Maintenance of Human T Cell Anergy: Blocking of IL-2 Gene Transcription by Activated Rap1

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In the absence of costimulation, T cells activated through their antigen receptor become unresponsive (anergic) and do not transcribe the gene encoding interleukin-2 (IL-2) when restimulated with antigen. Anergic alloantigen-specific human T cells contained phosphorylated Cbl that coimmunoprecipitated with Fyn. The adapter protein CrkL was associated with both phosphorylated Cbl and the guanidine nucleotide–releasing factor C3G, which catalyzes guanosine triphosphate (GTP) exchange on Rap1. Active Rap1 (GTP-bound form) was present in anergic cells. Forced expression of low amounts of Rap1-GTP in Jurkat T cells recapitulated the anergic defect and blocked T cell antigen receptor (TCR)– and CD28-mediated IL-2 gene transcription. Therefore, Rap1 functions as a negative regulator of TCR-mediated IL-2 gene transcription and may be responsible for the specific defect in IL-2 production in T cell anergy.

Ligation of TCR without costimulation results in a state of long-term functional unresponsiveness termed anergy (*1*). Anergic T cells do not transcribe the IL-2 gene when stimulated with specific antigen, even in the presence of costimulation. T cells in the anergic state cannot activate ZAP-70, Ras, ERK, JNK, or AP-1 when stimulated with antigen (*2–4*). However, they have constitutively increased concentrations of intracellular free Ca^{2+} and phosphatidylinositol 1,4,5-triphosphate, increased tyrosine phosphorylation of phospholipase C-γ1, and increased kinase activity of Fyn (*5*). Fyn, which is constitutively associated with the TCR, may be the only protein tyrosine kinase that has an active role in the maintenance of the anergic state. The mechanism by which this proximal signaling event results in a block of IL-2 transcription remains unclear.

To dissect this mechanism we used an alloantigen-specific human T cell clonal system (*6*). T cell clones specific for the human leukocyte antigen class II molecule HLA-DR7 were first cultured with NIH-3T3 fibroblasts transfected with either HLA-DR7 alone (t-DR7) to induce anergy or with HLA-DR7 and B7-1 (CD80) (t-DR7/B7-1) to induce productive stimulation (*7*). T cells were subsequently incubated with the Epstein-Barr virus (EBV)–transformed B lymphoblastoid cell line LBL-DR7, which is homozygous for HLA-DR7. T cell clones first cultured with t-DR7 were anergized and did not respond on rechallenge with LBL-DR7 cells. In contrast, T cell clones that were first cultured with t-DR7/B7-1 were capable of proliferating in response to rechallenge with LBL-DR7 cells (*6*).

Consistent with previous reports (*5*), Fyn from anergic T cells had increased constitutive tyrosine kinase activity compared with control cells or cells previously incubated with antigen and costimulation (productively stimulated) as determined by an in vitro kinase reaction (Fig. 1A). The increased kinase activity of Fyn was associated with a number of substrates, including a 116-kD Fyn-associated protein. This substrate, as well as Fyn, was also phosphorylated in vivo in anergic cells and remained unchanged after activation as determined by immunoblot analysis with antibody to phosphotyrosine (anti-phosphotyrosine) (Fig. 1B, top). In contrast, in control cells or cells productively stimulated, the kinase activity of Fyn was transiently increased only after activation (Fig. 1B, top). The effects of anergy on the kinase activity of Fyn were not a result of quantitative changes in protein expression; equal amounts of Fyn were immunoprecipitated from anergic, productively stimulated, and unstimulated cells (Fig. 1B, middle). Cbl, a 116-kD proto-oncoprotein, associates with Fyn in B and T cells (*8–10*); immunoblotting with Cbl-specific antiserum showed that the 116-kD protein immunoprecipitated with Fyn was Cbl (Fig. 1B, bottom). The prominent association of phosphorylated Fyn with Cbl in anergic T cells was confirmed in the reciprocal experiment in which anti-Cbl immunoprecipitated Fyn as revealed by immunoblot with monoclonal antibody to phosphotyrosine or antiserum to Fyn (*11*). The band was confirmed to be Cbl and not Cas or Cbl-b by immunoblot with peptide-specific antibodies or antisera for these proteins (*11*).

To determine the biochemical nature of the enhanced Fyn-Cbl associations in anergy, experiments were performed with glutathione S-transferase (GST) fusion

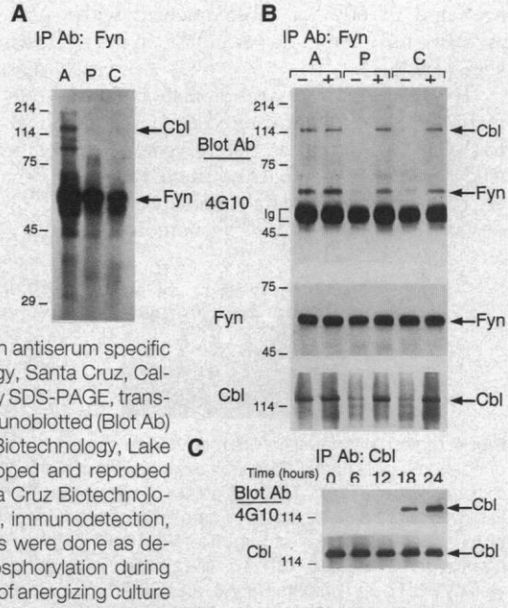

Fig. 1. Increased kinase activity of Fyn and increased phosphorylation of Fyn-associated Cbl in anergic cells. (**A**) Anergic (A), productively stimulated (P), and control (C) cells (10^7 cells per test) were examined for the constitutive kinase activity of Fyn by an in vitro kinase reaction as described (*6*). Reactions were analyzed by 10% SDS–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes, and exposed to x-ray film. (**B**) Anergic, productively stimulated, and control cells (10^7 cells per test) either unstimulated (–) or stimulated (+) (*33*) were lysed and immunoprecipitated with antiserum specific for Fyn (IP Ab:Fyn) (Santa Cruz Biotechnology, Santa Cruz, California). Immune complexes were resolved by SDS-PAGE, transferred on nitrocellulose membrane, and immunoblotted (Blot Ab) with phosphotyrosine mAb 4G10 (Upstate Biotechnology, Lake Placid, New York). Immunoblots were stripped and reprobed with antiserum specific for Fyn or Cbl (Santa Cruz Biotechnology). Cell lysis, immunoprecipitation, blotting, immunodetection, stripping, and reprobing of the immunoblots were done as described (*6*). (**C**) Kinetics of Cbl tyrosine phosphorylation during induction of anergy. At various time intervals of anergizing culture (*7*), tyrosine phosphorylation of Cbl was examined by immunoprecipitation with Cbl-specific antiserum and immunoblot with mAb 4G10. Membranes were stripped and reprobed with antiserum to Cbl. Identical results to those described here were obtained with two different clones. Molecular sizes are indicated in kilodaltons to the left of all panels.

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proteins GST-Fyn-SH2 or GST-Fyn-SH3. The Src homology 3 (SH3) domain was associated with Cbl from anergized, control, and productively stimulated cells, but only anergic cells had enhanced binding of Cbl to the SH2 domain (*11*). Larger amounts of Cbl could be detected in Fyn immunoprecipitations from anergic cells in comparison with productively stimulated and control cells (Fig. 1B, bottom), consistent with previous reports, which showed enhancement of Fyn-Cbl association upon phosphorylation (*9*, *12*). Kinetics experiments showed that tyrosine phosphorylation of Cbl was detectable by 18 hours of anergizing culture (Fig. 1C) and temporally coincided with the establishment of functional unresponsiveness (*11*).

Cbl does not have enzymatic activity but has an SH3-dependent interaction with Grb2 (*9*, *10*) and an SH2-dependent association with the Crk adapter proteins (*12–14*). Grb2 interacts with the guanidine nucleotide–releasing factor (GNRF) Sos to facilitate guanosine triphosphate (GTP) exchange on Ras (*15*). Crk proteins constitutively associate with C3G, a GNRF that may catalyze GTP exchange on a Ras family member Rap1 (*16*). Because the Ras pathway is blocked in anergy (*3*), we investigated whether phosphorylated Cbl might affect the activation of Ras and Ras-related proteins in anergic cells. In unstimulated T cell clones, Grb2 was associated with Cbl, and this association remained unchanged upon phosphorylation of Cbl (Fig. 2A). In contrast, CrkL associated and coprecipitated with Cbl only after phosphorylation of Cbl, indicating that this association was phosphorylation dependent. Among the three Crk proteins (CrkI, CrkII, and CrkL), CrkL was predominantly associated with phosphorylated Cbl (Fig. 2, A and B) (*12*); therefore, we focused on CrkL.

In anergic cells (Fig. 2C, left), Cbl immunoprecipitation and anti-phosphotyrosine immunoblot showed that phosphorylated Cbl remained unchanged after stimulation with anti-CD3 and anti-CD28. Phosphorylated Cbl coprecipitated with CrkL, which was constitutively associated with the GNRF C3G (Fig. 2C). Coprecipitation of C3G and phosphorylated Cbl could be detected by immunoprecipitations with antiserum to either Cbl or C3G after prolonged exposure of the blot (*11*); thus, the Cbl association with the CrkL-C3G complex was constitutive in anergic cells. Such ternary Cbl-CrkL-C3G complexes are present in Jurkat T cell lines that overexpress Cbl (*12*). In contrast, in control cells (Fig. 2C, right), Cbl became phosphorylated and associated with CrkL only after stimulation. Under these conditions, Cbl was phosphorylated less than in anergic cells, and the amount of Cbl that associated with CrkL after stimulation of control cells was less than the amount of Cbl constitutively associated with CrkL in anergic cells (Fig. 2C). Although C3G coprecipitated with CrkL in both anergic and control cells, CrkL-associated C3G in anergic but not control cells migrated with slower electrophoretic mobility (Fig. 2D). This slower electrophoretic mobility of C3G (*14*) may correspond to serine-threonine phosphorylation of C3G, similar to that induced to the related GNRF Sos after its serine-threonine phosphorylation (*17*).

C3G catalyzes GTP exchange of Rap1 (also known as Krev-1 and smg p21) (*18–20*). Therefore, the status of Rap1 activation before and after stimulation through TCR and CD28 was examined in anergic and control cells (*21*). Activated Rap1-GTP was constitutively present in anergic cells, whereas in controls it was only slightly induced after stimulation (Fig.

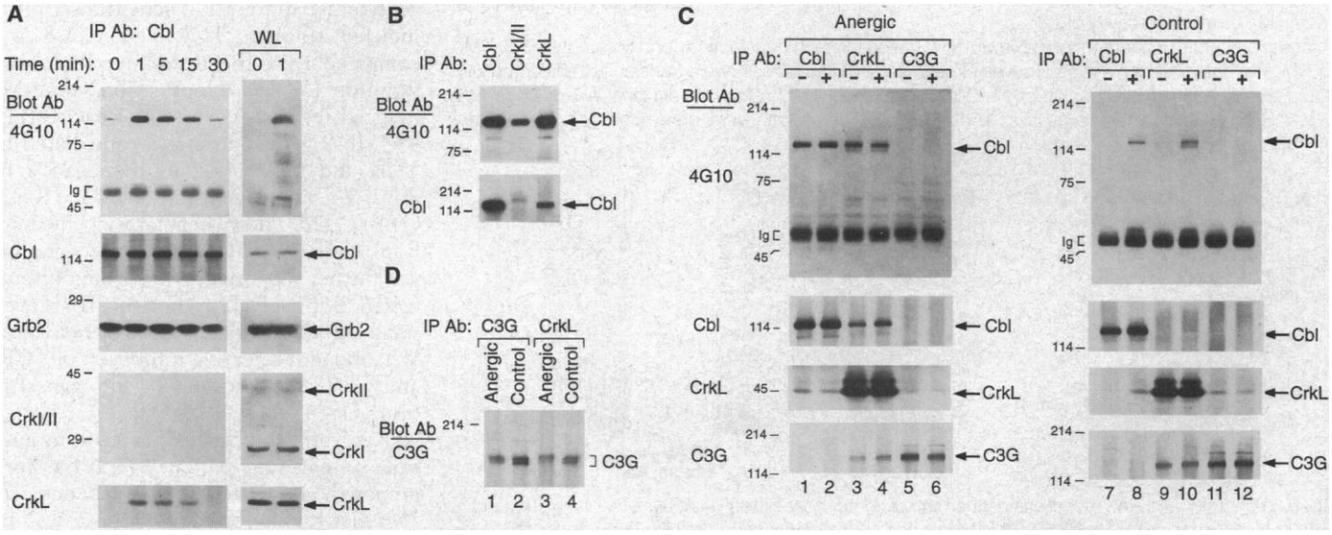

Fig. 2. (**A**) Constitutive association of Cbl with Grb2 and phosphorylation-dependent association of Cbl with CrkL in T cell clones. T cell clones (2×10^7 cells per test) were stimulated (*33*) for the indicated time intervals. Immunoprecipitations were done with antiserum to Cbl and analyzed by 10% SDS-PAGE along with whole-cell lysates (WL). Proteins were transferred to nitrocellulose membrane and immunoblotted with mAb 4G10, with antiserum specific for Cbl or CrkL (Santa Cruz Biotechnology), or with mAbs specific for Grb2 or CrkI/II (Transduction Laboratories, Lexington, Kentucky). In all subsequent experiments that examined Cbl phosphorylation, stimulation was done for 1 min, which generated maximum phosphorylation of Cbl. (**B**) Among the three Crk proteins phosphorylated Cbl predominantly associates with CrkL. Lysates (4×10^7 cells per test) from stimulated (*33*) T cell clones were immunoprecipitated with antiserum specific for Cbl or CrkL or mAb specific for CrkI/II. Immune complexes were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with mAb 4G10 or antiserum specific for Cbl. (**C**) Constitutive association of CrkL with C3G and phosphorylated Cbl in anergy. Lysates from anergic and control T cell clones (10^7 cells per test) either unstimulated (−) or stimulated (+) (*33*) were immunoprecipitated with antiserum specific for Cbl, CrkL, or C3G (Santa Cruz Biotechnology). Immune complexes were resolved by SDS-PAGE, transferred on nitrocellulose membrane, and immunoblotted with mAb 4G10. Blots were stripped and reprobed with antiserum specific for Cbl, CrkL, or C3G. (**D**) Shift in the electrophoretic mobility of CrkL-associated C3G in anergic cells. Lysates (10^7 cells per test) from anergic and control cells were immunoprecipitated with antiserum to either C3G or CrkL; immune complexes were resolved by electrophoresis on 8% SDS-PAGE and immunoblotted with antiserum to C3G. Molecular sizes are indicated in kilodaltons in all panels.

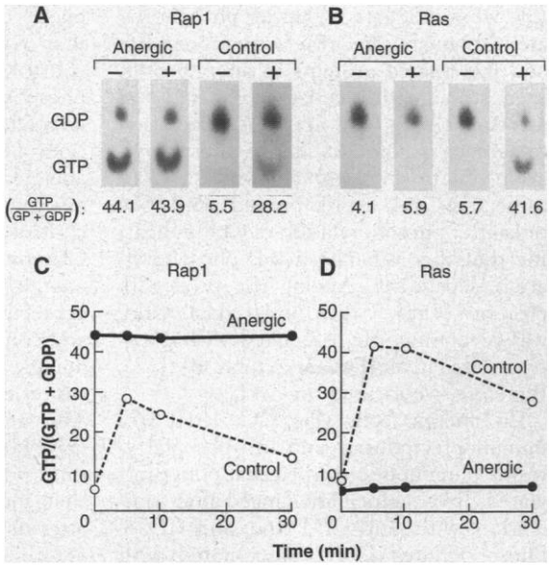

Fig. 3. (**A** and **B**) Activated Rap1 is constitutively present in anergic cells, whereas Ras cannot be activated. ^{32}P-labeled anergic or control cells (2×10^7 cells per sample) were unstimulated (−) or stimulated (+) (*33*) for 5 min. Labeled guanidine nucleotides bound to Rap1 or Ras were quantitated, and results were expressed as percent of GTP bound to Rap1 or Ras proteins relative to the total amount of guanidine nucleotides (GTP+GDP) complexed to these proteins. (**C** and **D**) Time course of Rap1 and Ras activation in anergic and control cells after stimulation. ^{32}P-labeled cells (2×10^7 cells per test) were unstimulated or stimulated for the indicated time intervals. Labeled guanidine nucleotides bound to Rap1 or Ras were quantitated, and results were expressed as in (A).

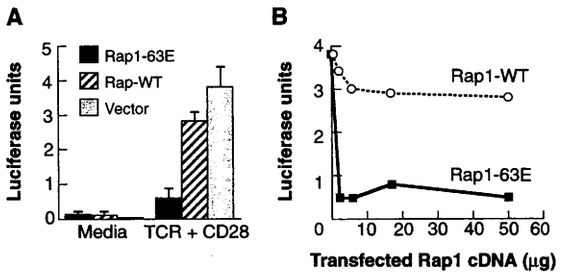

Fig. 4. Activated Rap1 functions as a negative regulator of TCR and CD28–mediated IL-2 transcription. (**A**) TAg Jurkat cells were transfected (*23*) with the reporter construct pIL-2-luciferase and the pAXEF vector containing either no insert (empty vector), Rap1-WT, or Rap1-63E cDNA. Cells were cultured with either media, mAbs to TCR and CD28, or PMA plus ionomycin (IM). Luciferase activity of cell lysates is presented as percent of maximum stimulation for each transfection condition, induced by PMA and IM. The data are the average of seven experiments. (**B**) Jurkat cells were transiently transfected with stable amounts of reporter construct pIL-2 luciferase and variable amounts of Rap1-WT or Rap1-63E cDNA. Stimulation, luciferase assays, and expression of results were done as in (A). Results are representative of two experiments.

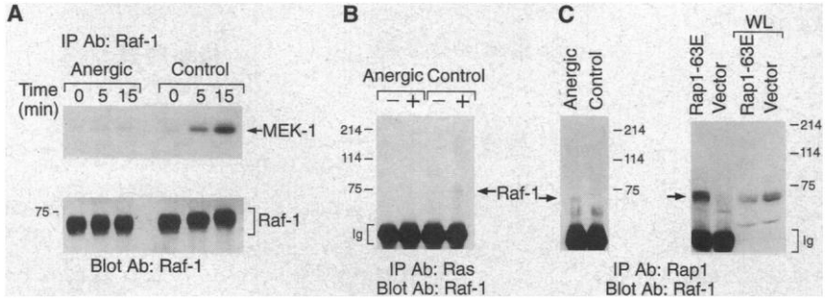

Fig. 5. In anergic cells Raf-1 kinase is neither activated nor associated with Ras after stimulation but is constitutively associated with Rap-1. (**A**) Anergic and control alloantigen-specific T cell clones (10^7 cells per test) were stimulated (*33*) for the indicated time intervals. Immunoprecipitation was done with Raf-1 antiserum (Santa Cruz Biotechnology), followed by an in vitro kinase reaction with MEK-1 (Santa Cruz Biotechnology) as exogenous substrate (*34*). Samples were analyzed by 8% SDS-PAGE, transferred to nitrocellulose membrane, and exposed to x-ray film (top panel). The same nitrocellulose membrane was immunoblotted with Raf-1 antiserum (bottom panel). Results are representative of three experiments. (**B**) Anergic and control T cell clones (10^7 cells per test) were either unstimulated (−) or stimulated (+) (*33*) for 5 min. Cells lysates were immunoprecipitated with the Ras mAb Y13-238 (Santa Cruz Biotechnology), which allows detection of Ras:Raf-1 complexes formed in vivo (*35*), and immunoblotted with Raf-1 antiserum. (**C**) Lysates from anergic and control T cell clones (10^7 cells per test), and from TAg-Jurkat cells (2×10^7 cells per test) transfected with either Rap1-GTP (Rap1-63E) or with empty vector were immunoprecipitated with Rap-1 antiserum and analyzed along with whole lysates (WL) of TAg-Jurkat on 8% SDS-PAGE. Immunoblots were probed with Raf-1 antiserum. Equivalent amounts of Raf-1, Ras, and Rap1 were present in both anergic and control cells (*11*). Molecular sizes are indicated in kilodaltons in all panels.

3A) and subsequently hydrolyzed (Fig. 3C). In contrast, consistent with previous reports (*3*), Ras was not activated after stimulation of the anergic cells (Fig. 3, B and D). Thus, although activation of ZAP-70, Ras, ERK, and JNK is blocked in anergy, a cascade of signaling events is initiated by increased Fyn activity that results in Cbl phosphorylation, recruitment of the CrkL-C3G complex, and Rap1 activation.

We examined whether the activated GTP-bound form of Rap1 might be responsible for the lack of IL-2 transcription upon antigen-specific restimulation in anergic T cells. To address this question, we transiently transfected the T cell line Jurkat with either wild-type Rap1 (Rap1-WT) or constitutively activated GTP-bound Rap1 (Rap1-63E) (*22*) and with a reporter construct of luciferase driven by the IL-2 promoter, to indicate IL-2 gene transcription (*23*). Transfected Jurkat cells were stimulated through TCR and CD28. Overexpression of Rap1-WT inhibited IL-2 transcription by 63%, whereas overexpression of Rap1-63E inhibited IL-2 transcription by 94% in comparison with control cells stimulated under identical conditions (*11*). To further examine the role of Rap1-GTP as a selective negative regulator of TCR-mediated IL-2 gene transcription, IL-2 gene transcription induced through TCR and CD28 was compared with the maximum IL-2 transcription for each transfection condition induced by PMA and ionomycin (Fig. 4A). IL-2 gene transcription mediated by TCR and CD28 was inhibited 25% by Rap1-WT and 83.7% by Rap1-63E (Rap1-GTP). This down-regulatory effect of Rap1-GTP on TCR-mediated signaling was further supported by titration of transfected Rap1 cDNA, showing that even small amounts of Rap1-63E, but not Rap1-WT, induced selective inhibition of TCR- and CD28-mediated IL-2 gene transcription (Fig. 4B).

Rap1 antagonizes Ras function in multiple systems (*19, 20, 22, 24*). It has been proposed, and there is some evidence (*18, 25*), that the biological properties of Rap1 are the result of direct competition for the same effectors as Ras and that Rap1 does not activate those effectors because it is not localized in the plasma membrane (*20, 26*). Raf-1 serine-threonine kinase, a downstream effector of Ras (*27*), is recruited by activated Ras to the plasma membrane (*28*), where it becomes activated in a Ras-independent manner. Activated Raf-1 phosphorylates and activates MEK-1, which leads to activation of the mitogen-activated protein (MAP) kinase cascade (*29*) and IL-2 gene transcription

in T cells (*30*). Raf-1 kinase was not activated after stimulation through TCR and CD28 in anergic cells as it was in control cells, although comparable amounts of protein were present (Fig. 4A). Similarly, Ras:Raf-1 association was not induced after stimulation in anergic cells as it was induced in control cells (Fig. 4B). In contrast, Raf-1 was constitutively associated and coprecipitated with Rap1 in anergic cells (Fig. 4C). Such complexes were also detected in Jurkat cells transfected with Rap1-63E (Rap1-GTP), but not in control transfectants (Fig. 4C).

We now show that in anergic T cells, Cbl was constitutively phosphorylated, crkL-C3G complexes were recruited, and Rap1, a negative regulator of IL-2 transcription, was activated. Cbl is one of the earliest phosphorylated substrates after optimal TCR-mediated stimulation (*9, 10, 12, 13*). Under these conditions, there is also activation of lck and ZAP-70, phosphorylation of TCR, recruitment of Shc-Grb2-Sos, activation of Ras, and transcription of IL-2 (*31*). In anergic cells, these events cannot be initiated (*2–4*) and activation of Rap1 predominates. Therefore, Cbl may balance the activation of GTP-binding proteins by activating a negative regulator of the Ras pathway. In support of this hypothesis, in *Caenorhabditis elegans* the Cbl-like molecule sli-1 is a negative regulator of *Let-23*, which affects activation of *Let-60*, a Ras homolog pathway (*32*).

Our results suggest that the key determinant of the functional outcome of TCR-initiated signals may be the ratio of Ras-GTP to Rap1-GTP. Increases of Ras-GTP result in a positive balance for IL-2 transcription even in the presence of Rap1-GTP. In contrast, in the absence of Ras activation, as occurs in anergy, Rap1-GTP predominates, which results in blockade of IL-2 transcription. The ability of Rap1-GTP to antagonize TCR-mediated IL-2 transcription suggests that stimulation of guanidine nucleotide exchange on Rap1 or inhibition of Rap1-GAP are potential targets of therapeutic approaches for the modification of T cell immune response and the generation of antigen-specific tolerance.

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21. The ^{32}P-labeled anergic or control cells (2×10^7 per sample) were unstimulated or stimulated by TCR and CD28 cross-linking for the indicated time intervals in Fig. 3. Rap1 was immunoprecipitated with Rap1-specific antiserum (Santa Cruz Biotechnology) conjugated on protein A Sepharose, and Ras was immunoprecipitated with the Y13-259 monoclonal antibody (mAb) agarose conjugate (Santa Cruz Biotechnology). Labeled guanidine nucleotides bound to Rap1 or Ras were eluted, separated by thin-layer chromatography (TLC), and quantitated by direct scanning for beta radiation (Alpha Innotech, San Leonardo, CA). ^{32}P labeling, lysis, immunoprecipitation, elution of guanidine nucleotides bound to the proteins, and calculation of the GTP/(GTP+GDP) ratio were performed as described (*30, 36*).
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23. TAg Jurkat T cells are a derivative of the human T cell leukemia line Jurkat stably transfected with the SV40 large T antigen (*37*). TAg Jurkat cells were transiently transfected by electroporation under previously established conditions (*37*) with 20 μg of reporter construct pIL-2-luciferase (2 kb of the promoter/enhancer sequence) and 50 μg of the pAXEF vector containing either no insert (empty vector), Rap1-WT, or Rap1-63E cDNA (*22*). Because the pAXEF vector contains an SV40 origin of replication, transfection into TAg Jurkat cells results in high-level gene expression. Transfection efficiency was determined by cotransfection of B7-2 cDNA, which showed that expression, detected by fluorescence activated cell sorting (FACS) analysis, was consistently 65 to 70% for all transfection conditions. Transfection of equivalent amounts of Rap1-WT or Rap1-63E cDNA resulted in equivalent overexpression of Rap1 above endogenous levels as determined by protein immunoblot. Forty hours after transfection 5×10^5 viable cells were cultured in duplicates with either media or with a combination of TCR mAb [2Ad2, immunoglobulin M (IgM), 1:200] plus CD28 mAb (3D10, IgG2a, 5 μg/ml) or a combination of phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) plus ionomycin (1 μM) for 6 hours. Cell lysates were prepared and measured for luciferase activity as described (*38*). For titration experiments Jurkat cells were transiently transfected with 20 μg of reporter construct pIL-2 luciferase and variable amounts (1.9, 5.6, 16.7, or 50 μg) of pAXEF vector containing Rap1-63E or Rap1-WT cDNA. The total amount of transfected cDNA was normalized to 50 μg by cotransfecting vector cDNA.
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Counteraction by MutT Protein of Transcriptional Errors Caused by Oxidative Damage

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Oxidized guanine (8-oxo-7,8-dihydroguanine; 8-oxo-G) is a potent mutagen because of its ambiguous pairing with cytosine and adenine. The *Escherichia coli* MutT protein specifically hydrolyzes both 8-oxo-deoxyguanosine triphosphate (8-oxo-dGTP) and 8-oxo-guanosine triphosphate (8-oxo-rGTP), which are otherwise incorporated in DNA and RNA opposite template A. In vivo, this cleaning of the nucleotide pools decreases both DNA replication and transcription errors. The effect of *mutT* mutation on transcription fidelity was shown to depend on oxidative metabolism. Such control of transcriptional fidelity by the ubiquitous MutT function has implications for evolution of RNA-based life, phenotypic expression, adaptive mutagenesis, and functional maintenance of nondividing cells.

Spontaneous errors in DNA, RNA, and protein synthesis, all of which can influence protein activity, occur at rates of about 10^{-9}, 10^{-5}, and 10^{-4} per residue, respectively (*1*). Errors in DNA synthesis can produce mutant genes, which lead to alteration of proteins derived from those genes in the mutant cell clone. However, the higher error rates of transcription and translation produce a wider variety and, in general, a greater number of error-containing proteins. Erroneous proteins may have altered functions that trigger an error propagation process (*2*) resulting in functional defects and, ultimately, cell death, possibly by error catastrophe (*3*).

To examine the cellular controls of the fidelity of information transfer from DNA to protein, we have used six *E. coli* strains, each containing a different single base substitution in codon 461, which encodes glutamic acid in the wild-type (WT) LacZ protein (*4*). The lowest levels of residual β-galactosidase activity (leakiness) were observed in the strains used to measure the mutations A:T to C:G and A:T to G:C (about 0.013 Miller unit). At the DNA level, A:T to C:G mutations are greatly increased in *mutT*-deficient bacteria. MutT protein hydrolyzes 8-oxo-dGTP, a major oxidation product, to 8-oxo-deoxyguanosine monophosphate (8-oxo-dGMP). 8-Oxo-dGTP is a potent mutagen in that it is readily incorporated into DNA and is inserted with equal frequency opposite template C or A (*5*). By removing 8-oxo-dGTP from the triphosphate pool, MutT protein reduces spontaneous mutations by a factor of 1000 (*6–8*).

8-Oxo-dGTP is produced by oxidation of dGTP mediated by free radicals. It is likely that oxygen free radicals oxidize ribonucleotides and deoxyribonucleotides with similar efficiencies and that the resulting molecules are structurally similar. MutT protein is known to hydrolyze some ribonucleotide analogs, such as 8-bromo-rGTP, albeit with low efficiency (*9*). If MutT protein can hydrolyze other ribonucleotide analogs such as 8-oxo-rGTP as well as it hydrolyzes 8-oxo-dGTP, it may have an important role in controlling the fidelity of information transfer from DNA to protein.

To examine the effect of *mutT* deficiency on the fidelity of these processes in vivo, we used P1-mediated transduction to introduce a *mutT* mutation into the LacZ$^-$ strains used to measure leakiness (i.e., partial phenotypic suppression). A 30-fold increase in leakiness in the strain requiring template A to pair with G (namely, A:T to C:G transversion strain) was observed; the other mutant strains were unaffected (Table 1).

The specific increase in β-galactosidase activities in the A:T to C:G tester strains in a *mutT*$^-$ background cannot easily be explained solely by generation of Lac$^+$ bacteria. The following formula helps to consider the contribution of revertants to the β-galactosidase activity of the population, M(pop), measured in Miller units: $M(pop) = M(lac^+)f(lac^+) + M(lac^-)f(lac^-)$ where $M(lac^+)$ and $M(lac^-)$ are β-galactosidase activities of Lac$^+$ and Lac$^-$ cells, and $f(lac^+)$ and $f(lac^-)$ their respective frequencies.

This formula can be applied to an overnight liquid culture where the frequency of Lac$^-$ is close to 1 and the frequency of A:T to C:G Lac$^+$ revertants is less than 10^{-5} in a *mutT*$^-$ context (*4*, *10*). Cultures containing more Lac$^+$ were discarded from the analysis. Appropriate reconstruction experiments were carried out that have validated these assumptions (*10*). Thus, if the increase in β-galactosidase concentration as a result of the *mutT* mutation (from 0.013 to 0.39) were due only to

Table 1. MutT protein counteracts the effect of oxidative metabolism on the residual β-galactosidase activity (leakiness) of an *E. coli lacZ* mutant. For clarity, only the values obtained for the A:T to C:G reversion mutation strain are shown. For tester strains representing all other possible base-pair changes, under both aerobic and anaerobic conditions, the *mutT*/WT ratio of β-galactosidase activities varied between 0.7 and 1.3, thus showing the specificity of *mutT* effect. Bacterial strains were constructed by transferring CC101-106 (*4*) episomes to NR3835 [Δ(*pro-lac*) *ara thi trpE9777*], which had previously been cured from its episome by treatment with acridine orange. A *mutT* mutation was introduced in the six strains by P1-mediated cotransduction with *leu*::Tn*10*. Liquid cultures were grown in rich 869 medium (5 g of NaCl per liter, 10 g of Bactotryptone per liter, 5 g of yeast extract per liter agar per liter) under aerobic or anaerobic conditions (the latter were obtained by using Generbox). β-Galactosidase activities were measured and are expressed as described by Miller (*11*). To measure low levels of β-galactosidase activities, longer incubation times were necessary (up to 1 day, always controlling for spontaneous hydrolysis of *o*-nitrophenyl-β-D-galactoside). Each value is the average ± standard error of at least four experiments. The relatively high variation in the *mutT*$^-$ background remains to be explained (one might want to invoke the role of secondary mutations or some kind of error propagation). Reconstruction experiments show that this variation is not due to errors associated with measurements of low levels of β-galactosidase activity.

β-Galactosidase	WT	*mutT*	*mutT*/WT
Aerobiosis	0.013 ± 0.002	0.39 ± 0.21	30
Anaerobiosis	0.009 ± 0.001	0.018 ± 0.002	2

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