long-term hyporesponsiveness to subsequent costimulation, a state known as T cell clonal anergy (1). In vitro induction of clonal anergy is achievable by treatment of cultured A.E7 T lymphocytes with immobilized anti-TCR mAb (anti-CD3) alone, whereas robust IL-2 production and proliferation are achieved if anti-CD28 mAb is included (2, 3). In contrast, the removal of IL-2 by washing the cells or the addition of neutralizing Ab to IL-2 results in anergy, even in a costimulatory environment (4, 5). In addition, administration of IL-2 is sufficient to rescue T cells from the anergic phenotype (6).

Comparative pharmacologic analysis has been used to characterize the contribution of IL-2R signaling to anergy evasion. Preventing T cell proliferation with rapamycin, a drug that blocks G$_1$-to-S phase cell cycle transition through interaction with the IL-2 signaling intermediate mammalian target of rapamycin, induces anergy in fully stimulated T cells. In contrast, hydroxyurea, a drug that arrests cell growth in the S phase but allows IL-2 signaling, does not promote the anergic state (7). It has been proposed that T cell anergy results from the up-regulation of anergy-specific factors following TCR engagement, but these factors are diluted or degraded following IL-2 signaling (7). In contrast, anergic cells, which do not receive IL-2 stimulation, appear to maintain high levels of these factors. Anergic cells exposed to exogenous IL-2 proliferate, presumably diluting or down-regulating these factors, and escape the hyporesponsive phenotype. It is unclear what molecular events are responsible for anergy induction following TCR stimulation and which events following IL-2R signaling can both prevent anergy induction and abrogate established anergy in unresponsive clones.

Productive stimulation of T cells results in IL-2 production, a process that requires the initiation of multiple parallel signaling pathways, including the activation of NFAT; MAPK members JNK, ERK, and p38; and NF-$\kappa$B. The individual contribution that each pathway makes to the production of IL-2 is complex and is currently the subject of intense study. Many components of these pathways are known, and have been compared with fully responsive and anergic T cell populations. For example, the activation of NFAT requires an early influx of calcium to the cytoplasm, an event that occurs comparably in normal and anergic populations and results in NFAT mobilization to the nucleus (8). However, the production and nuclear localization of AP-1 constituents *fos* and *jun* are defective in anergic cells (5, 8). In addition, impairments in the activation of MAPK pathway members Ras, JNK, and ERK have been described in anergic T cells, suggesting numerous points of regulation for AP-1 activity (9).

Several groups have now used screening methods to probe the identity of anergic factors that may mediate the described anergic phenotype, using differential display and microarray techniques in

models of anergy both in vitro and in vivo (10–14). A number of proteins likely to contribute to the induction of anergy have been described, including members of the E3 ubiquitin ligase family (15, 16). The presence of these likely leads to the degradation of signaling proteins necessary for entry into the cell cycle, thus preventing a degree of proliferation necessary for escape from anergy. At least one of these has been shown to be necessary for the induction of anergy (15).

We have used a genomic screen to identify genes selectively expressed in anergic A.E7 T cells but not in mock-stimulated or in fully stimulated control cell populations. We report that early growth response (Egr)[4] gene 2 expression persists in anergic cell populations, although its expression is much more transient in the presence of CD28 signaling. The Egr family of genes consists of four members (Egr-1–Egr-4) that are expressed rapidly following stimulation. A hallmark feature of the Egr family is a DNA-binding domain consisting of three zinc-finger motifs (17). This domain is known to bind the sequence GCGGGGGCG and its presence in gene promoter regions has been shown to facilitate transactivation of target genes (18). Egr-2 has been most widely studied in the context of the nervous system, and its targeting in knockout mice results in early lethality concurrent to defects in hindbrain patterning, peripheral nerve myelination, and bone formation (19). The ability of Egr-2 to up-regulate target genes has been shown to be regulated by corepressors such as members of the NGFI-A binding family and Ddx20 (19, 20). The role of Egr-2 in the immune compartment is not extensively described, although it has been shown that Egr-2 transactivation is dependent on members of the NFAT family in T lymphocytes, and Egr-2 may play a role in the NFAT-dependent regulation of Fas ligand (18). We show that Egr-2 is down-regulated ∼2 days after full stimulation, when active proliferation following IL-2 receptor signaling occurs. In contrast, anergic cells maintain elevated levels of the protein for as long as 10 days after initial stimulation. Exposure of anergic cells to IL-2 results in dilution or degradation of Egr-2 ∼3–5 days after addition of IL-2, when proliferation is most active. Depletion of Egr-2 through small interfering (si) RNA-mediated gene silencing disrupts anergy demonstrated by restored ERK activation, IL-2 production, and proliferation although control siRNA has no significant effect. These data suggest that Egr-2 expression is required for the full induction of anergy in T cells.

## Materials and Methods

### A.E7 cell line

The pigeon cytochrome *c* (PCC)-specific and I-E$^k$-restricted CD4$^+$ murine Th cell clone A.E7 (a kind gift from R. Schwartz, National Institutes of Health, Bethesda, MD) and their cultivation conditions have been previously described (7). Briefly, A.E7 (H2$^k$) cells were stimulated with irradiated B10.BR (H2$^k$) splenocytes and 8 μM whole PCC (Sigma-Aldrich, St. Louis, MO) for 48 h, expanded 20:1 in 10–15 U/ml rmIL-2, and cultured for a minimum of 12 days before use in all experiments.

### Anergy induction

A.E7 T cells were isolated over a Ficoll gradient (Lympholyte M; Accurate Chemical and Scientific, Westbury, NY) and anergy was induced by overnight incubation of 20–40 × 10$^6$ cells in a T75 or 100 × 10$^6$ cells in a T175 tissue culture flask (BD Falcon, Bedford, MA) that had previously been coated with anti-CD3 mAb (clone 145-2C11; BD Pharmingen, San Diego, CA) at a concentration of 1 μg/ml. Fully activated cells were supplemented with soluble anti-CD28 (clone 37.51; BD Pharmingen) at a concentration of 1 μg/ml. Mock-stimulated cells were incubated with immobilized IgG control Ab alone. After 12 h, cells were removed from the stimulus, washed, and rested in fresh medium for the indicated times.

### Proliferation and IL-2 assay

Proliferation was assayed by [$^3$H]thymidine incorporation into DNA and performed as described (7). Briefly, cells were cultured with irradiated syngeneic (B10.BR) splenocytes plus increasing doses of PCC Ag for 64–70 h and pulsed during the final 16 h with 1 μCi of [$^3$H]thymidine. IL-2 production was measured by ELISA using supernatants from unstimulated and anergic A.E7 cells stimulated with anti-CD3 and anti-CD28 Abs. Supernatants were harvested after 12–16 h of stimulation and assayed for IL-2 protein by sandwich ELISA using Abs purchased from BD Pharmingen. Biotinylated IL-2 detection Ab was probed with HRP-conjugated avidin D (Vector Laboratories, Burlingame, CA).

### Microarray procedures

A.E7 T cells that had been pretreated as described (mock-stimulated, anergized, or fully activated) were processed for RNA isolation with an RNeasy kit (Qiagen, Valencia, CA) at three time points: immediately following removal from a 12 h stimulus, after 2 days of rest, and after 5 days of rest. Fifteen micrograms of total RNA per sample was reverse transcribed with oligo(dT) primer using the SuperScript Choice system (Invitrogen Life Technologies, Rockville, MD) according to Affymetrix (Santa Clara, CA) recommended protocol. cDNA was used as a template for the in vitro transcription of biotinylated cRNA with the BioArray High Yield RNA Transcript Labeling kit (Enzo Life Sciences, Farmingdale, NY) according to manufacturer's instructions. Biotinylated cRNA was isolated by passing over an RNeasy Mini column (Qiagen), processed according to Affymetrix recommended protocol, and eluted with water. cRNA was precipitated, fragmented, and added to the hybridization mixture according to the Affymetrix recommended protocol (Affymetrix). Fresh mixture was prepared and hybridized to each of the murine genome U74v2 A, B, and C chips (Affymetrix) according to manufacturer's instructions. Data analysis was performed with Data Mining software supported by Affymetrix.

### Quantitative RT-PCR

Total RNA was prepared using an RNeasy kit (Qiagen). cDNA was synthesized using oligo(dT) primers and AMV reverse transcriptase (Roche, Indianapolis, IN) according to manufacturer's instructions. Quantitative real-time PCR was performed in a Light Cycler (Roche) using a SYBR Green I PCR kit (Roche) and specific primers to amplify a 300–500 bp fragment from Egr-2 or HPRT.

### Western blot analysis

For Western blotting, cells were washed once in cold PBS, lysed in SDS gel-loading buffer lacking 2-ME and bromophenol blue, boiled for 5–10 min, and quantified by bicinchoninic acid protein assay (Pierce, Rockford, IL). 2-ME and bromphenol blue were then added to the lysates and 20 (Egr-2) or 30 μg (ERK) of protein run on a 10% SDS gel. Protein was transferred to a PVDF membrane and Egr-2 blots were probed with anti-Egr-2 Ab (1/200; Covance, Richmond, CA), goat anti-rabbit secondary Ab conjugated to HRP (1/10,000; Chemicon International, Temecula, CA), and developed in chemiluminescence (PerkinElmer, Boston, MA). Blots were probed for actin with anti-actin Ab (1/50,000; Sigma-Aldrich), goat anti-mouse secondary Ab (1/10,000; Chemicon International), and developed in chemiluminescence (PerkinElmer). Phospho-ERK blots were probed with anti-phospho-ERK (1/1000; Cell Signaling Technology, Beverly, MA) or anti-ERK (1/1000; Cell Signaling Technology) Abs, goat anti-rabbit secondary (Chemicon International), and developed in ECL (Pierce) or chemiluminescence (PerkinElmer), respectively.

### CFSE proliferation and flow cytometry

Cells were washed and resuspended in 0.5 ml PBS. An equal volume of 1 μM CFSE (Molecular Probes, Eugene, OR) in PBS was added (final concentration of 0.5 μM). Cells were mixed and incubated at room temperature for 3 min. Labeling was quenched by addition of an equal volume of FBS, cells were washed twice in PBS, resuspended in complete medium and cell number determined by trypan blue exclusion. One-half million cells were added to 2.5 × 10$^6$ syngeneic (B10.BR) splenocytes plus 10 μM PCC in a total volume of 1 ml in a 48-well plate and incubated for 4 days. Dead cells were then stained with Live/Dead blue fluorescence (Molecular Probes) according to manufacturer's instructions and at least 10,000 live events were collected on an LSRII flow cytometer (BD Biosciences, San Jose, CA) using DigiFACS software (BD Biosciences). Postacquisition analysis was performed with FlowJo software (Tree Star, San Carlos, CA).

[4] Abbreviations used in this paper: Egr, early growth response; siRNA, small interfering RNA; PCC, pigeon cytochrome *c*.

*Ag stimulation of anergic or mock-stimulated A.E7 T cells and analysis of Egr-2 and Egr-1 expression by flow cytometry and Western blotting*

A.E7 T cells were anergized or mock-stimulated as described and rested for 5 days. Syngeneic (B10.BR) splenocytes were isolated, T cell-depleted, and $4 \times 10^6$ cells in 1 ml of medium were preloaded with 0, 0.3, or 10 $\mu$M PCC in a 12-well plate overnight. Two million anergic or mock-stimulated A.E7 T cells were added to each well and incubated with the splenocytes for 3 or 6 h. A.E7 T cells added to splenocytes in the absence of Ag were analyzed as 0 h of stimulation. Immediately following stimulation, the cells were placed on ice, Fc blocked, stained with allophycocyanin-conjugated anti-CD4 (1/25; BD Pharmingen), and fixed on ice with 3% formaldehyde for 30 min. Cells were washed with FACS buffer (0.1% BSA, 0.1% sodium azide in PBS) and then permeabilized with 0.5% Triton X-100 in PBS at room temperature for 10 min. Cells were washed with staining buffer (3% fetal clone, 0.1% sodium azide in PBS) and blocked at room temperature with blocking buffer (5% fetal clone, 0.1 M Tris pH 7.2, 0.01% Triton X-100) for 10 min. Anti-Egr-2 (1/200; Covance, Richmond, CA) or anti-Egr-1 (1/400; Santa Cruz Biotechnology, Santa Cruz, CA) was added for 30 min. Cells were washed with staining buffer, and stained with FITC-anti-rabbit secondary F(ab')$_2$ Ab fragment (1/200; Jackson ImmunoResearch Laboratories, West Grove, PA) in blocking buffer at room temperature for 30 min. Cells were then washed with staining buffer and kept at 4°C until data were collected on an LSRII flow cytometer (BD Biosciences) using DigiFACS software (BD Biosciences). Postacquisition analysis was performed with FlowJo software (Tree Star). At least 10,000 events within the CD4$^+$ gate (A.E7s) were analyzed for Egr-2 or Egr-1 expression. T cell-depleted splenocytes alone were stained and analyzed to verify depletion of CD4$^+$ cells.

For Western blotting, anergic and mock-stimulated cells were stimulated with anti-CD3 and anti-CD28 Abs and lysed in SDS buffer at the indicated times.

*Cell stimulation for ERK phosphorylation*

Five million resting or anergic cells, with or without siRNA as indicated, were stimulated with immobilized anti-CD3 mAb (clone 145-2C11; BD Pharmingen) precoated at 1 $\mu$g/ml and soluble anti-CD28 (clone 37.51; BD Pharmingen) at 1 $\mu$g/ml in a six-well tissue culture plate (BD Falcon). Immediately after addition of the cells, the plate was centrifuged at 300 ×

g for rapid contact and incubated at 37°C for 60 min. After the incubation, cells were removed from the stimulus and protein was isolated for Western blotting.

*siRNA transfection*

Two separate, nonoverlapping Egr-2-specific siRNAs (Dharmacon, Lafayette, CO) were determined to effectively deplete Egr-2 protein in anergic cells. The sequences are as follows: Egr-2–1, gugaccaccuuacuacucadtdt (sense), ugaguaguaagguggucacdtdt (antisense); and Egr-2–2, guuugccaggagugacgaadtdt (sense), uucgucacuccuggcaaacdtdt (antisense). These siRNA oligonucleotides were determined to have no significant overlap with homologous gene sequences. An irrelevant siRNA was synthesized as control with the following sequence: cagucgcguuugcgacuggdtdt, ccagucgcaaacgcgacugdtdt. A.E7 T cells were electroporated with 5 nM siRNA duplex in 0.5 ml of complete growth medium at a density of $60 \times 10^6$/ml in a 0.4-cm GenePulser cuvette (Bio-Rad, Hercules, CA). The electroporation was performed with a GenePulser electroporator II (Bio-Rad) at 310 mV, 950 mF. Cells were allowed to recover for 10 min on ice, added to complete growth medium, and rested for 4–6 h at 37°C before being anergized.

## Results

### Stimulation of A.E7 T cell clones through the TCR in the absence of costimulation results in anergy

One method of anergy induction in T lymphocytes is the delivery of signal through the TCR in the absence of costimulation through CD28 (2). To optimize conditions for the identification of genes with unique expression profiles in anergic T cells, we anergized cultured A.E7 T cells with immobilized anti-CD3 mAb and compared their responsiveness with cells that had been mock-stimulated (immobilized IgG control Ab) or fully activated (anti-CD3 plus soluble anti-CD28 mAb). We determined that 5 days of culture after removal from anti-CD3 was the earliest time at which anergic cells could be identified as phenotypically distinct from fully stimulated cells. (Fig. 1B). The anergized cells incorporated less [$^3$H]thymidine into DNA in response to subsequent challenge



**FIGURE 1.** Anergic T cells are hyporesponsive to full stimulation. T cell clonal anergy is induced with TCR stimulation alone, whereas the addition of CD28 costimulation results in a more responsive phenotype when assessed after at least 5 days of rest. *A–C*, A.E7 T cells were treated overnight with either 1) plate-bound IgG control Ab (control or mock-stimulated cells), 2) plate-bound anti-CD3 mAb (clone 145-2C11) (anergized cells), or 3) plate-bound anti-CD3 mAb plus soluble anti-CD28 mAb (clone 37.51) (fully activated cells). Following treatment, cells were removed from the stimulus and rested in culture. The cells were assayed for responsiveness to stimulation with irradiated syngeneic splenocytes plus whole PCC after 3, 5, or 7 days of rest by [$^3$H]thymidine incorporation. As a separate control, treated cells were also exposed to 10 U/ml IL-2 alone to demonstrate proliferative responsiveness to the cytokine (*left panels*, shown as a separate data point on the *right*). *D–F*, IL-2 production was determined by ELISA on supernatant from cells stimulated as above after 3, 5, or 7 days of rest and harvested at 14–16 h poststimulation.
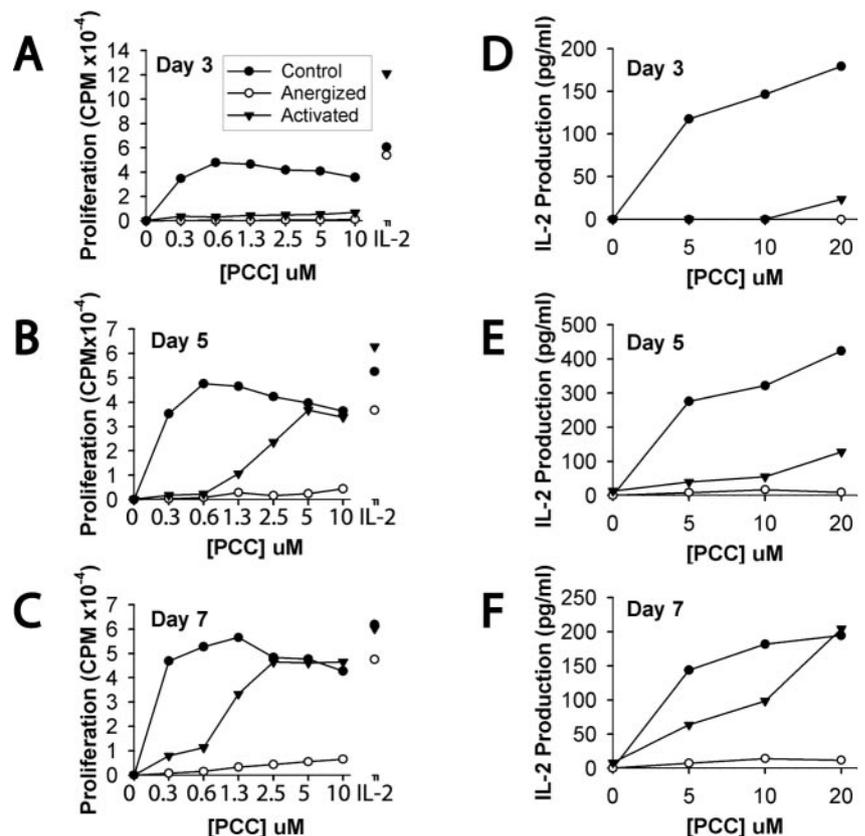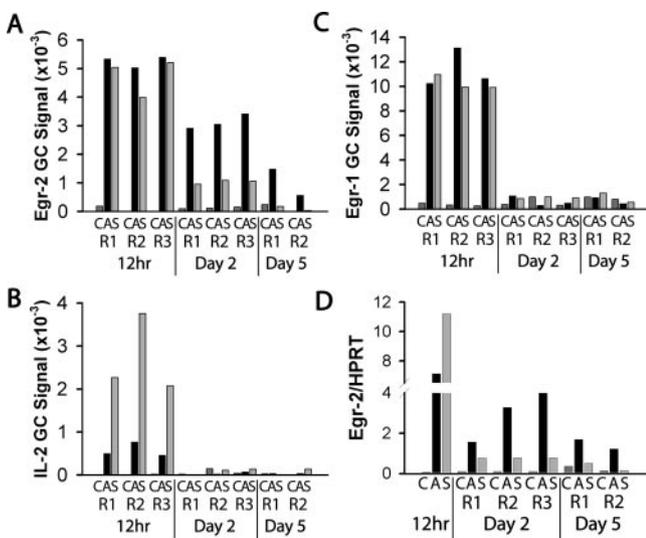
with PCC Ag plus irradiated syngeneic splenocytes compared with cells that had been initially fully activated. Similar data were obtained when IL-2 production was measured (Fig. 1, E and F). At earlier periods (e.g., 3 days after initial stimulation), both the cells treated with anti-CD3 alone and those treated with anti-CD3 plus anti-CD28 were unresponsive to subsequent Ag challenge (Fig. 1A and Ref. 7).

*Anergic T cells up-regulate and maintain long-term Egr-2 expression*

The mRNA transcription profiles were compared among anergized, fully stimulated, and untreated A.E7 cells using Affymetrix GeneChips. Cells were processed for mRNA isolation and cRNA synthesis at three time points: immediately after removal from a 12 h stimulus, 2 days after removal from stimulus, and 5 days after removal from stimulus. Replicate experiments were performed for each condition and time, and a portion of each pool of cells was again stimulated with Ag to verify the presence of their respective intended phenotypes. Analysis of data obtained from the expression arrays showed Egr-2 was more highly expressed in anergic cells compared with controls at days 2 and 5 (Fig. 2A). In contrast, at the earliest time (12 h) Egr-2 was highly expressed in both anergic and fully activated cells (Fig. 2A).

For comparison, expression of IL-2 and Egr-1 transcripts are shown (Fig. 2, B and C). As expected, IL-2 expression is significant only in fully activated T cells at the earliest time of 12 h. IL-2 expression in anergic cells is induced to some extent compared with mock-stimulated cells, but significantly less so (5-fold) than
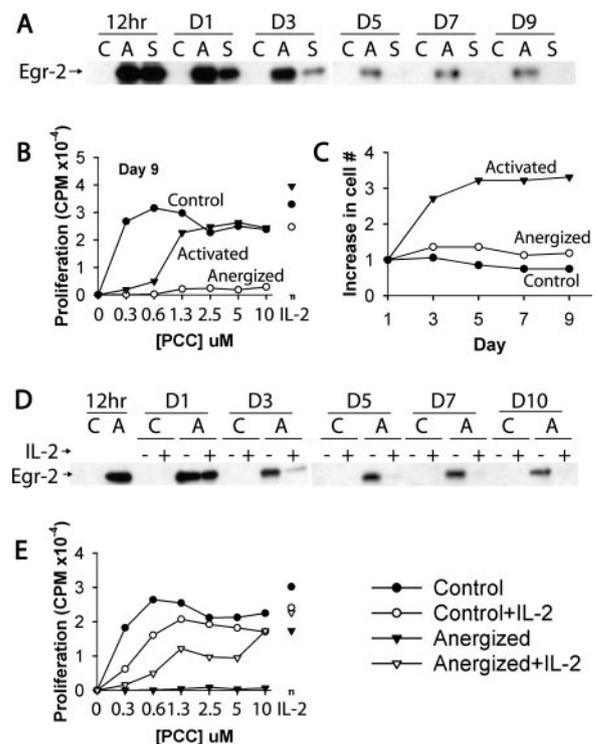
in fully activated cells (Fig. 2B). In contrast Egr-1, a gene known to be involved in early T cell activation and IL-2 induction (21), is induced equally in both anergic and fully activated T cells at 12 h but does not maintain expression past this early stage (Fig. 2C). Quantitative RT-PCR analysis of Egr-2 RNA from representative GeneChip experiments confirmed the GeneChip results (Fig. 2D).

*Egr-2 protein expression is diminished in proliferating cells following full activation or following IL-2 treatment of anergic cells*

Egr-2 protein was examined by Western blot in anergized, mock-stimulated, and fully activated cells. Protein was isolated immediately after removal of cells from the 12 h stimulus, and every other day for 9 days thereafter (Fig. 3A). After 12 h of stimulation, cells treated with either anti-CD3 alone or with both anti-CD3 and anti-CD28 exhibited high expression of Egr-2 protein. Importantly, cells that had been fully activated showed rapid loss of Egr-2 such that by day 5 little or none could be detected. In contrast, anergized



FIGURE 2. Egr-2 mRNA is initially induced in anergized and fully activated T cells but remains elevated only in anergic cells after 2 and 5 days of rest. Activation factors Egr-1 and IL-2 are not selectively expressed in anergic cells. A, A.E7 T cells were treated as in Fig. 1 to produce three cell populations: control or mock-stimulated cells (C), anergized cells (A), and fully activated cells (S). Following treatment, cells were processed for total RNA at the following times: immediately after removal from the stimulus (12 h), after 2 days of rest in medium (Day 2) or after 5 days of rest in medium (Day 5). Replicate experiments (R1, R2, R3) were performed for each condition and time and RNA was processed for hybridization to murine genome U74v2 Affymetrix GeneChips A, B, and C. Egr-2 GeneChip (GC) expression signal is reported as average difference values as determined by Affymetrix data mining software. B and C, GeneChip expression profiles for IL-2 (B) and Egr-1 (C). D, GeneChip results for Egr-2 were confirmed using quantitative RT-PCR to analyze RNA from representative GeneChip experiments. Transcript levels are reported as a ratio of Egr-2 to HPRT.



FIGURE 3. Egr-2 protein is expressed early in both anergized and fully activated T cells, quickly decreases in fully activated cells after 3–5 days, and is diminished in IL-2-treated proliferating anergic cells. A, Egr-2 protein levels were assessed by Western blot in cells that were mock-stimulated as a control (C), anergized (A), or fully activated (S) as in Fig. 1. Cells were processed immediately after removal from stimulus (12 h), and after 1, 3, 5, 7, and 9 days of rest (D1-D9) in medium. B, After 9 days of rest, the populations were challenged to assess their levels of responsiveness by [3H]thymidine incorporation as in Fig. 1. C, We assessed proliferation by counting the number of live cells by trypan blue exclusion on days 1, 3, 5, 7, and 9 after removal from the respective initial stimuli. Proliferation is reported as a ratio of the cell number of the indicated day to the cell number on day 1. D, Egr-2 protein levels were assessed by Western blot in cells that were control or mock-stimulated (C) or anergized (A) as above. Cells were processed immediately after removal from stimulus (12 h), and after 1, 3, 5, 7, and 10 days of rest in either medium alone or with exogenous IL-2 (10 U/ml) to induce proliferation and break anergy. E, After 10 days of rest, the populations were challenged to assess level of responsiveness by [3H]thymidine incorporation as in Fig. 1.

cells showed a markedly delayed initial decrement in Egr-2 expression, and these cells retained significant expression of the transcription factor throughout the 9-day period. Thus, Egr-2 expression inversely correlates with responsiveness of each cell population studied (Fig. 3*B*). We also assessed T cell proliferation by counting the number of live cells every other day for 9 days after removal from the stimulus. Viability was determined by trypan blue exclusion. As shown, Egr-2 expression is diminished in fully activated cells during proliferation (Fig. 3*C*).

Because the hyporesponsive phenotype is abrogated when anergic T cells are exposed to IL-2 during the rest period (6), we examined Egr-2 protein levels in mock-stimulated and anergic A.E7 T cells subsequently treated with IL-2. Cells were anergized with immobilized anti-CD3 or mock-stimulated with immobilized IgG control Ab. A portion of each cell population was processed for Western blotting immediately after removal from the 12 h stimulus, while the remaining cells were each divided into two subpopulations and incubated with or without 10 U/ml rmIL-2. Cells were extracted after 1, 3, 5, 7, and 10 days for protein and Egr-2 levels were assessed by Western blot (Fig. 3*D*). These data show Egr-2 protein is rapidly diminished in anergic cells following exposure to exogenous IL-2, and this is most evident between days 1 and 5. That IL-2 abrogates anergy was confirmed by measuring the responsiveness of these cells to Ag challenge, assessed by [$^3$H]thymidine incorporation on day 10 (Fig. 3*E*). Taken together, the data in Figs. 2 and 3 reveal that Egr-2 expression inversely correlates with the responsiveness of T cells to Ag stimulation, and that anergic cells retain comparatively elevated levels of this transcription factor.

### Egr-2 is expressed maximally in anergic cells following Ag challenge while expression of Egr-1 is repressed compared with responsive cells

Previously mock-stimulated or anergic cells were challenged again for 0, 3, or 6 h with T cell-depleted APCs that had been loaded with a high (10 $\mu$M) or low (0.3 $\mu$M) concentration of PCC. Cells were then harvested on ice, permeabilized, and stained for Egr-2 or Egr-1 for analysis by flow cytometry. As previously shown by Western blot, before stimulation (0 h) Egr-2 is most highly expressed in anergic cells (Fig. 4*A*) whereas Egr-1 is not expressed by either population (Fig. 4*B*).

Egr-2 is induced to maximal levels in previously mock-stimulated cells by 3 h and remains at high levels for at least 6 h. In anergic cells, Egr-2 begins at increased levels but was further induced to maximal levels with stimulation after 3 h (Fig. 4*A*). Maximal expression of Egr-2 after restimulation was equal in both mock-stimulated and anergic cells. Stimulation with both doses of Ag incurred the same pattern of protein expression, however a high Ag dose resulted in increased Egr-2 expression in both populations.

Egr-1 was up-regulated to maximal levels in previously mock-stimulated cells by 3 h but began to return to low levels after 6 h. This was true at both Ag doses. Anergic cells failed to up-regulate Egr-1 to levels of mock-stimulated cells when stimulated with both low and high Ag concentrations. Also in contrast to cells previously mock-stimulated, expressed levels of Egr-1 in anergic cells were quickly reduced to low levels after peak expression (Fig. 4*B*).

To confirm these data, protein levels were assessed by Western blot analysis. Egr-2 levels were up-regulated in both populations upon incubation with anti-CD3 and anti-CD28, and persisted over time (Fig. 4*C*) whereas Egr-1 protein levels increased early in the anergic population and was subsequently down-regulated over an 8-h time course. This was in contrast to the mock-stimulated population, in which Egr-1 expression persisted (Fig. 4*D*).

### Proliferation and IL-2 production was partially restored in anergic cells lacking Egr-2 at early, but not late stages of anergy

We tested whether Egr-2 is necessary for induction of the anergic phenotype by attenuating Egr-2 expression through siRNA-mediated gene silencing. A.E7 T cells were electroporated with Egr-2-specific siRNA constructs, with control siRNA, or without siRNA, allowed to recover for 4–6 h, and then incubated for 12 h with immobilized anti-CD3 mAb to induce anergy. Another sample of cells was electroporated without siRNA and left unstimulated to serve as nonanergized control T cells. To assay for transfection efficiency, we transfected a sample of cells with FITC-labeled siRNA. By flow cytometry, we determined transfection efficiency consistently ranges from 90 to 99%. Using fluorescence microscopy, we ruled out the possibility that the siRNA was simply sticking to the outside of the cell (data not shown). Five days after removal of T cells from the anergizing stimulus (anti-CD3 alone), Egr-2 protein levels were significantly lower in the presence of

**FIGURE 4.** Expression of Egr-1 is inhibited in fully stimulated anergic cells whereas Egr-2 is maximally expressed. *A* and *B*, Anergic or mock-stimulated A.E7 T cells were rested for 5 days, then exposed for 0, 3, or 6 h to CD4-depleted syngeneic splenocytes preloaded with either 0.3 or 10 $\mu$M PCC Ag. Each population was then fixed and stained with anti-CD4 Ab, and permeablized and stained with anti-Egr-2 (*A*) or anti-Egr-1 (*B*) Ab. Expression of these proteins was analyzed by flow cytometry. Populations shown are gated on anergic or mock-stimulated A.E7 T cells (CD4$^+$ population). *C* and *D*, Egr-2 and Egr-1 protein levels were assessed by Western blotting of mock-stimulated (C) and anergized (A) cells following anti-CD3 and anti-CD28 restimulation. Cells were treated, rested for 6 days, and stimulated for 4, 8, or 12 h at which point they were lysed in SDS buffer for Western blotting. Blots were stripped and reprobed with an Ab for $\beta$-actin to control for protein loading.
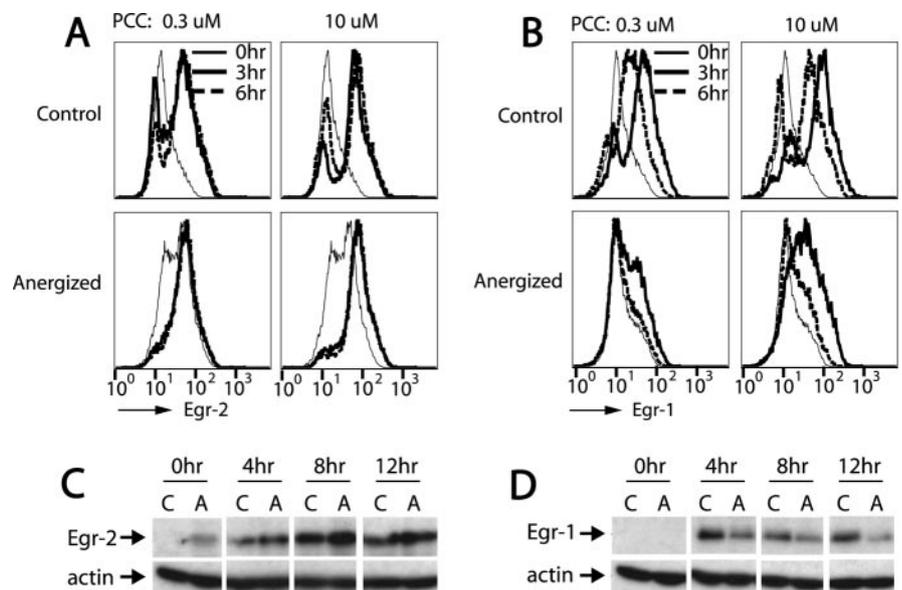
either of two siRNAs directed against different regions of Egr-2 message (Fig. 5C). Anergic cells electroporated with control siRNA or no siRNA retained high levels of Egr-2 protein (Fig. 5C). Remarkably, the siRNA-treated anergic cells with decreased Egr-2 protein expression were more responsive than anergic controls to Ag challenge. The increased proliferative response of cells with low Egr-2 protein levels was assessed by both [³H]thymidine incorporation into DNA (Fig. 5A) and by CFSE dilution (Fig. 6A). By this latter assay, 97% of resting cells divided at least once, whereas only 50% of anergic cells electroporated with control or no siRNA divided at least once. In contrast, 73% of Egr-2-depleted anergic T cells divided at least once. Additionally, the IL-2 production defect observed in anergic T cells is partially restored with siRNA-mediated depletion of Egr-2 (Fig. 5B). These data confirm an inverse correlation between Egr-2 expression and T cell responsiveness, and suggest a causative relationship between high Egr-2 expression and the anergic state.

To assess whether the effects of Egr-2 are present at late times, A.E7 T cells were anergized, rested for 5 days, and then electro-

porated with Egr-2-specific siRNA duplexes, with control siRNA, or without siRNA. Cells were then rested for 2 more days, then restimulated with Ag plus irradiated syngeneic splenocytes. Although Egr-2 protein was reduced in anergic cells treated with Egr-2 siRNA under these conditions (Fig. 5F), they remained unresponsive to Ag restimulation as assessed by [³H]thymidine incorporation (Fig. 5D) and IL-2 production (Fig. 5E).

Because some Egr-2 protein is expressed in cells stimulated with anti-CD3 and anti-CD28, the responsiveness of these cells was compared with that of anergic cells, and anergic cells treated with Egr-2 siRNA (Fig. 5G). Upon antigenic stimulation, anergic cells treated with Egr-2 siRNA manifest a response that is intermediate to the control anergic population and the fully stimulated population (Fig. 5G).

*ERK phosphorylation is restored in anergic cells lacking Egr-2*

Based on the restoration of the proliferative response and IL-2 production in anergized A.E7 T cells upon attenuation of Egr-2 expression during anergy induction, we evaluated the integrity of

**FIGURE 5.** siRNA-mediated gene silencing of early Egr-2 expression results in restored proliferation and IL-2 production in anergic cells. *A*, siRNA treatment before anergy induction results in Egr-2 protein knockdown. T cells were electroporated with the following: nothing (2-None), control siRNA (3-Con), or one of two distinct, nonoverlapping siRNAs specific for Egr-2 (4-Egr-2–1 or 5-Egr-2–2). A population of cells was electroporated and left unstimulated (Resting) as a responsive control (1-None). Five days later, cells were stimulated with PCC-pulsed irradiated syngeneic splenocytes for 68 h, the last 16 h of which included [³H]thymidine. *B*, IL-2 production is restored in anergic cells with decreased levels of Egr-2 treated before anergy induction. Cells were treated as above, rested for 5 days, and stimulated with anti-CD3 and anti-CD28 Abs. Supernatant was harvested and assayed by ELISA for IL-2. *C*, Egr-2 protein levels in cells treated with siRNA before anergy were assessed by Western blot 5 days after removal from the anergizing stimulus. The blot was sequentially probed with Egr-2 Ab and β-actin Ab. *D*, siRNA treatment 5 days following anergy induction results in Egr-2 protein knockdown, but confers no restoration of proliferative responsiveness. T cells were anergized for 12 h and removed from their stimulus. Five days after removal, cells were transfected with siRNA (Egr-2–1) as in *A*, rested for 2 days, and restimulated as in *A*. *E*, When Egr-2 is knocked down 5 days following anergy induction, no restoration of the cells' ability to make IL-2 is conferred. *F*, Egr-2 protein was assessed by Western blot in cells treated with Egr-2 siRNA 5 days following anergy induction. *G*, Proliferation of Egr-2 siRNA-treated anergic AE7 cells, compared with anergic cells treated with a control siRNA and anti-CD3⁺ anti-CD28-stimulated AE.7s. Egr-2 protein levels in the three populations of cells were assessed by Western blot with anti-Egr-2 Ab. Results are representative of at least three independent experiments.
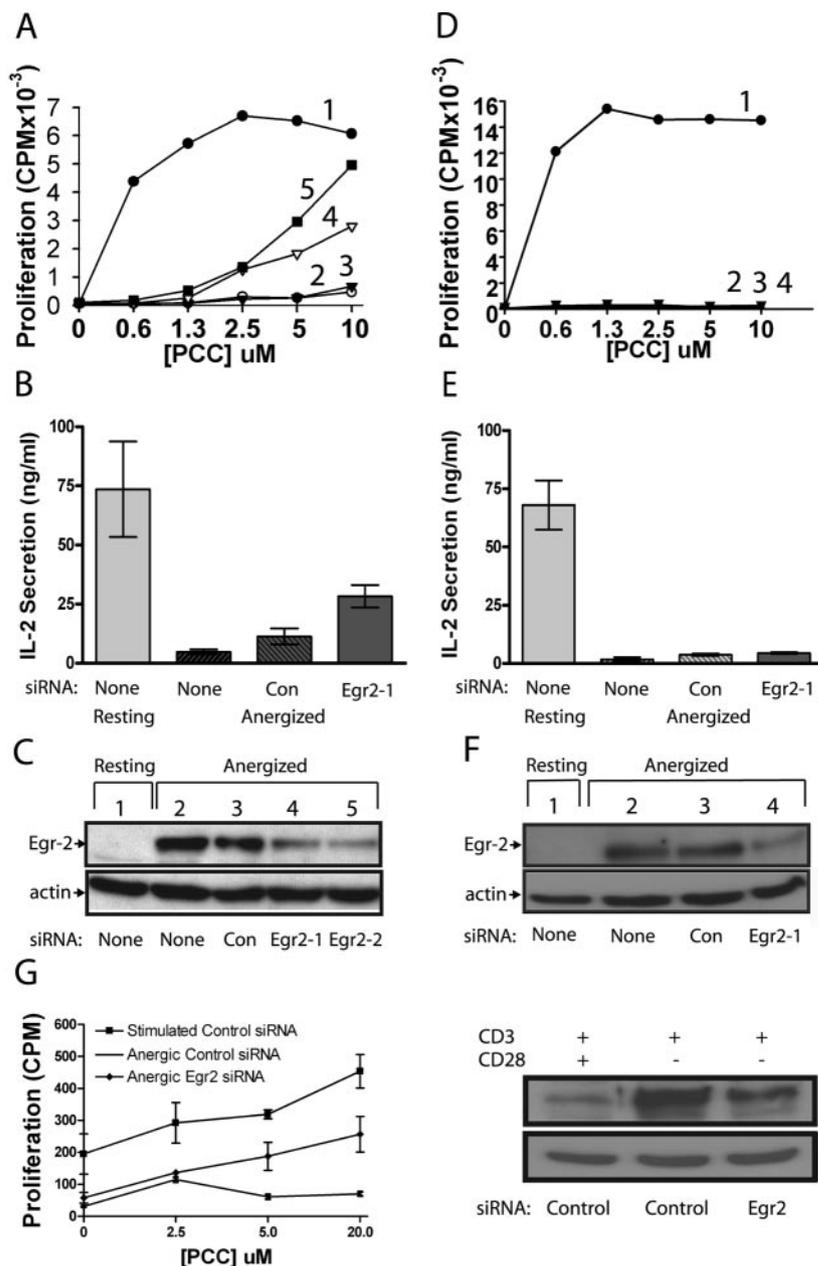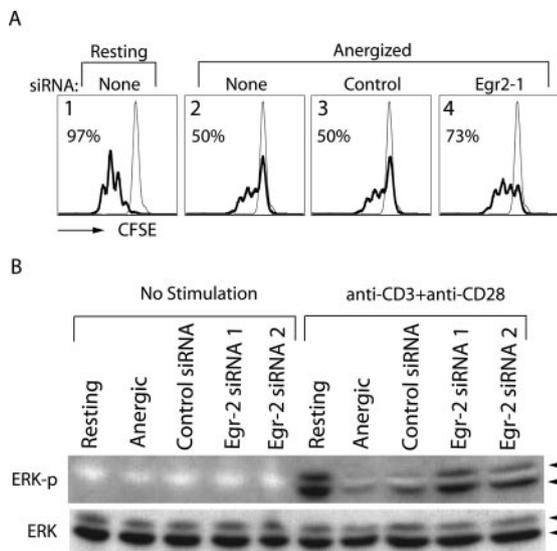
**FIGURE 6.** Depletion of Egr-2 enables cell division in anergic cells, concurrent with an increase in ERK phosphorylation in response to stimulation. *A*, Untreated, anergized, and Egr-2 siRNA-treated cells were labeled with CFSE and then stimulated with syngeneic splenocytes with or without PCC. CFSE fluorescence (thick histogram) in cells not exposed to Ag (0 divisions) and fluorescence (thin histogram) in cells stimulated with 10 $\mu$M PCC are represented. *B*, Cells were treated as in Fig. 5, rested for 6 days, and stimulated with anti-CD3 and anti-CD28 Abs. Cells were lysed and analyzed by Western blotting. The blot was sequentially probed with Abs against phospho-ERK and total ERK. Arrowheads mark ERK-1 (*upper blot*) and ERK-2 (*lower blot*). Results are representative of two independent experiments.

the MAPK signaling pathway in these cells. Defective phosphorylation (7, 22) and activation (9, 23) of ERK-1 and ERK-2 in anergic T cells in response to full stimulation has been well documented. We could confirm the decreased phosphorylation of ERK in anergized A.E7 T cells (Fig. 6*B*). This observed defect in ERK phosphorylation upon stimulation of anergic T cells with anti-CD3 plus anti-CD28 was reversed when Egr-2 expression was reduced through siRNA-mediated gene silencing (Fig. 6*B*). Thus depletion of Egr-2 in A.E7 T cells prevents the induction of three key TCR-mediated properties of anergic cells: the failure to proliferate, the failure to produce IL-2, and down-regulation of the MAPK cascade.

*Depletion of Egr-2 protein levels in CD3+ CD28-stimulated cells increases their responsiveness to stimulation*

As shown in Fig. 5*G*, it is possible to detect low levels of Egr-2 in fully stimulated cells, even at late times. To determine the effect this might have on cellular responsiveness, we treated A.E7 cells with Egr-2 siRNA and stimulated them with immobilized anti-CD3 and soluble anti-CD28. After removal from stimulus, cells were allowed to rest for 5 days, and then restimulated with irradiated syngeneic splenocytes pulsed with PCC Ag. In cells treated with Egr-2 siRNA, responsiveness was increased compared with those treated with a control siRNA (Fig. 7*A*), demonstrating further that the presence of Egr-2 dampens the ability of T cells to respond to full stimulation (Fig. 7*B*).

## Discussion

It has been well documented that stimulation of T cells through the TCR in the absence of costimulation can result in long-term hyporesponsiveness to subsequent stimulation, termed anergy. Although the anergic phenotype has been well studied, the molecular
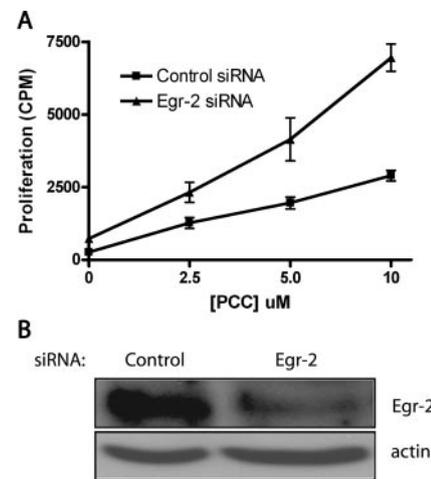


**FIGURE 7.** Depletion of Egr-2 in anti-CD3+anti-CD28 stimulated cells increases their response to restimulation. *A*, AE.7 cells were transfected with a control siRNA or an Egr-2-specific siRNA, or left untreated. Cells were then stimulated with immobilized anti-CD3 and soluble anti-CD28 Abs, rested for 5 days, and restimulated with PCC-pulsed irradiated syngeneic splenocytes for 68 h. For the last 16 h of restimulation, the cultures were pulsed with [3H]thymidine. *B*, A sample of cells from each population was lysed after the 5 day rest was analyzed by Western blotting with anti-Egr-2 Ab. Blots were stripped and probed with anti-actin for loading control. Results are representative of three independent experiments.

events responsible for the induction and maintenance of anergy are not fully known. Anergy is very likely a complex process involving a number of genes that serve to mediate the resistance to costimulation. The data presented in this report strongly indicate that the transcription factor Egr-2 functions in establishing the anergic state in A.E7 T cells. The expression profile of Egr-2 is consistent with the characteristics of a factor that confers anergy as predicted by Powell et al. (7) in that 1) it is initially up-regulated in both anergized and fully activated T cells before their proliferation (Figs. 2 and 3) and its expression is prevented by cyclosporin A (data not shown); 2) its expression decreases more rapidly in proliferating cells than anergized cells (Fig. 3*A*); and 3) high expression levels of Egr-2 in anergy are abrogated by IL-2 (Fig. 3*D*), which concurrently reverses the anergic state (Fig. 3*E*).

Interestingly, high expression of Egr-2 has been found in other studies to be associated with anergy, but the initial findings were not extended. Lechner et al. (12) screened for genes expressed in primary T cells anergized in vivo, and reported the induction of Egr-2 expression in the anergized T cell population. However, in these studies comparison was made with purified primary T cells stimulated with a mitogenic dose of anti-CD3 for 16 h, a time point before the down-regulation of Egr-2. Indeed these stimulated control cells also showed high Egr-2 expression (12). In a second study of gene expression profiles, Macian et al. (11) also observed up-regulation of Egr-2 following an anergizing stimulus but evaluated only early times after T cell anergy induction, before proliferation in the activated control population. Another study using microarrays to analyze tolerized B lymphocytes showed high Egr-2 expression compared with unstimulated control cells (24). All of these studies are consistent with the data presented in this report, and with the conclusion that Egr-2 plays a role in conferring the anergic state.

Our observation that the IL-2 activator Egr-1 is repressed in stimulated anergic cells is a novel one, and parallels the repression of the AP-1 components *fos* and *jun*. This effect may be yet another

mechanism of hyporesponsiveness in anergic cells. The fact that Egr-2 up-regulation occurs normally in stimulated anergic cells may explain why anergic cells require exposure to their Ag to maintain the hyporesponsive state. Tanchot et al. (25) describe a system of anergy induction to peptide in vivo in which the T cells survive and remain tolerant in vivo as long as Ag is present. If the anergic cells are transferred to a new host not expressing the peptide, the cells regain responsiveness. If the cells are transferred to a new host expressing the Ag, they are induced into an even deeper level of anergy (25). In another system described by Pape et al. (26), in which anergy is induced in TCR transgenic cells in a normal host with peptide injection in the absence of adjuvant, anergic cells lose unresponsiveness after a period of time. This loss of responsiveness is preceded by a clearing of Ag in the host. If the tolerizing Ag is repeatedly introduced the anergic cells remain hyporesponsive longer than with a single injection.

Over time, we observe this dissipation of anergy in the A.E7 cell line coincident with a loss of Egr-2 expression (data not shown). It is possible that Egr-2 maintains the transcription of anergy effector molecules long term, and that the loss of Egr-2 expression over time contributes to the impermanence of anergy. Because full stimulation of an anergic cell is able to refresh Egr-2 expression to maximal levels without inducing IL-2 transcription or proliferation, it is possible the presence of Ag in the in vivo models discussed earlier maintains hyporesponsiveness by repeatedly up-regulating Egr-2. Although we were unable to observe abrogation of anergy by silencing Egr-2 5 days after anergy induction, it is possible that key transcriptional targets of Egr-2 are present by this time point, and are not ablated quickly enough to restore normal responsiveness in our experiments.

Given its nature as a transcription factor, Egr-2 may be responsible for activating a network of genes whose proteins mediate the many different aspects of the anergic phenotype observed, including hyporesponsiveness, differential homing, and cytokine production (27, 28). The fact that other studies have observed Egr-2 induction in tolerized lymphocytes both in vitro and in vivo suggests that Egr-2 may function in anergy induced by a variety of mechanisms. Based on the data presented in this report, identifying the factors under the regulation of Egr-2 in these models should provide important insights into the mechanisms involved in the maintenance of immune tolerance. A number of recent papers have demonstrated the importance of E3 ubiquitin ligases in the induction of anergy (15, 16). The mechanisms by which these induce anergy has not been fully demonstrated, but it has been speculated that they induce anergy via the degradation of important signaling molecules such as protein kinase C$\theta$ and phospholipase C $\gamma$-1. It is possible that the up-regulation of E3 ubiquitin ligases also prevents IL-2 production by degrading proteins required for IL-2 message transcription. It will be interesting in future studies to determine whether the mechanisms related to Egr-2 function in T cells converge with those initiated by E3 ubiquitin ligases.

## Acknowledgments

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