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Inhibition of Cell Cycle Progression by Rapamycin Induces T Cell Clonal Anergy Even in the Presence of Costimulation

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Costimulation (signal 2) has been proposed to inhibit the induction of T cell clonal anergy by either directly antagonizing negative signals arising from TCR engagement (signal 1) or by synergizing with signal 1 to produce IL-2, which in turn leads to proliferation and dilution of negative regulatory factors. To better define the cellular events that lead to the induction of anergy, we used the immunosuppressive agent rapamycin, which blocks T cell proliferation in late G1 phase but does not affect costimulation-dependent IL-2 production. Our data demonstrate that full T cell activation (signal 1 plus 2) in the presence of rapamycin results in profound T cell anergy, despite the fact that these cells produce copious amounts of IL-2. Similar to conventional anergy (induction by signal 1 alone), the rapamycin-induced anergic cells show a decrease in mitogen-activated protein kinase activation, and these cells can be rescued by culture in IL-2. Interestingly, the rapamycin-induced anergic cells display a more profound block in IL-3 and IFN-γ production upon rechallenge. Finally, in contrast to rapamycin, full T cell activation in the presence of hydroxyurea (which inhibits the cell cycle in early S phase) did not result in anergy. These data suggest that it is neither the direct effect of costimulation nor the subsequent T cell proliferation that prevents anergy induction, but rather the biochemical events that occur upon progression through the cell cycle from G1 into S phase. ***The Journal of Immunology*****, 1999, 162: 2775–2784.**

When T cells encounter Ag in the absence of costimulation, they fail to proliferate (1). Furthermore, such cells fail to produce IL-2 or proliferate upon subsequent full rechallenge (2, 3). This state of nonresponsiveness is known as T cell clonal anergy. Initial experiments described anergy as a consequence of incubating T cell clones with chemically fixed APCs or planar membranes containing MHC class II molecules and peptide (4, 5). Subsequent experiments revealed that this state of hyporesponsiveness could also be induced by incubating T cell clones with anti-CD3 or Con A in the absence of APCs (6, 7). Together, these observations suggested that engagement of the TCR, signal 1, in the absence of a second signal resulted in the anergic state. Experimental support for this notion came from observations that the addition of allogeneic APCs to the incubation of the T cell clones with fixed APCs plus peptide was able to abrogate the induction of anergy (8). Because these allogeneic cells were unable to present peptide, it was assumed that they were providing in *trans* the second signal required for full activation and the prevention of anergy. These experiments suggested that anergy induction was not the result of an alteration of the Ag-MHC complex by chemical fixation, but rather the result of the destruction and/or inhibition of expression of a costimulatory molecule on the surface of the APC.

Vis-à-vis anergy, a simple interpretation of these data is that signal 2 directly prevents the induction of anergy. However, a number of subsequent experimental observations did not support this theory. DeSilva et al. showed that incubating T cell clones with live APCs plus Ag and anti-IL-2 along with anti-IL-2R Abs resulted in the induction of anergy (9). Beverly et al. also demonstrated the induction of anergy in the presence of costimulation by eliminating the APCs and changing the medium at 16 h after initiation of the cultures. These manipulations stopped IL-2 production and removed all the IL-2 that had been produced (10). The interpretation of both sets of experiments was that, despite costimulation, anergy was induced by preventing IL-2R engagement. This led Jenkins (11) and Beverly (10) to propose that anergy is induced when signal 1 is encountered in the absence of proliferation. In this model, costimulation prevents anergy induction indirectly by enhancing IL-2 production and promoting cell division. Consistent with this idea are the findings of Boussiotis et al., who showed that signaling through the common γ-chain could prevent anergy induction in human T cells (12), as well as the work of Gilbert and Weigle, who proposed that anergy was the result of G1a cell cycle blockade (13). The model also implies that TCR engagement (in addition to providing the positive signals necessary for IL-2 production) results in the production of negative regulatory factors. The abilities of cyclosporin and cycloheximide to inhibit anergy induction supports this concept as these agents prevent NF-AT-induced transcription and protein synthesis, respectively, which might be necessary for the production of these negative regulatory factors (5, 6). In the absence of proliferation, these factors accumulate and thus the cell fails to respond upon restimulation.

Despite these data, the precise mechanism(s) leading to the induction of anergy have not yet been elucidated, and several groups have focused on the direct ability of costimulation to prevent anergy. Based on studies using CD28 knockout mice, Bachmann et al. suggest that signaling through CD28 has both costimulatory (signal 2c) and tolerance prevention (signal 2t) abilities (14). Furthermore, Becker et al. have demonstrated the up-regulation and increased binding of the negative regulatory transcription factor Nil-2a when T cells are stimulated by signal 1 alone (15). Nil-2a has been shown to bind to the IL-2 promoter at the negative regulatory element (NRE) and inhibit expression of reporter constructs (16). In the presence of costimulation, Nil-2a does not appear to be up-regulated (15). Thus, this group proposed that

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costimulation directly inhibits anergy induction by preventing the enhancement of Nil-2a function following TCR occupancy.

In the present work we sought to better define the events responsible for anergy induction by using the immunosuppressive agent rapamycin. Rapamycin is a macrolide antibiotic with a similar biochemical structure to cyclosporin A (CSA)[2] and FK506 (17). Indeed, the intracellular target for rapamycin is FK506 binding protein (FK506BP). However, unlike CSA and FK506, rapamycin does not inhibit IL-2 production (18). Rather, it inhibits the ability of lymphocytes to proliferate in response to IL-2. It is thought that the rapamycin-FK506BP complex does this by binding to and inhibiting the mammalian target of rapamycin (mTOR), a serine/threonine kinase that is believed to be a critical step in the pathway leading to the down-regulation of the cell cycle inhibitor Kip-1 following IL-2R engagement (17). As a result, the cell fails to progress from G1 into S phase and does not proliferate. Therefore. we incubated the $CD4^+$ Th1 clone A.E7 overnight with or without costimulation in the presence or absence of rapamycin. Our data show that even when given full signal 1 and signal 2, A.E7 cells are rendered anergic if cultured in the presence of rapamycin. Furthermore, the rapamycin-induced anergy appears to be more complete than conventional anergy in that there is also a marked decrease in IFN-γ and IL-3 production upon restimulation. On the other hand, A.E7 T cells stimulated with signal 1 plus 2 in the presence of hydroxyurea, an agent which arrests proliferation in S phase, are not rendered anergic. These data suggest that it is neither direct costimulation nor proliferation per se that prevents anergy induction, but rather the biochemical events that occur upon progression through the cell cycle from G1 to S phase.

Materials and Methods

Cell culture and anergy induction

A.E7 is a $CD4^+$ Th1 clone specific for the pigeon cytochrome *c* (PCC) peptide 81–104. It was grown and maintained as previously described (19). Briefly, cells were stimulated for 48 h with whole PCC and irradiated (3000 rad) B10.A splenocytes as a source of APCs. The cells were next expanded 20:1 in 10 U/ml of rIL-2. After a minimum of 12 days in culture, when the IL-2 was consumed and the cells were rested, the live cells were isolated from a Ficoll gradient and utilized. Anergy induction was achieved by overnight incubation of $20-40 \times 10^6$ A.E7 cells in a T75 tissue culture flask (Costar, Cambridge, MA) that had previously been coated with anti-TCR-β Ab H57-597 (20) at a concentration of 10 μg/ml. Some cultures were also supplemented with ascitic fluid containing the anti-CD28 mAb 37.51 (21) (a kind gift from Dr. James Allison) at a final dilution of 1:5000. CSA (Calbiochem, Cambridge, MA) was added to some cultures at a final concentration of 100 or 1000 nM, while rapamycin (a generous gift of Dr. S. Sehgal, Wyeth-Ayerst, Princeton, NJ) was added to other cultures at the same concentrations. Both the cyclosporin and the rapamycin were dissolved in ethanol. Preliminary studies revealed that adding an equivalent volume of ethanol (vehicle alone) did not affect cell cultures (data not shown). Hydroxyurea (Sigma, St. Louis, MO), dissolved in water, was added to some cultures at a concentration of 2 mM. None of the drugs used caused a decreased viability as determined by trypan blue exclusion during the overnight incubation period. After the overnight incubation, the cells were removed from the flask, washed, and recultured in fresh medium for 5–14 days before rechallenge. At this time, the cells were reisolated and assayed for their ability to proliferate or produce IL-2.

Proliferation assay

Anergized or control clones were assessed for their ability to proliferate to PCC by adding 2×10^4 A.E7 cells to 50×10^4 B10.A irradiated splenocytes (3000 rad) and increasing doses of PCC in 96-well plates in triplicate. After 48 h, the cells were pulsed with [^{3}H]thymidine and harvested 16 h later, and thymidine incorporation was determined using a betaplate reader. Controls included A.E7 cells cultured in the presence of 50 U/ml of exogenous IL-2, which were pulsed and harvested as above.

Cytokine production and measurement

Conditions were determined for optimal IL-2 production by the clones. A total of 50×10^4 anergized or control cells were added to 24-well plates, precoated with 10 μg/ml anti-TCR, with a 1:5000 dilution of anti-CD28 in a total volume of 0.5 ml. The supernatant fluids were harvested after 16 h and frozen at −20°C until they were used. IL-2 was assessed by measuring the proliferation of the IL-2-dependent CTLL cell line (American Type Culture Collection, Manassas, VA) (22) as described previously (23). Each IL-2 determination was calculated from eight twofold serial dilutions of supernatant fluids. In addition, IL-2, IFN-γ, and IL-3 were assayed by ELISA (Endogen, Woburn, MA) according to the manufacturer's instructions. Each value was calculated from either two or three serial dilutions of supernatant fluid.

Assessment of extracellular regulatory kinase (ERK) activation

Anergized and control cells (4×10^6 in 50 μl) were stimulated or mock stimulated in anti-TCR (10 μg/ml)-coated 24-well plates floated in a 37°C water bath for 10 min. At that time, the cells were lysed in 150 μl of SDS sample buffer that was supplemented with 1 mM sodium vanadate. PAGE with a 10% gel was performed on 25 μg of each sample, and the samples were then transferred to nitrocellulose. Western blot analysis was performed using 1 μg/ml of anti-phospho-ERK (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-ERK (Upstate Biotechnology, Lake Placid, NY). Secondary Ab consisted of alkaline-phosphatase-labeled anti-mouse (Santa Cruz) (1:10,000) or anti-rabbit (Sigma) (1:5000) Abs (24). The blots were developed using Vistra ECF substrate (American Life Sciences, Arlington Heights, IL) and the blue fluorescence mode of the STORM Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Results

Rapamycin inhibits proliferation to IL-2 but not IL-2 production by A.E7 cells

The strategy behind using rapamycin was that it would enable us to stimulate A.E7 cells with signal 1 plus 2, allow for the production and secretion of IL-2 as well as engagement of the IL-2R, but prevent IL-2-induced proliferation. Previously, Dumont et al. demonstrated that rapamycin inhibited proliferation but not IL-2 production by murine T cells (18). Therefore, initial experiments were performed to confirm that rapamycin was able to inhibit IL-2-induced proliferation in the A.E7 clone and not affect IL-2 production. In these experiments, similar doses of CSA were used for comparison. Fig. 1*A* formally demonstrates that CSA does not inhibit proliferation of the A.E7 cells in response to exogenous rIL-2 (50 U/ml), while rapamycin inhibits proliferation in a dose-dependent fashion. The ID_{50} for rapamycin was ~4 nM. On the other hand, we see the exact opposite effect for IL-2 production. A.E7 cells were cultured with plate-bound anti-TCR and soluble anti-CD28 in 24-well plates overnight, and supernatant fluids were collected and assayed by ELISA for IL-2 production. As seen in Fig. 1*B*, CSA inhibited IL-2 production completely at a concentration of 100 nm. On the other hand, for the same concentrations of rapamycin tested, IL-2 production was similar to that of the control cells. This overnight stimulation protocol is precisely the same as that we used to induce anergy. Of note, when the clones were stimulated with anti-TCR and anti-CD28 overnight and then pulsed for an additional 12 h with [^{3}H]thymidine, proliferation resulted in the uptake of 24,500 cpm, while in the presence of 1,000 nM CSA or rapamycin there was only 242 and 267 cpm, respectively. In this case, the CSA inhibited proliferation by inhibiting IL-2 production, while the rapamycin inhibited proliferation by endogenously produced IL-2.

Rapamycin induces anergy even in the presence of costimulation

To induce anergy, A.E7 cells are normally incubated with plate-bound anti-TCR overnight (induction phase). The cells are then harvested, washed, and rested in fresh medium. After a minimum

[2] Abbreviations used in this paper: CSA, cyclosporin A; mTOR, mamalian target of rapamycin; MAP, mitogen-activated protein; TRE, 12–0-tetradeconoylphorbol-13-acetate-responsive element; FK506BP, FK506 binding protein; PCC, pigeon cytochrome *c*; ERK, extracellular regulatory kinase.

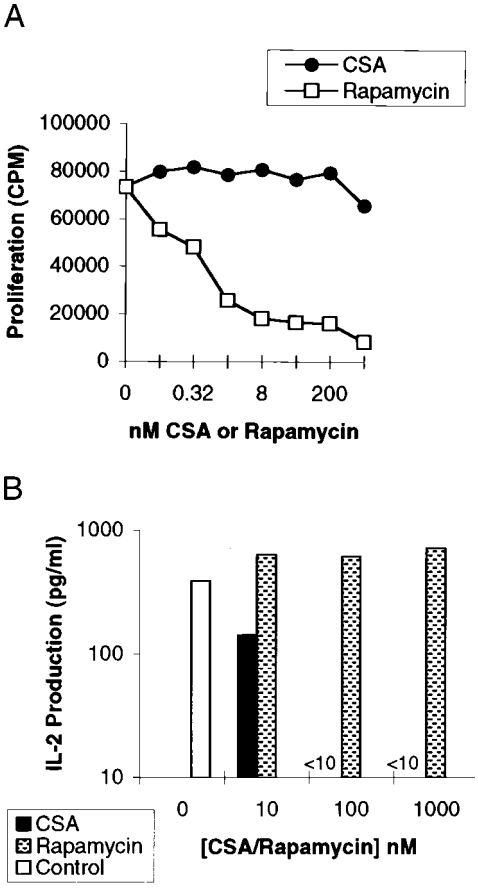

FIGURE 1. *A*, Rapamycin but not CSA inhibits proliferation of A.E7 cells to exogenous IL-2. A.E7 cells were incubated with 50 U/ml of IL-2 in the presence of increasing doses of either CSA or rapamycin. After 48 h, [^{3}H]thymidine was added to each well and the cells were harvested and assayed for proliferation after an additional 16 h of culture. Comparable data were obtained in three other experiments. *B*, CSA but not rapamycin inhibits IL-2 production. A.E7 cells were incubated overnight with plate-bound anti-TCR and soluble anti-CD28 in the presence of increasing doses of either CSA or rapamycin. Supernatant fluids were harvested and assayed for IL-2 by ELISA.

of 5 days, the cells are rechallenged with either APCs plus Ag or anti-TCR plus anti-CD28. In the following experiments, anti-CD28 with or without CSA or rapamycin was added to the induction phase. Of note is the fact that the rapamycin and CSA are only present during the 16-h overnight incubation and not during the resting or rechallenge phase. After the overnight culture to induce anergy, the cells are washed and the drugs are removed.

A.E7 cells stimulated in the presence of signal 1 alone proliferate less effectively upon rechallenge with APCs and Ag than cells that received signal 1 plus 2 during the induction phase; this is conventional anergy (Fig. 2*A*). As has been previously shown, the presence of CSA during the induction phase inhibits the development of the anergic state (5). In other experiments, we have determined that the enhanced proliferation of the CSA-treated cells when compared with the signal 1 plus 2-treated cells is actually the same level of proliferation as nonmanipulated cells (data not shown). In contrast, the presence of rapamycin during the induction phase with signal 1 alone does not interfere with anergy induction, but rather results in strong anergy (Fig. 2*B*). In fact, in this particular experiment, the presence of rapamycin appears to enhance conventional anergy. We have noted this enhancement in a number of experiments, though not all, (see Fig. 3 for example),

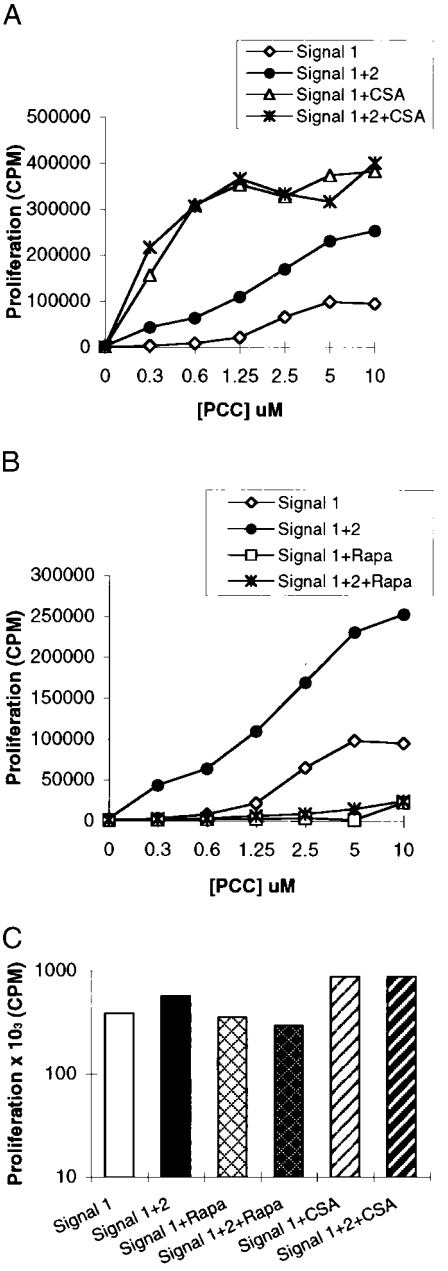

FIGURE 2. *A*, CSA inhibits anergy induction. A.E7 cells were incubated overnight with plate-bound anti-TCR alone (signal 1) or plate-bound anti-TCR plus soluble anti-CD28 (signal 1 plus 2) in the presence or absence of CSA. The cells were harvested, washed, and rested for 7 days in fresh medium. The cells were then tested for their ability to proliferate to Ag and APCs. Note, the CSA is only present during the induction phase of anergy and not during the rest period or rechallenge phase. In this and subsequent figures, the legend refers to the conditions of the induction phase. *B*, Rapamycin augments anergy induction even in the presence of costimulation. In the same experiment as shown in *A*, A.E7 cells were incubated overnight with plate-bound anti-TCR alone or plate-bound anti-TCR plus soluble anti-CD28 in the presence or absence of rapamycin. The signal 1 and signal 1 plus 2 groups are the same as in *A* to facilitate easy comparison. Once again, the rapamycin was only present during the induction of anergy and not during the rest or rechallenge phases. *C*, Rapamycin-induced anergic cells proliferate to exogenous IL-2. The cells that were anergized in *A* and *B* were tested for their ability to proliferate to 50 U/ml of exogenous IL-2. A.E7 cells were cultured for 48 h and pulsed with [^{3}H]thymidine for an additional 16 h and then harvested and assayed for incorporation of radioactivity into DNA. Proliferation of cells in the absence of IL-2 was <1000 cpm for all conditions except signal 1 plus 2, which was 2400 cpm (not shown). Comparable data were obtained in three other experiments.

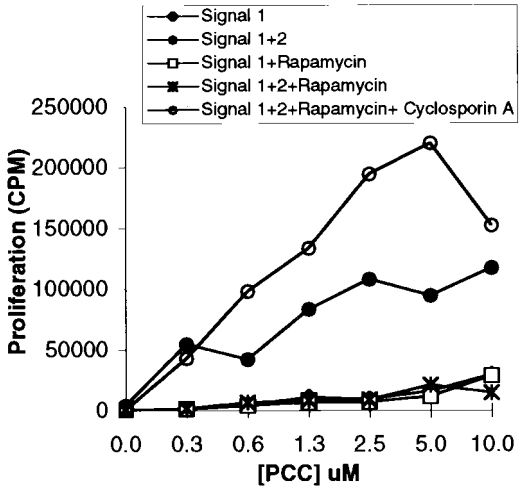

FIGURE 3. CSA inhibits rapamycin-induced anergy. A.E7 cells were anergized as in Fig. 2. In an additional culture, A.E7 cells were incubated overnight with plate-bound anti-TCR, soluble anti-CD28, and 100 nM of both CSA and rapamycin. The cells were rested and rechallenged as described in Fig. 2. These data are representative of two separate experiments.

particularly if the signal 1-induced anergy is not optimal. Rapamycin in the absence of anti-TCR had no effect on the cell cultures (data not shown). Most important, though, is the observation that the presence of rapamycin promotes anergy induction even in the presence of full costimulation. Thus, in the presence of rapamycin, costimulation does not inhibit the induction of anergy. Finally, a characteristic of anergic T cell clones is that they are able to proliferate to exogenous IL-2, because they constitutively express low levels of the high-affinity IL-2R. As seen in Fig. 2*C*, the cells anergized in the presence of rapamycin proliferated to exogenous IL-2 as well as the conventionally anergized cells and slightly less effectively than the cells that were given signal 1 plus 2 without rapamycin. Also, as expected, the cells that were initially incubated with CSA proliferated to the exogenous IL-2. In addition to their ability to respond to exogenous IL-2, the rapamycin-induced anergic cells were able to up-regulate CD25 and CD69 in response to TCR stimulation as well as normal T cell clones and their conventionally anergized counterparts (data not shown). The TCR level was also comparable (data not shown).

Cyclosporin inhibits rapamycin-induced anergy

As seen in Fig. 2, CSA inhibits anergy induction while the presence of rapamycin results in anergy induction even in the presence of signal 2. Thus, the question arises as to what happens when the two drugs are both present during the induction phase. Fig. 3 shows once again that the presence of rapamycin during the induction phase promotes anergy even in the presence of costimulation. On the other hand, when CSA is also added to this culture, anergy induction is abrogated. CSA's ability to inhibit anergy dominates over the ability of rapamycin to induce anergy. This is presumably because CSA blocks anergy induction proximally by inhibiting the production of negative regulatory factors. The results are consistent with the idea that rapamycin works distally, possibly by inhibiting proliferation and/or the inactivation of the negative regulatory factors.

Hierarchy of anergy induction

Cells that were stimulated during the induction phase with both signal 1 and signal 2 appeared to be less responsive than nonmanipulated resting cells. These cells appeared to be partially anergized. To test this directly, we compared the proliferative response of cells that had been stimulated overnight with signal 1 plus 2, rested, and rechallenged, to cells that were "mock" stimulated, rested, and rechallenged. As seen in Fig. 4*A*, cells that were stimulated with signal 1 plus 2 during the induction phase proliferated less effectively upon rechallenge than cells that were not stimulated at all (mock stimulation). This observation is consistent with the results of Beverly et al., who showed that anergy could be induced in the presence of signal 1 plus 2 if APCs and the medium were removed and the cells washed free of IL-2 (10). In our system, after 16 h of signal 1 plus 2, the stimulating Abs and medium are also removed. It might be that despite the high levels of IL-2 produced during the 16 h with signal 1 plus 2, there is not sufficient time to induce the cells to proliferate or completely inactivate the

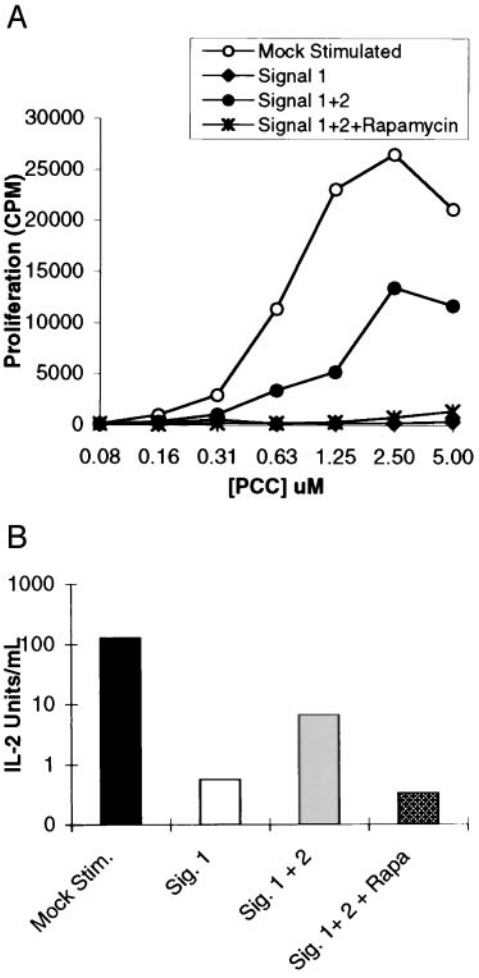

FIGURE 4. *A*, Stimulation with signal 1 plus 2 can result in hyporproliferation upon rechallenge. A.E7 cells were anergized as described in *Materials and Methods*. However, this time a mock culture (A.E7 cells that were cultured without any Abs) was included during the induction phase. These results are representative of three experiments. *B*, Induction of anergy in the presence of rapamycin as well as signal 1 plus 2 alone results in decreased IL-2 production upon rechallenge. The cells were anergized, rested, and then tested for their ability to produce IL-2 upon rechallenge by incubating them in the presence of plate-bound anti-TCR and soluble anti-CD28 for 16 h. Supernatant fluids were harvested and assayed for IL-2 using a CTLL bioassay as described in *Material and Methods*. This pattern of results was obtained in three other experiments (see Fig. 5*B* for example).

negative regulatory factors. In addition, under such culture conditions, the cells are continuously being stimulated by signal 1 and thus presumably continuously replenishing negative factors. By adding rapamycin to this culture, the anergy induced is more complete and rivals that of the cells anergized conventionally with signal 1 alone. This is consistent with the idea that rapamycin completely blocks the ability of IL-2 to prevent anergy during the initial stimulation phase. As is the case for conventional anergy, the partial anergy induced by signal 1 plus 2 and the profound anergy induced by signal 1 plus 2 plus rapamycin is not secondary to down-regulation of the TCR as determined by flow cytometry (data not shown).

A hallmark of anergic cells is their inability to produce IL-2 upon rechallenge (2). Thus, we wanted to confirm that the lack of proliferation upon rechallenge of the rapamycin-induced anergic cells also correlated with a decrease in their ability to produce IL-2. Cells were anergized, rested, and then assayed for their ability to produce IL-2 upon rechallenge with plate-bound anti-TCR and soluble anti-CD28. As seen in Fig. 4*B*, there was a >220-fold decrease in the IL-2 produced by the conventionally anergized cells. Likewise, the cells anergized in the presence of signal 1 plus 2 plus rapamycin demonstrated a >370-fold reduction in IL-2 production. In addition, consistent with their decreased proliferative ability upon rechallenge, the cells that were stimulated with signal 1 plus 2 during the induction phase also showed a decrease in IL-2 production when compared with the nonmanipulated cells (19-fold), though their response was still >20-fold that of the fully anergized cells.

IL-2 rescues cells from rapamycin-induced anergy

The anergic state of T cell clones is reversed by culturing the cells in exogenous IL-2 and allowing them to proliferate. We wanted to determine whether the anergic state induced in the presence of rapamycin was also reversible. Anergy was induced in the presence and absence of costimulation and the presence and absence of rapamycin. The cells were harvested and split into either fresh medium or fresh medium containing 50 U/ml of IL-2. The cells were cultured for 12–14 days and then harvested. As seen in Fig. 5*A*, induction with signal 1 or signal 1 plus 2 plus rapamycin results in hypoproliferation in response to rechallenge with APCs and Ag when compared with the cells that were induced with signal 1 plus 2. However, culturing these cells in IL-2 during the rest period reverses the anergy. In addition, we see that culturing cells in IL-2 that were initially stimulated with signal 1 plus 2 rescues them from their state of partial anergy. As shown in Fig. 5*B*, the clones were tested for their ability to produce IL-2 after being rested in the presence or absence of exogenous IL-2. Note the hierarchy of IL-2 production in the control groups in this experiment: the nonmanipulated cells produced the greatest amount of IL-2, followed by the cells stimulated with signal 1 plus 2 and then the cells stimulated with signal 1 alone or 1 plus 2 plus rapamycin. Thus, 14 days after their exposure to signal 1 plus 2 and rapamycin the clones remain anergic. In contrast, when these cells were stimulated with IL-2, following removal of rapamycin, the anergic state was reversed. In terms of IL-2 production, the reversal was not as complete as that seen for the reversal of proliferation (Fig. 5*A*). In this experiment, there was a 5.4-fold increase in IL-2 production after culture of anergic cells in IL-2, while in three other experiments we have seen between a 5- and 12-fold increase. The failure to completely reverse anergy and achieve maximum IL-2 production upon rechallenge might relate to the deeper state of anergy induced with rapamycin (Fig. 2*B* and Fig. 7 to be discussed below).

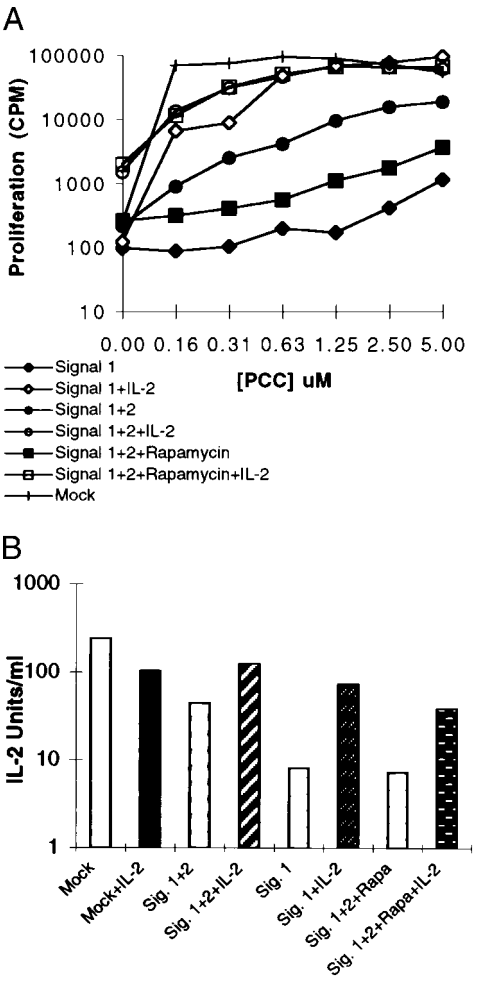

FIGURE 5. Culturing cells in IL-2 rescues them from anergy. *A*, After being anergized, the cells were washed and split into two cultures containing fresh medium with or without 50 U/ml of IL-2 and cultured for 12 days. The cells were washed and tested for their ability to proliferate to Ag. *B*, Cells were anergized and cultured in the presence or absence of IL-2 and then tested for their ability to produce IL-2. Comparable results were obtained in three separate experiments. Note, in both *A* and *B*, "+ IL-2" refers to the presence of IL-2 during the rest culture before rechallenge.

The mitogen-activated protein (MAP) kinase pathway is blocked in rapamycin-induced anergy

Anergic cells have a defect in their 12–0-tetradeconoylphorbol-13-acetate responsive element (TRE)-mediated transcription upon rechallenge (25, 26). Recently, it was shown that there is a block in the MAP kinase pathway in anergic cells (27, 28), which could be responsible for the decrease in TRE-mediated transcription. To determine whether the induction of anergy in the presence of costimulation and rapamycin resulted in a block in the MAP kinase pathway, Western blot analysis was performed on extracts derived from cells anergized under various conditions. As shown in Fig. 6, mock-anergized, conventionally anergized, partially anergized (signal 1 plus 2), and rapamycin-anergized cells were either left unstimulated or stimulated with plate-bound anti-TCR for 10 min. For all conditions, there was relatively little phosphorylated ERK found in the extracts from the unstimulated cells. The extracts from the mock stimulated cells showed a marked increase in phosphorylated ERK following TCR signaling. In comparison, there was less phoshorylated ERK in the extracts derived from the conventionally anergized, the rapamycin-anergized, as well as the partially anergized cells. In this particular experiment, the relative

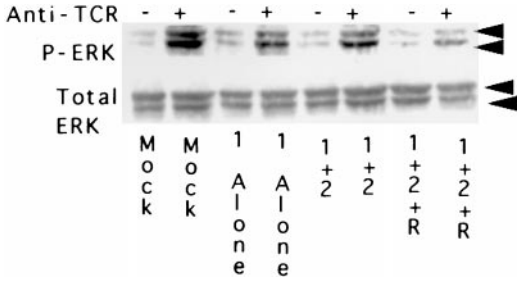

FIGURE 6. Rapamycin-induced anergy results in a block in the MAP kinase pathway. Extracts were derived from cells anergized under various conditions and then either left unstimulated or stimulated with plate-bound anti-TCR for 10 min. The *upper panel* was blotted with anti-phospho-ERK, while the *bottom panel* is the same blot stripped and reblotted with anti-ERK. The upper and lower arrows indicate ERK 1 and 2, respectively. Extracts from nonanergic A.E7 cells are denoted by "Mock," conventionally anergized cells are denoted as "1 alone," partially anergized cells are denoted as "1 + 2," and rapamycin-anergized cells are denoted as "1 + 2 + R."

decrease in ERK phosphorylation follows the hierarchy of anergy induction (rapamycin-anergized < conventionally anergized < signal 1 plus 2). However, in other experiments we have seen a less profound decrease in phosphorylated ERK in the rapamycin-anergized extracts, even though such cells were profoundly anergic. The range of inhibition relative to the phosphorylated ERK induced in the mock cells was ~60–95%. Thus, our data show that cells anergized in the presence of costimulation and rapamycin display a similar biochemical block in the MAP kinase pathway to that seen in cells anergized by signal 1 alone.

Rapamycin-induced anergy results in a more profound block in IL-3 and IFN-γ production

Despite the profound block in IL-2 production by anergic cells, they still produce other cytokines upon rechallenge, albeit in decreased amounts. Typically, the anergic clones in our laboratory display a 50% decrease in IFN-γ production and a 10-fold decrease in IL-3 production under optimal activation conditions (10). To examine the production of these two cytokines by the rapamycin-induced anergic cells, supernatant fluids from 16-h stimulated cells were assayed by ELISA for IFN-γ and IL-3 levels. These are the same supernatant fluids from Fig. 5*B* that demonstrated 240 U/ml IL-2 for the stimulated cells vs 8 U/ml and 7 U/ml for the signal 1 and rapamycin-induced anergic cells, respectively. As can be seen in Fig. 7*A*, there is a ~50% decrease in IFN production as measured in the signal 1-induced anergic supernatants, when compared with the nonanergic supernatant fluid. On the other hand, there is a >10-fold reduction in IFN production in the supernatant fluid derived from the rapamycin-induced anergic cells. Likewise, as seen in Fig. 7*B*, there is a typical sixfold reduction in IL-3 production between the supernatant fluids derived from the conventionally anergized cells and the nonanergic cells, while the supernatant fluids derived from the rapamycin-induced anergic cells showed nearly a 25-fold difference. Thus, it appears as if the cells anergized in the presence of costimulation and rapamycin have a more profound block in the production of these other cytokines than the decrease seen in cells that are anergized by stimulation with signal 1 alone. This observation was also made for cells treated with signal 1 and rapamycin in the absence of costimulation, suggesting that costimulation is not necessary for the deeper state of rapamycin-induced anergy (data not shown). Furthermore, as is the case for signal 1-induced anergy, the hierarchy of inhibition (IL-2 > IL-3 > IFN-γ) is also maintained in the rapamycin-induced anergy. Interestingly, this hierarchy of inhibition is also observed for the signal 1 plus 2-induced partial anergy. As seen in Fig. 7, *A* and *B*, there is essentially no inhibition of IFN-γ in the supernatant fluid from the cells incubated initially with signal 1 plus 2, while there is a 2.5-fold decrease in the production of IL-3 and a 5-fold decrease in the production of IL-2 (Fig. 5*B*).

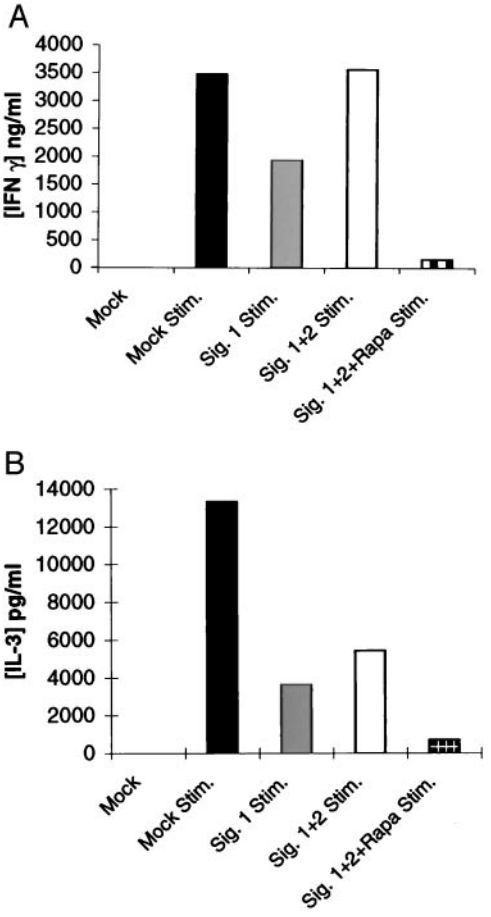

FIGURE 7. Rapamycin-induced anergy results in decreased IFN-γ and IL-3 production. Cells were anergized, rested in fresh medium, and stimulated with plate-bound anti-TCR and anti-CD28 ascites. Supernatant fluids were assayed by ELISA for IFN-γ (*A*) and IL-3 production (*B*). Cytokine production was <2.5 pg/ml of IL-3 and undetectable for IFN-γ for all unstimulated cultures (only unstimulated "Mock" culture is shown). Comparable results were observed in three experiments.

Cell cycle progression from G1 to S phase prevents the induction of anergy

In as much as we have been able to demonstrate the induction of T cell clonal anergy for cells cultured in the presence of signal 1 plus 2 and rapamycin, the data thus far support the Jenkins and Beverly hypothesis that TCR engagement in the absence of proliferation leads to anergy induction. Because rapamycin blocks IL-2-induced proliferation at the level of mTOR, it was possible that it was not proliferation and subsequent dilution of negative regulatory factors that prevented anergy induction, but rather the biochemical events that are the result of progression through the cell cycle. If indeed such were the case, then we would predict that by incubating the clones with signal 1 plus 2 and hydroxyurea, which blocks proliferation in early S phase, anergy would not be induced. Fig. 8*A* demonstrates that increasing concentrations of hydroxyurea inhibit IL-2-induced T cell proliferation as potently as rapamycin (see Fig. 1*A*). In addition, the clones still produce IL-2 in the

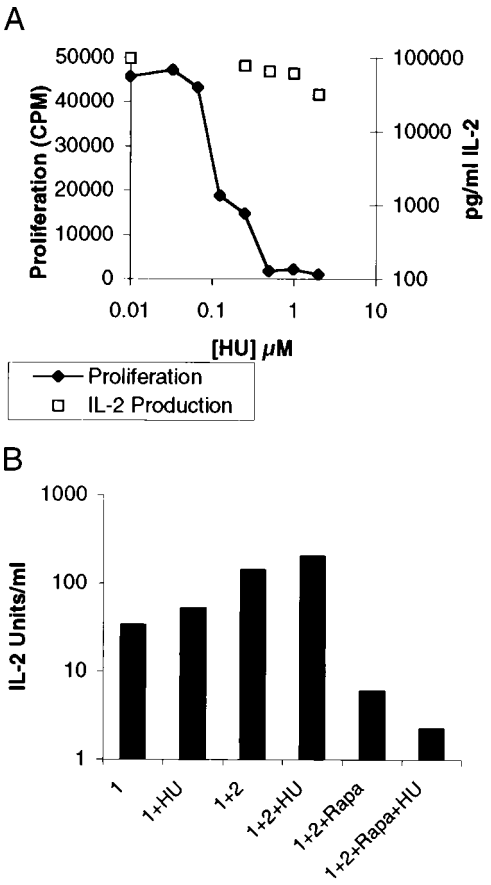

FIGURE 8. *A*, Hydroxyurea inhibits IL-2-mediated T cell proliferation. A.E7 T cells were incubated with 50 U/ml IL-2 and increasing concentrations of hydroxyurea in a 96-well plate. The cultures were pulsed with [^{3}H]thymidine after 48 h and harvested at 64 h. In a separate culture, A.E7 cells were stimulated with plate-bound anti-TCR and anti-CD28 in the presence of increasing concentrations of hydroxyurea. After 16 h, supernatant fluid was harvested and IL-2 production was determined using ELISA. *B*, The effect of hydroxyurea on anergy induction. A.E7 T cells were stimulated overnight with either anti-TCR alone or anti-TCR plus anti-CD28 in the presence or absence of hydroxyurea, rapamycin, or both. The cells were washed and rested 7 days and then rechallenged with anti-TCR and anti-CD28 for 16 h. Supernatant fluid was collected and assayed for IL-2 production using the CTLL bioassay.

presence of hydroxyurea, although there is a slight attentuation of IL-2 production at the highest concentration of the drug (Fig. 8*A*). Cell cycle analysis by propidium iodide staining confirmed that for the A.E7 cells rapamycin blocks IL-2-induced proliferation in late G1, while hydroxyurea blocks cell cycle progression in S phase (data not shown). As seen in Fig. 8*B*, like rapamycin, hydroxyurea does not inhibit anergy when the clones are induced with anti-TCR alone. However, in contrast to rapamycin, when the clones are stimulated with signal 1 plus 2 in the presence of hydroxyurea they do not become anergic. These findings are consistent with the data of Gilbert and Weigle, who also found that hydroxyurea did not cause anergy in negative controls for their butyrate-induced anergy model (13). Thus, inhibition of proliferation in early S phase does not result in anergy if the cells are stimulated through the TCR in the presence of costimulation. Furthermore, in sharp contrast to CSA (Fig. 3), hydroxyurea does not inhibit rapamycin-induced anergy (Fig. 8*B*). This is consistent with the idea that rapamycin acts proximally to hydroxyurea and that the mechanism responsible for preventing anergy lies between G1 and S phase. Overall, these data are consistent with a model in which CSA blocks the up-regulation of "anergic factors" and rapamycin blocks their degradation/inactivation, while hydroxyurea, despite its ability to block proliferation, neither blocks the up-regulation nor down-regulation of the factors that promote anergy.

Discussion

T cell clonal anergy results from TCR engagement in the absence of costimulation. Despite this observation, the precise role that costimulation plays in preventing anergy has yet to be defined. Some propose that CD28 signaling directly serves to prevent anergy (14, 15), while others argue that costimulation prevents anergy inasmuch as it facilitates the production of IL-2 and subsequent T cell proliferation (10, 11). Taking advantage of the ability of rapamycin to block IL-2-induced proliferation without inhibiting costimulation-mediated IL-2 production, we sought to discriminate between these two models. Our data are consistent with the idea that signaling through CD28 only prevents anergy under normal conditions by enhancing IL-2 production. On the other hand, we have also presented evidence that it is not proliferation in and of itself that inhibits anergy, but rather the biochemical events that occur as a result of progression through the cell cycle from G1 into S. Thus, although proliferation and dilution may contribute to the prevention of anergy, we propose that anergy induction can be prevented by the consequences of IL-2R engagement distal to the serine/threonine kinase activity of mTOR.

Clearly, we have not formally ruled out the possibility that rapamycin prevents the signaling of an as-yet unidentified CD28 "anti-anergy" or signal 2t pathway (14). In fact, it has been shown that rapamycin can inhibit CD28-mediated down-regulation of IκB and up-regulation of CTLA4 (29, 30). However, at the very least, as shown in Fig. 1*B*, in the presence of rapamycin, the CD28-mediated signaling pathway responsible for the up-regulation of IL-2 production appears to be intact. Furthermore, our findings are consistent with the observations of Gilbert and Weigle, who used the histone deacetylase inhibitor *n*-butyrate to induce anergy (13). They proposed that anergy is the result of TCR stimulation and G1a sequestration. However, unlike rapamycin, histone deacetylase inhibitors also inhibit IL-2 production (13, 31), and, thus, the ability of *n*-butyrate to induce anergy could also be consistent with the model of Jenkins and Beverly (10, 11). Finally, in terms of whether or not regulation of the transcription factor Nil-2a contributes to the antagonism of the anergic state, using electrophoretic mobility shift assays we could not demonstrate the down-regulation of Nil-2a binding to the NRE in A.E7 cells stimulated with anti-TCR and anti-CD28 Abs (data not shown). Thus, at this time, the role, if any, that Nil-2a plays in anergy induction in the A.E7 clone is not clear.

The anergic state induced in the presence of rapamycin shares many similarities with conventionally anergized cells. They both display a block in the MAP kinase pathway as determined by decreased ERK phosphorylation (27, 28) and they both were reversed by adding exogenous IL-2. On the other hand, unlike conventionally anergized cells, the rapamycin-induced anergic cells appeared to have a more profound block in the production of the cytokines IL-3 and IFN-γ. It is not clear whether this is due to more complete anergy, as a result of the ability of rapamycin to fully prevent the G1 to S phase transition, or the ability of rapamycin to affect an additional pathway. It is also of note that both IL-3 and IFN-γ contain AP-1 sites in their promoters (32, 33). Because it has been shown that there is a decrease in TRE-mediated transcription in anergic cells, it might be that the differences seen in the production of these cytokines in anergy is related to the relative contribution

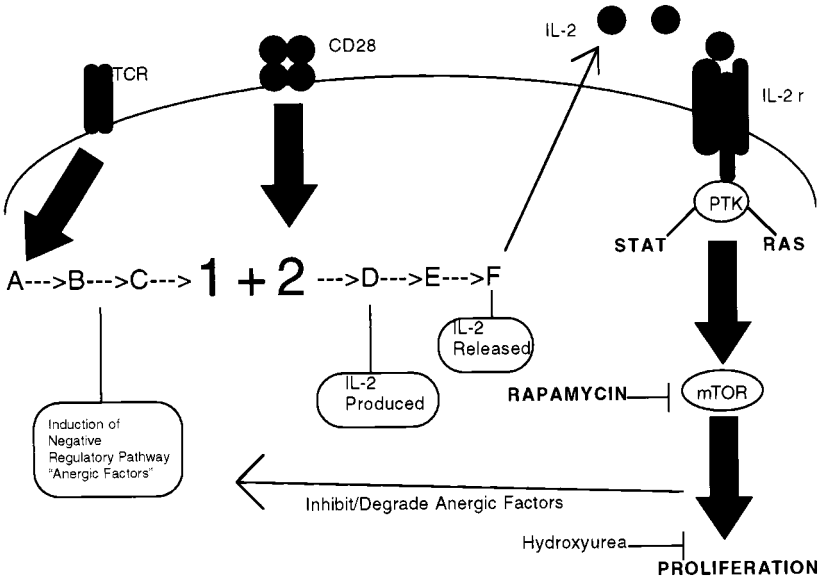

FIGURE 9. Model of anergy induction. This model adapts the kinetic proofreading model of T cell activation (35, 36) to incorporate a number of observations concerning anergy induction in $CD4^+$ T cell clones. See *Discussion* for details.

of these sites to TCR-induced activation. The consistency of the hierarchy of inhibition between IL-2, IL-3, and IFN-γ is striking and is observed under conditions of partial anergy (induced by 16 h of signal 1 plus 2 and removal of IL-2), conventional anergy (signal 1 alone), and rapamycin-induced anergy. Interestingly, this same pattern is observed when examining the effect of costimulation on cytokine production (Ref. 34 and our unpublished observations). That is, the production of IL-2 by A.E7 is costimulation dependent, while IL-3 and, to a greater degree, IFN-γ are produced in response to signal 1 alone (34). This hierarchy may ultimately provide insight into the precise molecular mechanisms of cytokine induction and inhibition in Th1 cells.

Fig. 9 depicts a general model for anergy induction. We have adapted concepts from the kinetic proofreading model of TCR signal transduction to incorporate a number of observations concerning the anergy induction process (35, 36). TCR engagement leads to a series of reactions (A $\rightarrow$ B $\rightarrow$ C) that ultimately lead to full signal 1-induced changes. At reaction B, the negative regulatory pathway leading to anergy is initiated. It has been shown that certain variant peptides with lower affinities for a particular TCR can induce anergy even in the presence of costimulation, a so-called partial agonist anergy (2, 37, 38). A partial agonist may have an affinity for the TCR that only permits signaling up to B, never leading to full signal 1. Thus, in this case, the negative regulatory factors are produced but there is insufficient signaling to produce IL-2 and the subsequent G1 to S phase transition. This is consistent with the data of Madrenas et al., who showed that anergy induction for partial agonists could be overcome by the addition of exogenous IL-2 (39). In the case of conventional anergy, TCR engagement results in both the initiation of the negative regulatory pathway as well as full signal 1. However, in the absence of costimulation, little or no IL-2 is produced, and, as a result, there is no entry into S phase. In rapamycin-induced anergy, there is production of the negative regulatory factors as well as full signal 1 and signal 2. This leads to IL-2 production, release, and IL-2R engagement. However, the cell is blocked from proceeding through the cell cycle by rapamycin. As a result, there remains a build up of negative regulatory factors, and the cell is hyporesponsive upon rechallenge. In this model, hydroxyurea blocks proliferation distal to the events responsible for the abrogation of anergy, and, as a result, even though cells incubated in the presence of signal 1 plus 2 and hydroxyurea do not proliferate, signaling through the IL-2R leads to the prevention of anergy. Finally, signal 1 plus 2 followed by the removal of IL-2 results in a small anergic effect. We postulate that this is due to the fact that the cells are continuously being stimulated through the TCR and thus the IL-2 produced during the 16-h culture period is unable to accomplish the complete dissipation of the negative regulatory factors.

Several groups have shown that anergic T cells display a block in the MAP kinase pathway upon rechallenge (27, 28, 40). Our current studies have demonstrated a similar block in cells that have been anergized in the presence of costimulation and rapamycin. However, in previous studies using IL-2 promoter-driven reporter constructs, our laboratory has shown that the block in the Ras pathway alone cannot fully account for all of the inhibition of IL-2 production seen in anergy (23). Rather, this inhibition appears to be due in part to *cis*-dominant repression mediated at the level of the IL-2 promoter. The target of this repression appears to be centered around the -180 and -150 regions of the enhancer/promoter (23) and may be mediated by cAMP-response-element-binding protein (CREB) family member proteins (41) and (our unpublished observations). We believe that persistent TCR engagement leads to the induction/activation of such negative transcriptional regulators. Upon rechallenge of anergic cells (in the setting of decreased induction of positive transcription factors such as Jun and Fos), these unopposed negative factors would serve to shut down IL-2 transcription. Recently, Bodor and Habener have suggested that the decrease in IFN-γ and IL-2 production secondary to increases in cAMP is the result of the up-regulation of the negative transcription factor inducible cAMP early repressor (ICER) (42). ICER is an isoform of cAMP-response-element modulator (CREM) that has the ability to bind to DNA, but lacks a transactivating domain (43). It remains to be seen if this protein contributes to the decrease in IL-2 and IFN-γ production seen in anergy.

Because rapamycin does not appear to inhibit IL-2-induced $p21^{ras}$ activation or the induction of c-*myc* mRNA, presumably these biochemical consequences of IL-2R engagement do not play

a role in inhibiting anergy (44). A role for signaling via the common γ-chain of the IL-2R is supported by findings in human T lymphocytes that activating Abs against this chain can antagonize anergy induction (12). The prevention of anergy induction by IL-2 must involve mTOR, the serine/threonine kinase blocked by rapamycin (17, 44). At this time, it is unclear if mTOR is directly or indirectly involved in the degradation or inactivation of negative factors up-regulated by TCR engagement. In this regard, mTOR is an obligate participant in the activation of $p70^{s6k}$, which is proposed to be essential for G1 cyclin-cdk activation and progression of the cell from G1 into S phase (44). Although the precise mechanism whereby $p70^{s6k}$ promotes this transition is not known, it is believed that progression through the cell cycle is mediated in part by the kinase's ability to enhance translational initiation (44). This is mediated in part by its ability to phosphorylate and inactivate translational repressors. Likewise, $p70^{s6k}$ might serve the same function in terms of inactivating the negative regulatory factors that maintain anergy. In addition, $p70^{s6k}$ has been shown to phosphorylate and increase the transcriptional activity of CREMτ (45). It remains to be seen if this kinase has additional effects on other CREB family members, for example those that might be involved in the *cis*-dominant repression of IL-2 transcription.

TCR engagement results in the transition of the cells into the late G1 phase of the cell cycle (17). Further progression is blocked by the accumulation of the inhibitor Kip-1 (44). Signaling through the IL-2R results in the degradation of Kip-1 through the ubiquitin-proteasome pathway and the subsequent assembly of G1 cyclin-cdk complexes (46). This in turn leads to progression into S phase and cellular proliferation. The presence of rapamycin inhibits IL-2-mediated degradation of Kip-1. Similarly, we propose that rapamycin promotes anergy by blocking cell cycle progression and promoting the build up of negative regulatory factors. As is the case for the inhibitor Kip-1, it might be that progression through the cyclin-dependent kinase pathway leads to the degradation/inactivation of the negative regulatory factors that mediate anergy. In this regard, it has been shown that the transcriptional repressor ICER is degraded by the ubiquitin-proteasome pathway (47). If such is the case, then Kip-1 itself may be central to the maintenance of the anergic state by its ability to inhibit the cyclin-dependent kinase cascade. Thus, anergy might be viewed as part of the mechanism of check point cell cycle growth arrest adopted by the lymphocyte for an immunological purpose. Current studies are focused on determining the level at which anergy is prevented in the cyclin-dependent kinase cascade between G1 (rapamycin) and S phase (hydroxyurea).

Finally, the data described herein have potential clinical implications. As seen in Fig. 2*A* and as has been demonstrated previously, CSA inhibits anergy induction (5, 6). Indeed, in a mouse allograft model, it has been shown that long-term graft acceptance in the absence of long-term immunosuppression can be induced by the infusion of CTLA4 Ig and anti-CD40 ligand Abs in the peritransplant period (48). However, if CSA is added to this regimen, the grafts are rejected. These observations suggest the potential importance of signal 1 in the induction of graft tolerance in this model. By contrast, in a swine transplant model, it has been shown that peritransplant transfusion of donor-derived, dendritic cell-depleted PBMC under the cover of rapamycin results in long-term graft survival without long-term pharmacologic immunosuppression (49). This finding is consistent with our in vitro data that T cell activation in the presence of rapamycin can lead to tolerance. Ultimately, it might be possible to design immunosuppressive regimens that do not inhibit TCR-mediated signaling and thus facilitate graft tolerance in the absence of long-term immunosuppression.

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References

1. Schwartz, R. H. 1992. Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell 71: 1065.*
2. Schwartz, R. H. 1996. Models of T cell anergy: is there a common molecular mechanism? *J. Exp. Med. 184:1.*
3. Schwartz, R. H. 1997. T cell clonal anergy. *Curr. Opin. Immunol. 9:351.*
4. Jenkins, M. K., and R. H. Schwartz. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J. Exp. Med. 165:302.*
5. Quill, H., and R. H. Schwartz. 1987. Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. *J. Immunol. 138:3704.*
6. Jenkins, M. K., C. A. Chen, G. Jung, D. L. Mueller, and R. H. Schwartz. 1990. Inhibition of antigen-specific proliferation of type 1 murine T cell clones after stimulation with immobilized anti-CD3 monoclonal antibody. *J. Immunol. 144: 16.*
7. Mueller, D. L., M. K. Jenkins, and R. H. Schwartz. 1989. An accessory cell-derived costimulatory signal acts independently of protein kinase C activation to allow T cell proliferation and prevent the induction of unresponsiveness. *J. Immunol. 142:2617.*
8. Jenkins, M. K., J. D. Ashwell, and R. H. Schwartz. 1988. Allogeneic non-T spleen cells restore the responsiveness of normal T cell clones stimulated with antigen and chemically modified antigen-presenting cells. *J. Immunol. 140:3324.*
9. DeSilva, D. R., K. B. Urdahl, and M. K. Jenkins. 1991. Clonal anergy is induced in vitro by T cell receptor occupancy in the absence of proliferation. *J Immunol 147:3261.*
10. Beverly, B., S. M. Kang, M. J. Lenardo, and R. H. Schwartz. 1992. Reversal of in vitro T cell clonal anergy by IL-2 stimulation. *Int. Immunol. 4:661.*
11. Jenkins, M. K. 1992. The role of cell division in the induction of clonal anergy. *Immunol. Today 13:69.*
12. Boussiotis, V. A., D. L. Barber, T. Nakarai, G. J. Freeman, J. G. Gribben, G. M. Bernstein, A. D. D'Andrea, J. Ritz, and L. M. Nadler. 1994. Prevention of T cell anergy by signaling through the γc chain of the IL-2 receptor. *Science 266:1039.*
13. Gilbert, K. M., and W. O. Weigle. 1993. Th1 cell anergy and blockade in G1a phase of the cell cycle. *J. Immunol. 151:1245.*
14. Bachmann, M. F., K. McKall-Faienza, R. Schmits, D. Bouchard, J. Beach, D. E. Speiser, T. W. Mak, and P. S. Ohashi. 1997. Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation. *Immunity 7:549.*
15. Becker, J. C., T. Brabletz, T. Kirchner, C. T. Conrad, E. B. Brocker, and R. A. Reisfeld. 1995. Negative transcriptional regulation in anergic T cells. *Proc. Natl. Acad. Sci. USA 92:2375.*
16. Williams, T. M., D. Moolten, J. Burlein, J. Romano, R. Bhaerman, A. Godillot, M. Mellon, F. J. d. Rauscher, and J. A. Kant. 1991. Identification of a zinc finger protein that inhibits IL-2 gene expression. *Science 254:1791.*
17. Abraham, R. T., and G. J. Wiederrecht. 1996. Immunopharmacology of rapamycin. *Annu. Rev. Immunol. 14:483.*
18. Dumont, F. J., M. J. Staruch, S. L. Koprak, M. R. Melino, and N. H. Sigal. 1990. Distinct mechanisms of suppression of murine T cell activation by the related macrolides FK-506 and rapamycin. *J. Immunol. 144:251.*
19. Beverly, B., K. Brorson, S. Umlauf, and R. H. Schwartz. 1993. Technology to study gene transcription in normal T lymphocyte clones. In *Methods in Molecular Genetics*. K. Adolph, ed. Academic Press, Orlando, FL, p. 331.
20. Kubo, R. T., W. Born, J. W. Kappler, P. Marrack, and M. Pigeon. 1989. Characterization of a monoclonal antibody which detects all murine αβ T cell receptors. *J. Immunol. 142:2736.*
21. Gross, J. A., T. St. John, and J. P. Allison. 1990. The murine homologue of the T lymphocyte antigen CD28: molecular cloning and cell surface expression. *J. Immunol. 144:3201.*
22. Ashwell, J. D., R. E. Cunningham, P. D. Noguchi, and D. Hernandez. 1987. Cell growth cycle block of T cell hybridomas upon activation with antigen. *J. Exp. Med. 165:173.*
23. Kitagawa-Sakakida, S., and R. H. Schwartz. 1996. Multifactor *cis*-dominant negative regulation of IL-2 gene expression in anergized T cells. *J. Immunol. 157: 2328.*
24. Wei, S., A. M. Gamero, J. H. Liu, A. A. Daulton, N. I. Valkov, J. A. Trapani, A. C. Larner, M. J. Weber, and J. Y. Djeu. 1998. Control of lytic function by mitogen-activated protein kinase/extracellular regulatory kinase 2 (ERK2) in a

human natural killer cell line: identification of perforin and granzyme B mobilization by functional ERK2. *J. Exp. Med. 187:1753.*
25. Kang, S. M., B. Beverly, A. C. Tran, K. Brorson, R. H. Schwartz, and M. J. Lenardo. 1992. Transactivation by AP-1 is a molecular target of T cell clonal anergy. *Science 257:1134.*
26. Mondino, A., C. D. Whaley, D. R. DeSilva, W. Li, M. K. Jenkins, and D. L. Mueller. 1996. Defective transcription of the IL-2 gene is associated with impaired expression of c-Fos, FosB, and JunB in anergic T helper 1 cells. *J. Immunol. 157:2048.*
27. Fields, P. E., T. F. Gajewski, and F. W. Fitch. 1996. Blocked Ras activation in anergic $CD4^+$ T cells. *Science 271:1276.*
28. Li, W., C. D. Whaley, A. Mondino, and D. L. Mueller. 1996. Blocked signal transduction to the ERK and JNK protein kinases in anergic $CD4^+$ T cells. *Science 271:1272.*
29. Lai, J. H., and T. H. Tan. 1994. CD28 signaling causes a sustained down-regulation of IκBα which can be prevented by the immunosuppressant rapamycin. *J. Biol. Chem. 269:30077.*
30. Finn, P. W., H. He, Y. Wang, Z. Wang, G. Guan, J. Listman, and D. L. Perkins. 1997. Synergistic induction of CTLA-4 expression by costimulation with TCR plus CD28 signals mediated by increased transcription and messenger ribonucleic acid stability. *J. Immunol. 158:4074.*
31. Takahashi, I., H. Miyaji, T. Yoshida, S. Sato, and T. Mizukami. 1996. Selective inhibition of IL-2 gene expression by trichostatin A, a potent inhibitor of mammalian histone deacetylase. *J. Antibiot. 49:453.*
32. Nimer, S. D., and H. Uchida. 1995. Regulation of granulocyte-macrophage colony-stimulating factor and interleukin 3 expression. *Stem Cells 13:324.*
33. Young, H. A. 1996. Regulation of interferon-γ gene expression. *J. Interferon Cytokine Res. 16:563.*
34. Jenkins, M. K., D. M. Pardoll, J. Mizuguchi, T. M. Chused, and R. H. Schwartz. 1987. Molecular events in the induction of a nonresponsive state in interleukin 2-producing helper T-lymphocyte clones. *Proc. Natl. Acad. Sci. USA 84:5409.*
35. McKeithan, T. W. 1995. Kinetic proofreading in T-cell receptor signal transduction. *Proc. Natl. Acad. Sci. USA 92:5042.*
36. Rabinowitz, J. D., C. Beeson, D. S. Lyons, M. M. Davis, and H. M. McConnell. 1996. Kinetic discrimination in T-cell activation. *Proc. Natl. Acad. Sci. USA 93:1401.*
37. Sloan-Lancaster, J., B. D. Evavold, and P. M. Allen. 1993. Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells. *Nature 363:156.*
38. Ryan, K. R., and B. D. Evavold. 1998. Persistence of peptide-induced $CD4^+$ T cell anergy in vitro. *J. Exp. Med. 187:89.*
39. Madrenas, J., R. H. Schwartz, and R. N. Germain. 1996. Interleukin 2 production, not the pattern of early T-cell antigen receptor-dependent tyrosine phosphorylation, controls anergy induction by both agonists and partial agonists. *Proc. Natl. Acad. Sci. USA 93:9736.*
40. Boussiotis, V. A., G. J. Freeman, A. Berezovskaya, D. L. Barber, and L. M. Nadler. 1997. Maintenance of human T cell anergy: blocking of IL-2 gene transcription by activated Rap1. *Science 278:124.*
41. Butscher, W. G., C. Powers, M. Olive, C. Vinson, and K. Gardner. 1998. Coordinate transactivation of the interleukin-2 CD28 response element by c-Rel and ATF-1/CREB2. *J. Biol. Chem. 273:552.*
42. Bodor, J., and J. F. Habener. 1998. Role of transcriptional repressor ICER in cyclic AMP-mediated attenuation of cytokine gene expression in human thymocytes. *J. Biol. Chem. 273:9544.*
43. Molina, C. A., N. S. Foulkes, E. Lalli, and P. Sassone-Corsi. 1993. Inducibility and negative autoregulation of CREM: an alternative promoter directs the expression of ICER, an early response repressor. *Cell 75:875.*
44. Karnitz, L. M., and R. T. Abraham. 1996. Interleukin-2 receptor signaling mechanisms. *Adv. Immunol. 61:147.*
45. de Groot, R. P., L. M. Ballou, and P. Sassone-Corsi. 1995. Positive regulation of the nuclear activator CREM by the mitogen-induced p70 S6 kinase. *Immunobiology 193:155.*
46. Pagano, M., S. W. Tam, A. M. Theodoras, P. Beer-Romero, G. Del Sal, V. Chau, P. R. Yew, G. F. Draetta, and M. Rolfe. 1995. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science 269:682.*
47. Folco, E. J., and G. Koren. 1997. Degradation of the inducible cAMP early repressor (ICER) by the ubiquitin-proteasome pathway. *Biochem. J. 328:37.*
48. Larsen, C. P., E. T. Elwood, D. Z. Alexander, S. C. Ritchie, R. Hendrix, C. Tucker-Burden, H. R. Cho, A. Aruffo, D. Hollenbaugh, P. S. Linsley, K. J. Winn, and T. C. Pearson. 1996. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature 381:434.*
49. Granger, D., A. Matas, M. Jenkins, A. Moss, S. Chen, and P. Almond. 1994. Prolonged survival without posttransplant immunosuppression in a large animal model. *Surgery 116:236.*