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Sustained Activation of the Raf-MEK-ERK Pathway Elicits Cytokine Unresponsiveness in T Cells

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Activation of T cells via the TCR and other costimulatory receptors triggers a number of signaling cascades. Among them, the Ras-activated Raf-mitogen-activated protein/extracellular signal-related kinase (ERK) kinase (MEK)-ERK signaling cascade has been demonstrated to be crucial for both T cell development and activation. It has previously been demonstrated that high doses of Ag or anti-CD3 mAb are able to induce in T cells a nonresponsive state to subsequent treatment with cytokines such as IL-2. The precise biochemical mechanisms underlying this effect are not fully characterized. In this study, we demonstrate that cytokine nonresponsiveness is accompanied by the induction of the cyclin-dependent kinase inhibitor p21Cip1 that is mediated, at least in part, by the activation of the Raf-MEK-ERK pathway. Furthermore, we demonstrate that selective activation of the Raf-MEK-ERK signaling pathway in T cells is sufficient to induce cytokine nonresponsiveness in both a T cell clone and naive primary T cells. In this case, nonresponsiveness is accompanied by the induction of p21Cip1 and the prevention of p27Kip1 down-regulation, leading to inhibition of cyclin E/cyclin-dependent kinase 2 activity. These data suggest that anti-CD3 mAb-induced cytokine nonresponsiveness may be a consequence of hyperactivation of the Raf-MEK-ERK pathway, leading to alterations in the expression of key cell cycle regulators. These observations may provide a novel insight into the mechanisms of induction of peripheral tolerance.

cytokines and growth factors, which is accompanied by cell cycle arrest, in a number of primary, established, and tumor cell types (38–44). In mouse NIH 3T3 and in human IMR-90 fibroblasts, the level of MAP kinase activation determines whether cells will proliferate or be arrested in the G1 phase of the cell cycle. In general, low levels of MAP kinase activation promote cell proliferation, whereas higher levels promote cell cycle arrest that appears to be mediated by the induced expression of CDK inhibitors (CKI) such as p21Cip1 and p16INK4a (38, 39). We therefore decided to test whether nonresponsiveness in T cells involves activation of the Raf-MEK-ERK pathway and whether this could be mimicked by selective activation of this pathway in T cells.

In this work, we demonstrate that the cytokine nonresponsive
ness induced in T cells in response to high doses of anti-CD3 mAb is inhibited, in part, by a specific and selective inhibitor of MEK, PD98059. This observation is further supported by the fact that conditional activation of the ERK pathway induced a cytokine unresponsive state that was similar to that induced by treatment with high dose anti-CD3 mAb. Biochemical analyses revealed that the nonresponsive state correlates with the robust induction of the CKI p21Cip1 and inhibition of cyclin E/CDK2 activity. Moreover, anti-CD3 mAb-induced expression of p21Cip1 was MEK1 dependent. These data suggest that engagement of the TCR can elicit an inhibitory signal that is most likely transmitted through the Raf-MEK-ERK pathway. Such mechanisms may participate in the generation of negative signals that lead to the induction of peripheral tolerance.

Materials and Methods

Cell culture

D10G4.1 cells were cultured in the presence of IL-2 (10 ng/ml) and stimulated with conalbumin and irradiated splenocytes from CBA/J mice (The Jackson Laboratory, Bar Harbor, ME) every 2 wk (45). Primary naive T cells were isolated from DO11.10 transgenic mice that express TCR specific for the chicken OVA323–339 peptide in the context of I-A^d (46). Isolation and maintenance of the naive T cells were performed as described elsewhere (47). Briefly, splenocytes were enriched for CD4^+ cells by negative selection using magnetic bead cell sorting with a mixture of Abs to B220, Mac1, and CD8 (all Abs from PharMingen (San Diego, CA) and BioMag beads from PerSeptive Biosystems, Cambridge, MA). CD4^+ Mel14^+ naive T cells were further purified by fluorescence-activated cell sorting using a FACStarplus flow cytometer (Becton Dickenson, Palo Alto, CA). Sorted naive T cells were then cultured in RPMI with 10% FCS, 50 U/ml IL-2, 2 mM L-glutamine (Life Technologies, Gaithersburg, MD), 1 mM sodium pyruvate (JRH Biosciences, Lenexa, KS), 10 mM HEPES (Life Technologies), 100 U/ml penicillin, 100 mg/ml streptomycin (Life Technologies), and 10 ng/ml IL-2. Cells were stimulated with irradiated BALB/c splenocytes and 0.6 mg of OVA323–339 peptide (Biosynthesis, Lewisville, TX) every 7 to 10 days. The retroviral packaging cell line, Phoenix E, from Dr. Gary Nolan (Stanford University, Stanford, CA), was maintained in DMEM with 10% FCS and supplements. For T cell activation analysis, 96-well flat-bottom plates (Falcon) or 150-mm tissue culture dishes (Falcon) were coated with different concentrations of anti-CD3 mAb (145-2C11; PharMingen) overnight at 4°C and were washed three times with PBS before adding cells. The specific and selective MEK inhibitor PD98059 was a generous gift from Dr. John Lyons (Onyx Pharmaceuticals, Richmond, CA). PMA (Upstate Biotechnol-

logies, Lake Placid, NY) and ionomycin (Calbiochem, La Jolla, CA) were used at final concentrations of 20 ng/ml and 1 mM, respectively. Cells were treated with different concentrations of either 4-hydroxytamoxifen (4-HT) or ICI 182,780 (ICI), which activate the

retroviral constructs and retroviral infection

The retroviral vector used in the experiments was a modified version of a pMX-based vector (48). The internal ribosomal entry site from encephalomyocarditis virus and the sequences encoding the enhanced green fluorescent protein (eGFP, kindly provided by Dr. Hergen Spits, the Netherlands Cancer Institute) (49) were introduced into the vector (generated and provided by Alice Mui, DNAX Research Institute, Palo Alto, CA). A ki-

nase-active form of the ΔRaf-1:ER (39, 50, 51) and a similarly constructed kinase-inactive version of the protein, ΔRaf301:ER (39), were introduced into the vector by standard subcloning techniques (Fig. 2A). The human ER domain encoding in the original Raf:ER constructs was replaced by a mutated form of the mouse ER domain (ER*) that is sensitive to 4-HT and the ICI series of compounds, but is insensitive to 17-β-estradiol and Phenol Red in the cell culture medium (52). The ΔRaf-1:ER* and ΔRaf301:ER*-encoding retrovirus constructs were used for infection of T cell clones and primary T cells. Phoenix E cells were transfected by the lipofectamine-based method, and viral supernatants were harvested as described elsewhere (53). Retroviral infection of primary T cells was performed as described by Ferber et al. (54). Briefly, 2 days after OVA323–339/APC stimulation, primary T cells were infected with viral supernatants in the presence of 0.5 mg/ml polybrene plus IL-2. eGFP-positive cells were isolated by fluorescence-activated cell sorting 4 days after infection. Alternatively, transfected virus producing Phoenix E cells were irradiated and cocultured with D10G4.1 T cells for 1 to 2 days before expanding the cells with fresh medium. Infected T cells were selected by sorting for cells double positive for eGFP and CD4 with a FACStarplus flow cytometer (Becton Dickinson).

RNase protection assay

D10 cells were harvested 4 h after various treatments, and RNA was extracted with Qiagen RNeasy columns. A total of 5 mg of RNA per condition was used for each array. Multiple toxigenic templates (PharMingen) were used to generate 32P-labeled riboprobes. Probe labeling, hybridization, RNase digestion, and final denaturing polyacrylamide gel electrophoresis were performed, as described elsewhere (55).

DNA synthesis assays

DNA synthesis was measured by plating cells on 96-well plates at 1–2 10^5 cells/well. Cells were preincubated with ICI 182,780, a gift of Dr. Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, Cheshire, U.K.) or 4-HT (Upstate Biotechnologies) in the absence of IL-2 for 24 h. Cells were then treated with IL-2 (100 U/ml; DNAX) or left untreated for an additional 12 or 36 h. Methyl-[3H]thymidine was added to final concentration of 1.5 Ci/ml for the final 4 h of culture. Incorporation of methyl-[3H]thymidine was assayed using a Skatron cell harvester and a Betaplate scintillation machine, as described previously (39).

Flow cytometry

Cell viability was measured in 96-well microtiter dishes exactly in parallel to the assays set up to measure DNA synthesis, as described above. An equal volume of PBS containing 20 mg/ml of propidium iodide was added to each well and analyzed using a FACScan II (Becton Dickinson) with CellQuest software. Three thousand individual cells were assessed for each sample. The percentage of viable cells was calculated by quantifying the propidium iodide-negative cells. Each measurement was performed in triplicate.

For cell cycle analysis, cells were fixed in solution containing 0.1% sodium citrate, 0.1% Nonidet P-40, and 50 mg/ml of propidium iodide for 30 min on ice and assayed by a FACScan II. Alternatively, cells cultured in IL-2 were labeled with 0.1 mg/ml 5-bromo-2-deoxyuridine (BrdU; Sigma) for 30 min, washed with warm PBS, and then cultured under different conditions. At different time points, cells were collected, fixed, and stained with a FITC-coupled anti-BrdU Ab and propidium iodide, as described in the manufacturer’s protocol (Boehringer Mannheim, Indianapolis, IN). Samples were analyzed using a FACScan II.

Preparation of cell lysates and Western blotting

Cells were harvested and lysed in ELB buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, and 0.1% (v/v) Nonidet P-40 with standard protease and phosphatase inhibitors (50)). Abs for immunoprecipitation and Western blotting were as follows: anti-ERK1, anti-ERK2, anti-cyclin E, anti-cyclin A, and anti-CDK2 (all from Santa Cruz Biotechnology, Santa Cruz, CA); anti-p21Cip1 (PharMingen); anti-p27Kip1 (Transduction Laboratories, Lexington, KY); anti-cyclin D2 was a gift of Drs. David Parry and Emma Lees (DNAX). HRP-conjugated anti-mouse and anti-rabbit Igs (Amersham, Arlington Heights, IL) were used at 1/5,000 to 1/10,000 dilutions, Western blots were visualized using the enhanced chemiluminescence technique (Amersham).

Protein kinase assays

Antiseras were used to immunoprecipitate ERK1 and ERK2 from 100–200 mg of cell lysate, and a protein kinase assay was performed using myelin basic protein as a substrate according to published protocols (50). The
kinase activity of ERK1 and ERK2 was quantitated using a Molecular Dynamics Storm PhosphorImager (Sunnyvale, CA). Cyclin E-associated kinase assays were performed as described elsewhere (39). Briefly, 500 μg of cell lysate per sample was subjected to immunoprecipitation with an anti-cyclin E polyclonal Ab (Santa Cruz Biotechnology). Immune complexes, collected with protein A-Sepharose 4B (Sigma), were washed three times with lysis buffer and once with kinase reaction buffer (50 mM Tris, pH 7.4, 10 mM MgCl₂, 1 mM DTT). The kinase reaction mixtures were incubated in 20 μl of the same buffer containing 10 μCi of [γ-32P]ATP, 12.5 μM ATP, and 2 μg histone H1 (Boehringer Mannheim) as substrates for 30 min at 30°C. The kinase reactions were denatured in SDS sample buffer and analyzed by SDS-PAGE and transferred onto an Immobilon P membrane (Millipore, Bedford, MA). Histone H1 phosphorylation was quantitated using a Molecular Dynamics Storm PhosphorImager. Proteins in the immune complexes were detected by probing the membrane with appropriate Abs, as described above.

Results

The Raf-MEK-ERK pathway is involved in anti-CD3 mAb-mediated IL-2 unresponsiveness

For this study, primary naive CD4⁺ T cells as well as various T cell clones were utilized. However, we focused primarily on a Th2 clone D10G4.1 (D10) as a model cell line in which to dissect the biochemical mechanism of anti-CD3 mAb and Raf-induced cytokine unresponsiveness. Treatment of D10 cells with increasing concentrations of anti-CD3 mAb induced a bell-shaped mitogenic response with approximately a 50% reduction in DNA synthesis when more than 1 μg/ml of immobilized anti-CD3 mAb was utilized (Fig. 1, top panels). Furthermore, the inhibition of cell proliferation was not due to a lack of cytokine expression, because exogenously added IL-2 did not abrogate the inhibition of DNA synthesis observed at high concentrations of anti-CD3 mAb. Furthermore, increased doses of anti-CD3 mAb had no obvious effect on cell survival, as cell viability remained greater than 65% even at the highest dose of anti-CD3 mAb (Fig. 1, bottom panels). Therefore, the decreased thymidine incorporation observed at high doses of anti-CD3 mAb was not due to cell death. Similar inhibition of DNA synthesis was observed in primary naive CD4⁺ T cells stimulated with varying doses of anti-CD3 mAb (A. O’Garra, data not shown).

Based on previous observations that activation of the MAP kinase pathway is able to induce nonresponsiveness to cytokines and growth factors in nonlymphoid cells (38–44), we decided to test whether the ERK pathway might be involved in the cell cycle inhibition elicited by high dose anti-CD3 mAb treatment of T cells (D10, Th2 cells). To investigate this possibility, we utilized a selective and specific MEK inhibitor, PD098059 (56). The addition of 10 μM PD098059 to the D10 cell culture partially abrogated the inhibition of thymidine incorporation observed when D10 cells were treated with high concentrations of anti-CD3 mAb (Fig. 1). Similar results were obtained in two independent experiments using D10 cells. The inability to fully reconstitute the levels of DNA synthesis with higher doses of PD098059 as shown in Fig. 1 was most likely a reflection of the fact that in addition to its ability to induce cell cycle arrest, the Raf-MEK-ERK pathway is also required for cell proliferation. Indeed, the use of higher concentrations of PD098059 significantly inhibited the cell proliferation induced by low concentrations of anti-CD3 mAb (data not shown). These data are consistent with previous observations in both human and mouse fibroblasts, in which it has been shown that the Raf-MEK-ERK pathway is required for normal cell proliferation, but is also capable of eliciting cell cycle arrest and senescence when activated in a sustained fashion (38, 39, 43). These data suggest that in T cells, the Raf-MEK-ERK pathway is required for normal cell proliferation as well as in the induction of cytokine nonresponsiveness by anti-CD3 mAb.

Activation of Raf elicits cytokine nonresponsiveness and cell cycle arrest in T cells

To determine whether activation of the Raf-MEK-ERK pathway might elicit IL-2 unresponsiveness in T cells, we expressed a conditionally active form of Raf (∆Raf-1:ER*) in two T cell clones (D10 and HDK1) using retroviral infection. Retroviral constructs encoding either a catalytically active form of the kinase domain of human Raf-1 (∆Raf-1) or its kinase-inactive counterpart (∆Raf301) were fused to a modified form of the hormone-binding domain of mouse estrogen receptor that is insensitive to 17β-estradiol, but retains sensitivity to 4-HT and the ICI series of compounds, as described in Materials and Methods. ∆Raf:ER expression constructs were introduced into T cells by retrovirus infection. To select for infected cells, the retroviral construct was also engineered to encode an enhanced form of GFP. Cells infected with these viruses express ∆Raf:ER and eGFP proteins from a single bi-cistronic mRNA with the translation of the 5’ coding region for eGFP promoted by the presence of the internal ribosomal entry site (IRES) from encephalomyocarditis virus. Virus-infected cells were FACS sorted according to the expression of eGFP, which allowed the isolation of cells also expressing the appropriate ∆Raf:ER proteins.

To maximize the efficiency of virus infection, D10 T cells were cocultured with the virus-producing cell line, as described in Materials and Methods, and infected T cells were isolated by FACS sorting cells that were positive for the expression of eGFP and the T cell marker CD4. We have previously demonstrated that the level of activation of ∆Raf-1:ER is dose dependent on the amount of hormone added to the cell culture media (50). To characterize these cells, we first compared the kinetics of ERK activation upon the activation of ∆Raf-1:ER by ICI 182,780 (ICI) or by the addition of the phorbol ester PMA. As shown in Fig. 2B, ERK activity was induced upon either the activation of ∆Raf-1:ER or PMA treatment, and the maximal levels observed were comparable between the two stimuli. However, ∆Raf-1:ER-induced ERK activity

![Graph](image-url)
was maintained at elevated levels, whereas that induced by PMA showed transient kinetics of induction returning to low levels 24 h after PMA addition. In addition, the level of induced ERK activity could be titrated by varying the ICI and anti-CD3 mAb concentrations, which provided a convenient way to correlate biological responses with the levels of ERK activity (Fig. 2C). As expected, D10 cells expressing equivalent amounts of the kinase-inactive ΔRaf301:ER displayed no ERK activation in response to ICI treatment (data not shown), which served as a negative control for all subsequent experiments. In addition, the kinase-inactive

**FIGURE 2.** Response of D10G4.1 cells to selective Raf activation. A, Diagram of the retrovirus constructs used to express conditionally active ΔRaf:ER proteins in T cells. B, D10 cells expressing ΔRaf-1:ER were treated with ICI or PMA for different times, and the kinetics of ERK activation was compared using a protein kinase assay, as described in Materials and Methods. C, D10 cells expressing ΔRaf-1:ER were treated with different concentrations of ICI or anti-CD3 mAb, and ERK activity was measured as in B. D, D10 cells expressing either ΔRaf-1:ER or kinase-inactive ΔRaf301:ER were either untreated (no tx) or treated with ethanol (ETOH), or with 300 nM ICI (ICI) or with immobilized anti-CD3 mAb (αCD3) for 16 h and stained with a PE-conjugated anti-CD69 Ab. E, ΔRaf-1:ER-expressing D10 cells were treated with ethanol, ICI, ICI + ionomycin (ICI + Iono), PMA, ionomycin (Iono), or PMA + ionomycin (PMA + Iono) for 4 h, as indicated. RNA samples were prepared from these cells and probed using an RNase protection assay with a panel of cytokine-specific antisense riboprobes, as indicated. As positive and negative controls, RNA prepared from PMA-treated EL4 cells and yeast tRNA, respectively, were subjected to similar analysis side by side.
\( \Delta \text{Raf301:ER} \) did not display any evidence of a dominant-negative effect either in the absence or presence of 4-HT (data not shown).

To determine the biological consequences of Raf-MEK-ERK activation in these cells, we determined whether any T cell activation markers were induced upon \( \Delta \text{Raf-1:ER} \) activation. Initially, we examined the expression of CD69, the induction of which has been reported to occur in a Ras-dependent manner (57). Similar to the controls, untreated D10 cells expressing either \( \Delta \text{Raf-1:ER} \) or \( \Delta \text{Raf301:ER} \) expressed little or no CD69 in the resting state (Fig. 2D and data not shown). Upon addition of ICI, the expression of CD69 was significantly induced on the surface of \( \Delta \text{Raf-1:ER} \)-expressing cells, whereas no change in CD69 expression was detected on the surface of cells expressing the kinase-inactive \( \Delta \text{Raf301:ER} \) (Fig. 2D). Both populations showed similar induction of CD69 expression in response to anti-CD3 mAb stimulation; hence, neither of the forms of \( \Delta \text{Raf:ER} \) expressed in these cells is capable of eliciting a dominant-negative effect on CD69 expression, an observation consistent with previous results. These data indicate that \( \Delta \text{Raf-1:ER} \) is able to activate the ERK pathway, leading to the expression of at least one T cell activation marker, namely CD69.

To further examine the response of T cells to \( \Delta \text{Raf-1:ER} \) activation, we analyzed the effects of Raf activation on the expression of cytokine genes as monitored by an RNase protection assay (Fig. 2E). Upon PMA treatment or Raf activation, D10 cells were induced to express IL-5, IL-6, IL-10, and IL-13 mRNAs. Elevation of intracellular calcium with ionomycin led to induced expression of IL-4, IL-10, and IL-13 mRNAs, and synergized with \( \Delta \text{Raf-1:ER} \) activation or PMA to superinduce the expression of the IL-5, IL-6, IL-10, and IL-13 mRNAs. As expected, we observed no induction of IL-2 or IFN-\( \gamma \) mRNAs, because D10 cells are prototypical Th2 cells that do not express such Th1 cytokines. In these experiments, it was apparent that Raf activation had almost identical effects on cytokine gene expression as PMA treatment of cells. Indeed, the level of cytokine mRNA expression elicited either by Raf alone or in conjunction with ionomycin was very similar to that elicited by PMA. Hence, sustained Raf-MEK-ERK activation is not leading to supraphysiological induction of cytokine mRNAs at least when compared with treatment of cells with PMA.

Because D10 cells do not produce IL-2 and because \( \Delta \text{Raf-1:ER} \) activation was insufficient to induce IL-4 expression in these cells, we added exogenous IL-2 to determine whether activation of \( \Delta \text{Raf-1:ER} \) could render D10 cells unresponsive to IL-2. In the absence of exogenous IL-2, D10 cells displayed a low basal level of DNA synthesis. Activation of \( \Delta \text{Raf-1:ER} \) was sufficient to induce a modest increase in thymidine incorporation (Fig. 3A). As seen previously in NIH 3T3 cells (39), D10 cells displayed a bell-shaped mitogenic response to activation of \( \Delta \text{Raf-1:ER} \), suggesting that low levels of Raf-MEK-ERK activation induced proliferation, whereas higher levels did not. The addition of IL-2 to resting D10 cells stimulated high levels of thymidine incorporation (Fig. 3A). Under these conditions, activation of \( \Delta \text{Raf-1:ER} \) inhibited IL-2-induced DNA synthesis. The addition of ICI had no effect on either the proliferation or viability of parental D10 cells or cells expressing the kinase-inactive \( \Delta \text{Raf301:ER} \) (Fig. 3A), indicating that Raf kinase activity is required for both \( \Delta \text{Raf-1:ER} \)-induced cell proliferation and arrest. The lower total level of \([^{3} \text{H}] \) thymidine incorporation in the \( \Delta \text{Raf301:ER} \)-expressing cells in this experiment was not reproducible in other iterations of this experiment and most likely reflects a smaller number of cells in the proliferation assay. The surface expression of the IL-2R (\( \alpha \)-, \( \beta \)-, and \( \gamma \)-chains) was not changed upon activation of \( \Delta \text{Raf-1:ER} \) (data not shown).

Thus, the lack of mitogenic response to IL-2 was not due to the down-regulation of the IL-2R. Because D10G4.1 are Th2 cells, they proliferate in response to IL-4. Consequently, we examined the proliferative response of these cells to IL-4. We found that, similar to the response to IL-2, \( \Delta \text{Raf-1:ER} \) activation inhibited IL-4-induced cell proliferation (data not shown). Importantly, cell viability was not significantly altered by \( \Delta \text{Raf-1:ER} \) activation (Fig. 3A and data not shown), suggesting that the ability of Raf to inhibit IL-2- and IL-4-induced proliferation did not result from increased apoptosis. On the contrary, in the absence of cytokine, Raf activation appeared to provide a survival signal, as demonstrated by a moderate increase in cell viability (Fig. 3A). Similar retroviral infection and proliferation assays were also performed in an IL-2-dependent Th1 clone, a cytokine-independent T cell line, and an IL-3-dependent pro-B cell line. In all cases, we observed that activation of \( \Delta \text{Raf-1:ER} \) led to inhibition of cell cycle progression (data not shown).
Raf activation induces G1 arrest in T cells

High levels of MAP kinase activation have been shown to induce cell cycle arrest in nonlymphoid cells (38–40, 42–44). To examine whether ΔRaf-1:ER activation could elicit cell cycle arrest in T cells, D10 cells expressing ΔRaf-1:ER were cultured in the presence or absence of ICI and/or IL-2, and the effects of ΔRaf-1:ER activation were compared. Cell cycle status was assessed by staining cell nuclei with propidium iodide (Fig. 3B).

In the absence of stimulation, ~70% of cells displayed a 2N DNA content indicative of cells in the G0/G1 phase of the cell cycle. The remaining 30% of cells were either in the S or G2/M phases of the cell cycle. Following 12 h of IL-2 treatment, the percentage of cells in G0/G1 was decreased to 50%, with a corresponding increase in the numbers of cells in S and G2/M phase, consistent with the expected mitogenic effects of IL-2. However, activation of ΔRaf-1:ER significantly inhibited the mitogenic response of D10 cells to IL-2 treatment, as under these conditions the percentage of cells in G0/G1 phase was maintained at 77%, and the percentage of cells in S and G2/M phase was reduced from 50% to 23%. Importantly, cells expressing ΔRaf301:ER, the kinase-inactive form of the protein, displayed no such inhibition of IL-2-induced mitogenesis, indicating that Raf activity is required for the observed inhibition of cell cycle progression (Fig. 3B).

In a separate experiment, we examined the inhibition of IL-2-induced mitogenesis by treatment of parental D10 cells with high doses of anti-CD3 mAb. In this experiment, 22% of untreated cells were in the S or G2/M phases of the cell cycle before IL-2 treatment, and this was increased to 59% after IL-2 addition. Prior treatment of these cells with high dose anti-CD3 mAb reduced the percentage of cells in S and/or G2/M from 58% to 38% (Fig. 3C). Hence, high dose anti-CD3 mAb also induces a G0/G1 arrest in these cells.

Additionally, we performed a pulse-chase experiment, in which D10 cells were pulse labeled with BrdU for a short period of time. Following the removal of BrdU, the cells were cultured in the presence of IL-2, and their transit time through the cell cycle into the next S phase was assessed. In the absence of ΔRaf-1:ER activation, cells reentered S phase ~12 h following the initial BrdU labeling. However, when ΔRaf-1:ER was activated, cells had failed to reenter S phase even up to 14 h after the initial BrdU labeling (data not shown). These data further support the hypothesis that ΔRaf-1:ER elicits a G1 cell cycle arrest in T cells.

Raf activation inhibited IL-2-dependent cell cycle progression in naive primary T cells

We wished to address whether the observations made in D10 cells also held true for primary T cells cultured in vitro. To this end, we isolated a population of naive CD4+ Mel-14high T cells from DO11.10 TCR transgenic mice and cultured them in the presence of the OVA 323–339 antigenic peptide and irradiated APC (46, 47, 58). T cells were then infected with retrovirus encoding either kinase-active ΔRaf-1:ER or kinase-inactive ΔRaf301:ER. Infected cells were selected by FACS, expressing eGFP, and stained with propidium iodide. Treatment of the ΔRaf301:ER-expressing populations of DO11.10 T cells with IL-2 led to a 10-fold increase in the population of cells with greater than 2N DNA content that were presumably in the S/G2/M phase of the cell cycle. However, ΔRaf-1:ER activation caused a 50% inhibition of the response to IL-2, an effect that was not observed with the kinase-inactive ΔRaf301:ER (Fig. 4B). Taken together, these data strongly suggest that, similar to high dose exposure to Ag, ΔRaf-1:ER activation is capable of eliciting cell cycle arrest in naive primary T cells.
Effects of Raf activation on cell cycle regulatory proteins

The experiments described above suggested that ΔRaf-1:ER activation could elicit G1 arrest in T cells that could not be overcome by exogenous IL-2. We have previously described a similar situation in mouse NIH 3T3 fibroblasts in which Raf activation induced a potent cell cycle arrest mediated, in large part, by the induced expression of the CKI, p21Cip1 (39). To determine whether either ΔRaf-1:ER or anti-CD3 mAb-induced G1 arrest in T cells is mediated by alterations in the expression or activity of key components of the cell cycle machinery, we examined the expression and activity of some of the proteins that are known to control the G1 to S phase transition in mammalian cells.

D10 cells expressing ΔRaf-1:ER were treated with two different concentrations of ICI (30 or 300 nM) to activate ΔRaf-1:ER or with a high dose of anti-CD3 mAb in the presence or absence of IL-2. Different concentrations of ICI were used in an effort to activate the MAP kinase pathway to different extents, as indicated in Fig. 1C. However, similar results were obtained with both concentrations of ICI used. After 12 h, cell extracts were prepared and the expression of cell cycle regulators and the activity of cyclin E/CDK2 were assessed.

Treatment of D10 cells with IL-2 alone led to increased expression of both cyclin E and cyclin A, a modest increase in the expression of p21Cip1, and decreased expression of p27Kip1 (Fig. 5A). Cyclin E/CDK2 activity was measured in the same extracts and, as expected, was shown to be strongly induced by IL-2 treatment. Consistent with this was the observation that cyclin E/CDK2 complexes from IL-2-treated cells contained little or none of the CKI, p21Cip1, and p27Kip1 as compared with untreated cells (Fig. 5B).

Activation of ΔRaf-1:ER alone or treatment of cells with a high dose of anti-CD3 mAb led to induction of cyclin D2 and cyclin E, but not cyclin A (Fig. 5A). Moreover, activation of ΔRaf-1:ER or treatment with anti-CD3 mAb led to highly elevated expression of p21Cip1. In addition, these treatments largely prevented the reduced expression of p27Kip1 that normally occurs following IL-2 treatment. The apparent consequence of these events was that the activation of cyclin E/CDK2 complexes by IL-2 was significantly inhibited (Fig. 5B). Consistent with this was the fact that we could readily detect the presence of both p21Cip1 and p27Kip1 in cyclin E/CDK2 immune complexes (Fig. 5B). These data suggest that the observed effects of ΔRaf-1:ER and high dose anti-CD3 mAb on p21Cip1 and p27Kip1 expression may be responsible for the cell cycle arrest observed in response to these agents.

To determine whether the induction of p21Cip1 by high dose anti-CD3 mAb was mediated by the Ras-activated Raf-MEK-ERK pathway, D10 cells were treated with anti-CD3 mAb for 4 or 7 h in the absence or presence of 20 or 40 µM of the MEK-specific inhibitor PD098059 (Fig. 5C). Consistent with the partial ability of PD098059 to reverse the antiproliferative effects of high dose anti-CD3 mAb, the induced expression of p21Cip1 was inhibited, although not entirely abrogated, by the presence of the MEK inhibitor. Equal loading of the gel lanes in Fig. 5, A and C, was confirmed by probing the Western blots with an antisera that recognizes ERK1 and ERK2, which, due to the low resolving power of these polyacrylamide gels, are not resolved according to their phosphorylation status. These data support a model in which high dose anti-CD3 mAb induces cell cycle arrest in T cells by activating the Raf-MEK-ERK pathway leading to induced expression of p21Cip1 and a failure to down-regulate the expression of p27Kip1.

It has previously been suggested that the formation of complexes between D-type cyclins and CDK2 may be involved in the transmission of an antiproliferative signal in mammalian cells (59). Because ΔRaf-1:ER and anti-CD3 mAb, but not IL-2, induced cyclin D2 expression, we assessed whether cyclin D2 formed a complex with CDK2 in cells arrested as a consequence of ΔRaf-1:ER activation or treatment with anti-CD3 mAb. Interestingly, we...
were readily able to detect CDK2 in immunoprecipitates of cyclin D2 from cells that were arrested as a consequence of ∆Raf-1-ER activation or high dose anti-CD3 mAb treatment (Fig. 5D). Although not definitive proof of the hypothesis, these data are consistent with the suggestions of others that the formation of cyclin D2/CDK2 complexes may play a role in the inhibition of cell cycle progression.

Discussion

The mitogenic response of resting T cells is mediated initially by interactions of Ag bound to MHC with the TCR, which elicits a plethora of early signaling events (60, 61). This initial signaling interaction leads to the expression of a series of activation markers as well as the expression of cytokines such as IL-2 and its cognate high affinity receptor. The autocrine effects of secreted cytokines lead to the induction of D- and E-type G1 cyclins and their cognate CDKs and the down-regulation of the CKI p27^Kip1, allowing cells to transit from G1 into S phase (60, 61).

Previous observations indicated that high intensity signaling through the Raf-MEK-ERK pathway could render cells resistant to the effects of growth factors and cytokines (38–44). Such observations prompted us to explore a possible role for this pathway in the cytokine nonresponsive phenotype that is elicited in T cells by either a high dose of Ag or treatment with anti-TCR mAb. In this work, we demonstrate that activation of the ERK pathway either through the TCR or by specific activation of the Raf-MEK-ERK pathway leads to a cytokine unresponsive state. This inability of T cells to respond to IL-2 was not associated with cell death or a lack of IL-2R expression, but appeared to be due to cell cycle arrest. We found that both anti-TCR mAb and Raf-induced cell cycle arrest were associated with enhanced expression of the CKI p21^{Cip1} and that the level of induced p21^{Cip1} expression correlated with the signal strength elicited by TCR or Raf activation. Importantly, the level of induction of p21^{Cip1} elicited by Raf and by anti-TCR mAb treatment was similar, suggesting that the effects of Raf on T cell cycle were not a consequence of supraphysiological induction of p21^{Cip1} expression. Second, the induction of p21^{Cip1} elicited by anti-TCR mAb treatment was mediated, at least in part, by the activation of the Raf-MEK-MAP pathway, which is consistent with the ability of Raf to induce p21^{Cip1} and thereby induce cell cycle arrest. The induced association of p21^{Cip1} with the cyclin E/CDK2 complexes led to decreased activity of this complex consistent with the observed G1 cell cycle arrest, as has been demonstrated previously (39). In addition to observing the induced expression of p21^{Cip1}, both Raf and anti-CD3 mAb also prevented the normal down-regulation of p27^{Kip1} expression from cyclin E/CDK2 complexes that occurs in response to IL-2 treatment. Typically, down-regulation of p27^{Kip1} expression is associated with the initial activation of CDK2 that leads to phosphorylation of p27^{Kip1}, thereby targeting it for degradation. Because the consequence of induced p21^{Cip1} expression is the inhibition of cyclin E/CDK2 complexes, it is possible that the failure to down-regulate p27^{Kip1} expression is secondary to the induction of p21^{Cip1}. Nonetheless, the induced expression of p21^{Cip1}, the sustained expression of p27^{Kip1}, or most likely a combination of both may be important in the induction of the cytokine nonresponsive state in T cells. This stands in contrast to the situation in NIH 3T3 and primary mouse embryo fibroblasts in which the induction of p21^{Cip1} appears to be most important to the induction and maintenance of a growth-factor nonresponsive state. Indeed, in this situation, NIH 3T3 cells arrest with high levels of p21^{Cip1}, but low levels of p27^{Kip1} expression. Furthermore, in p21^{Cip1}−/− mouse embryonic fibroblasts, activation of ∆Raf-1-ER failed to cause cell cycle arrest, even though the cells continue to express p27^{Kip1} (39). Similarly, p21^{Cip1}, but not p27^{Kip1}, was found to be associated with cyclin E/CDK2 in a B lymphoma cell line that was arrested in the cell cycle by treatment with anti-IgM (26). Therefore, we believe that the induction of p21^{Cip1} by the Raf-MEK-ERK pathway may be the trigger for cell cycle arrest in a number of circumstances.

It has been suggested that the assembly of complexes containing cyclin D2 and CDK2 might be involved in the induction of cell cycle arrest in fibroblasts and in a T cell lymphoma (59, 62). It is interesting, therefore, that in T cells arrested either by high dose anti-CD3 mAb or by ∆Raf-1-ER activation, there was a robust induction of cyclin D2 as well as the formation of cyclin D2/CDK2 complexes. These complexes were not seen upon cytokine withdrawal or in response to mitogenic stimulation. It is not clear how such complexes are assembled, but the recent demonstration that p21^{Cip1} serves as an assembly factor for D-type cyclins and their cognate CDKs suggests that the induced expression of this CKI may play a role. Even less clear is the potential role of the cyclin D2/CDK2 complex in the induction and maintenance of the nonresponsive state, although the availability of cyclin D2 knockout mice makes such analyses feasible in the future.

It has been reported that Ag-induced IL-2 unresponsiveness can be attributed to activation-induced cell death both in vivo and in vitro (24, 63). When we examined the D10 cells and primary T cells from DO11.10 mice following either anti-CD3 mAb treatment or ∆Raf-1-ER activation, there was no obvious induction of apoptosis within the first 24 h of treatment. Indeed, activation of ∆Raf-1-ER appeared to protect cells from the apoptotic cell death that normally occurs after the withdrawal of cytokines. However, at later times following Raf activation, we observed increased cell death in these cultures. It is possible that the initial response to Raf activation is cell cycle arrest that is followed at later times by apoptosis. Our results are consistent with the observation that in Jurkat T cells and PBL, activation-induced apoptosis occurs following late G1 arrest (64).

The availability of a system for the conditional activation of the Raf-MEK-ERK pathway in T cells should provide a useful tool to study how costimulatory signals such as the Ca^{2+}-calcineurin-NF-AT pathway, and other MAPK pathways such as the c-Jun N-terminal kinase pathway interact to synergistically regulate nuclear events that control immune cell function. Indeed, we and others have previously demonstrated the feasibility of such studies by demonstrating that the activation of c-Fos and MAP kinase phosphatase-1 expression in fibroblasts requires the synergistic action of the MAP kinase pathway and a calcium signal (65, 66). The availability of conditional systems for the regulation of MEK kinase, P13-kinase, and Akt will further facilitate such studies (67–69). Moreover, it will be interesting to determine whether the activation of costimulatory receptors such as CD28 or other signaling molecules might modify the cell cycle arrest elicited by the activation of the Raf-MEK-ERK pathway. Such an approach is all the more interesting in view of the recent observation that pathways regulated by the Rho GTPase can antagonize the induction of p21^{Cip1} and thereby prevent the cell cycle arrest induced by activated Ras in Swiss 3T3 cells (70). Indeed, the Rho GTPase has previously been shown to be important for thymic development (71, 72).

In this study, we have demonstrated that the constitutive activation of the Raf-MEK-ERK signaling pathway was sufficient to render T cells insensitive to subsequent mitogenic stimulation. This finding may provide a partial explanation for the induction of anergy in lymphoid cells as a consequence of high dose Ag or anti-TCR stimulation. It is possible that the sustained activation of...
ERKs in the absence of costimulatory signals results in the sustained expression of CKI such as p21Cip1 and p27kip1. It is interesting to note, therefore, that memory T cells from aged mice with autoimmune disease exhibit a predominant G1 arrest and are refractory to anti-CD3 mAb-induced proliferation and apoptosis. It has been suggested that the unresponsive state of T cells in these refractory to anti-CD3 mAb-induced proliferation and apoptosis. It has been suggested that the unresponsive state of T cells in these mice may be a consequence of cumulative chronic self Ag stimulation, and so it is interesting to note that the phenotype correlates with increased expression of p21Cip1 and p27kip1. (73). Furthermore, it has been demonstrated that anergic B cells from mice engineered to express anti-hen egg lysozyme IgM and IgD and solublen hen egg lysozyme display low level constitutive activation of the ERK pathway (29). However, it is unclear whether the cell cycle arrest elicited under these conditions is mediated by any of the CKI implicated in this study.

In a previous study, we demonstrated that the activation of the Raf-MEK-ERK pathway in primary human fibroblasts elicits an irreversible cell cycle arrest that is accompanied by the expression of a variety of cellular senescence markers. The induction of irreversible arrest in this context was associated with the induced expression of p16ink4a that sequesters CDK4 and CDK6 into an inactive complex (38, 74). In contrast, the cell cycle arrest induced by ΔRaf-1:ER in D10 T cells was fully reversed by the removal of the activating agent, indicating that Raf is most likely not inducing a senescence-like response in these cells. It is not clear whether this observation is a peculiarity of lymphoid cells or reflects a fundamental difference between mouse and human cells, but this phenomenon warrants further investigation in both primary and established mouse and human T cells. As described above, the availability of conditional forms of various signaling molecules and efficient systems for gene transfer into lymphoid populations will significantly facilitate such analyses.

In conclusion, we show in this study that selective activation of the Raf-MEK-ERK pathway in T cells can mimic the antiproliferative signals transduced by the TCR. In both cases, the induced expression of p21Cip1 is implicated in the cell cycle arrest that ensues following these treatments. Further study will be necessary to elucidate the involvement of other mechanisms in TCR-induced nonresponsiveness and how such mechanisms relate to peripheral tolerance.

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