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Telomerase Regulation during Entry into the Cell Cycle in Normal Human T Cells

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Telomerase activity is involved in telomere length maintenance. Leukocytes, unlike many human somatic tissues, have detectable telomerase activity. These cells provide a normal human cell type in which to study telomerase. We studied the regulation of telomerase activity and the telomerase RNA component as leukocytes were stimulated to enter the cell cycle. In primary human leukocytes stimulated with phytohemagglutinin, telomerase activity increased >10-fold as naturally quiescent cells entered the cell cycle. Antibodies to the T cell receptor (TCR)/CD3 complex and the costimulatory CD28 receptor induced telomerase activity in a T cell-enriched population of cells. Rapamycin, an immunosuppressant that blocks TCR/CD3 signal transduction pathways and cdk2 activation, blocked telomerase induction. Hydroxyurea, an inhibitor of S phase, did not block cdk2 kinase activity or telomerase activation. In summary, telomerase is regulated in G1 phase as normal human T cells enter the cell cycle.

INTRODUCTION

In mammals, cell-cycle progression is often regulated by external signals that are transmitted to a cell through a variety of well-defined signal transduction pathways. Understanding the processes that are regulated by the external signals provides valuable insight into the regulation of cell proliferation. Telomerase activity is tightly regulated during mammalian development and may be required for the long-term proliferation of some cell types. To begin to understand the pathways that regulate telomerase in normal human cells, we studied telomerase activity during human T cell activation.

Telomerase is a ribonucleoprotein DNA polymerase that synthesizes telomere repeats onto chromosome ends (Greider and Blackburn, 1985, 1987; Morin, 1989; Prowse *et al.*, 1993). In most eukaryotes telomere DNA consists of many tandem repeats of simple sequence motifs; for example, human telomeres contain ~10 kilobase pairs (kbp) of tandem TTAGGG repeats (reviewed in Henderson, 1995). The addition of telomere sequence repeats by telomerase balances telomere shortening that occurs with each cell division, presumably caused by the inability of DNA polymerases to completely replicate the chromosome ends (Watson, 1972; Olovnikov, 1973).

When telomere elongation is compromised in yeast by deleting telomerase components or other regulators of telomere length, telomeres shorten and cell viability decreases dramatically (Lundblad and Szostak, 1989; Singer and Gottschling, 1994; McEachern and Blackburn, 1995). This loss in cell viability is likely due to chromosome instability. Chromosomes without telomeres are unstable, lead to rearrangements (McClintock, 1941, 1942; reviewed in Greider, 1991) and chromosome loss (Haber and Thorburn, 1984), and can signal cell-cycle arrest (Sandell and Zakian, 1993), presumably because they are recognized as damaged DNA.

During the growth of immortal single-cell organisms, such as ciliates and yeast, telomere length is established as an equilibrium between telomere shortening and telomere elongation by telomerase (reviewed in Greider and Harley, 1996). In mammals, telomere length and telomerase activity are developmentally regulated. Telomere length varies between different tissues in adult mice, and these differences are established during postnatal development (Prowse and Greider, 1995). The RNA component of telomerase is present at high levels in newborn mice

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but is specifically down-regulated during postnatal growth (Blasco *et al.*, 1995). Although many adult human somatic tissues do not express telomerase, some adult tissues, such as lymphocytes, do express active enzyme (Broccoli *et al.*, 1995; Counter *et al.*, 1995; Hiyama *et al.*, 1995).

Telomerase activity is present in many human tumors and immortal cell lines (reviewed in Harley *et al.*, 1994; Kim *et al.*, 1994; Bacchetti and Counter, 1995). Evidence from cultured cell lines suggests that telomerase activation may be required for the indefinite proliferation of immortal cancer cells (Counter *et al.*, 1992, 1994; Shay *et al.*, 1993). Thus, telomerase inhibitors have been proposed as potential anticancer agents (reviewed in Harley *et al.*, 1994; Bacchetti and Counter, 1995).

Telomere shortening with aging is observed in a variety of human cell types, including white blood cells (Harley *et al.*, 1990; reviewed in Harley, 1995a; Hastie *et al.*, 1990; Lindsey *et al.*, 1991; Vaziri *et al.*, 1993). In addition to total leukocyte populations, telomere shortening is seen in hematopoietic progenitor cells (Vaziri *et al.*, 1994) and in $CD4^+$ naive and memory cells (Weng *et al.*, 1995). In the $CD4^+$ T cells, the naive cells have longer telomeres than the memory T cells, and long telomeres correlated with a higher replicative capacity in culture (Weng *et al.*, 1995).

Although normal leukocytes express telomerase activity (Broccoli *et al.*, 1995; Counter *et al.*, 1995; Hiyama *et al.*, 1995), telomere length shortens in leukocytes in vivo (Vaziri *et al.*, 1993). Telomere shortening during human aging has also been observed in hematopoietic progenitor cells ($CD34^+$, $CD38^{-/lo}$) that express telomerase (Vaziri *et al.*, 1994; Hiyama *et al.*, 1995). Either the telomerase that is present in these cells is insufficient to maintain telomeres, or some additional essential component of telomere length maintenance is absent in these cells.

Although the identification of telomerase activity in normal leukocytes raises new considerations for antitelomerase cancer therapies, it also provides an opportunity to study telomerase regulation in a noncancerous cell type. Signal transduction pathways that regulate leukocyte development and proliferation are well established. Unstimulated leukocytes isolated directly from human blood are arrested in the G0 phase of the cell cycle (reviewed in Crabtree, 1989; Ullman *et al.*, 1990). Cross-linking of cell surface receptors on T cells by lectins or antibodies initiates a signal transduction cascade that alters gene expression and leads to entry into the cell cycle and DNA synthesis (Ullman *et al.*, 1990; Crabtree and Clipstone, 1994). To begin to understand telomerase regulation in normal human cells, we analyzed telomerase activity and the telomerase RNA component in T cells stimulated to enter the cell cycle.

MATERIALS AND METHODS

Isolation and Culturing of Leukocytes

Peripheral blood leukocytes were isolated from heparin-treated blood by density gradient centrifugation in Ficoll and sodium diatrizoate solution (Lymphocyte Separation Medium; Boehringer Mannheim, Indianapolis, IN). Whole blood was layered on the Ficoll solution and centrifuged for 20 min at 1800 rpm. The gradient interface, containing peripheral blood mononuclear cells, was collected, and the cells were washed in phosphate-buffered saline (PBS). The percentage of $CD2^+$ cells (T cells) and $CD20^+$ cells (B cells) was determined by cell surface staining and flow cytometry with anti-CD2 antibodies (clone RPA-2.10; Pharmingen, San Diego, CA) and CD20 antibodies (clone 2H7; Pharmingen). The leukocytes were typically cultured at 5–10 $\times$ 10^5/ml in RPMI media (Life Technologies, Gaithersburg, MD) supplemented with 2 mM glutamine and 20% fetal bovine serum (FBS; HyClone, Logan, UT). For the stimulation/depletion/restimulation experiment, the leukocytes were cultured at 5 $\times$ 10^6 cells/ml from day 0 to day 3. At the time of addition of the IL-2, the cells were diluted 10-fold in serum-containing media.

Stimulation and Inhibition of Leukocyte Proliferation

Phytohemagglutinin (M form; Life Technologies) and interleukin 2 (Lymphocult-T; Biotest, Denville, NJ) were prepared according to the manufacturer's instructions. Interleukin 2 was added to the culture medium at a final concentration of 20 U/ml. Rapamycin (kind gift of the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD) was dissolved in 100% ethanol and added to the medium at a final concentration of 0.05–500 nM. Hydroxyurea (Sigma Chemical, St. Louis, MO), dissolved in sterile distilled water, was added to the medium at a final concentration of 10 mM.

Enrichment for Quiescent T Cells and CD3 and CD28 Stimulation

A leukocyte fraction enriched for quiescent T cells was prepared as described (Firpo *et al.*, 1994). Peripheral blood leukocytes from a Ficoll gradient interface were washed with PBS, pelleted, resuspended in 1 ml of 150 mM NaCl and 30% Percoll (Pharmacia, Piscataway, NJ) solution, and layered onto a discontinuous Percoll gradient. The gradient contained 2.5 ml of each of the following Percoll solutions (bottom to top): 60, 55, 50, 45, and 40%. Colored density beads (Pharmacia) were included in an adjacent gradient as density markers. After centrifugation at 1500 $\times$ *g* for 20 min, the dense fraction (including the 55%/60% Percoll interface and 1.5 ml of the 60% fraction) was removed and diluted to <15% Percoll in PBS. To deplete B cells, the cells were pelleted, resuspended in RPMI + 20% FBS, and loaded onto a sterile, nylon wool column in one void volume. After incubation for 1 h at 37°C, the nonadherent cells were collected by washing the column with several void volumes of media. Cell number was determined at each step of purification. From 50 ml of whole blood, 5 $\times$ 10^7 cells were obtained from the Ficoll interface, 3.3 $\times$ 10^6 cells from the dense fraction of the Percoll gradient, and 1.5 $\times$ 10^6 cells from the nylon wool column. The cells were pelleted, resuspended in media, and cultured in the presence of anti-CD3 and anti-CD28 antibodies. The anti-CD3 antibodies (OKT3 ascites; kind gift of Dr. Dafna Bar Sagi, State University of New York, Stony Brook) were diluted 1:10 in PBS and used to coat the wells of a 96-well tissue culture plate by incubation at 37°C for 1 h, followed by washing with PBS. Anti-CD28 antibodies were added to the media at a final concentration of 10 ng/ml.

Cell Lysis and Polymerase Chain Reaction (PCR)-enhanced Telomerase Assay

Cells (1×10^6) were lysed in 100 μl of CHAPS buffer [10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.5% CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate), 10% glycerol, 5 mM β-mercaptoethanol, 1 mg/ml pepstatin, 10 mg/ml leupeptin, and 0.1 M phenylmethylsulfonyl fluoride]. Insoluble cellular components were removed by centrifugation at 14,000 × *g* for 10 min. Each extract was divided and incubated in the presence or absence of DNase-free RNase (1.0 μg RNase/20 μg total protein) for 15 min at 37°C. Telomerase activity in each extract was measured with a modified version of the Telomeric Repeat Amplification Protocol (TRAP; Kim *et al.*, 1994). The modified telomerase assay was performed as follows: 5 μl of cellular extract was mixed with 5 μl of a 2× telomerase reaction mix containing (in mM) 100 Tris acetate, pH 8.5, 100 potassium acetate, 6 $MgCl_2$, 2 spermine, 2 EGTA, 10 β-mercaptoethanol, 4 thymidine triphosphate (dTTP), 4 dATP, 4 2-deoxyguanosine-5'-triphosphate (dGTP), and 2 unlabeled telomerase substrate (TS) oligonucleotide (Kim *et al.*, 1994). The TS oligonucleotide served as a substrate for telomerase addition of deoxynucleotides. The reaction samples were incubated at 30°C for 60 min. Forty microliters of PCR amplification mix (20 mM Tris-HCl, pH 8.3, 68 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 0.5% Tween, 0.4 mM dCTP, and 20 fM ^{32}P-end labeled TS oligonucleotide) were added to the 10-μl telomerase reaction. The specific activity of TS oligonucleotide in the amplification step was 2×10^4 cpm/pmol. To assure "hot start" PCR, the samples were sealed with wax, and 0.1 μg of the second PCR primer (a modified version of the CX primer [Kim *et al.*, 1994; N. Kim, personal communication]) and *Taq* polymerase (2 U) were spotted onto the wax barrier. As a control for PCR efficiency, 0.1 fmol of a synthetic telomerase product (TS+5; [5' AATCCGTCGAGCAGAGTTAGGGTTAGGGTTAGGGTTAGGTTAG 3']) was added to RNase-treated samples. The samples were incubated at 94°C for 2 min followed by 18–20 cycles of PCR (94°C/30 sec, 50°C/30 sec, 72°C/90 sec). Five microliters of the PCR products were mixed with 5 μl of deionized formamide. The samples were incubated at 90°C for 1 min, and the amplified telomerase products were resolved on a 12% acrylamide/7 M urea/0.6 × trisborate EDTA (TBE) gel.

Telomerase activity was quantified by phosphorimaging. The signal intensity from each lane of the gel was quantified by defining a rectangular boundary around all visible bands in the lane with the most intense signal. A rectangle of equivalent size and shape was used for quantifying the signal from each lane on a gel. The signal present in the "no extract" lane was subtracted as background.

Measurement of DNA Content and Percentage of Cellular Debris by Flow Cytometry

One million cells were resuspended in 500 μl of PBS plus 1% calf serum. Five ml of ethanol (75–95%) were added to fix the cells. The cells were stored at 4°C overnight (or up to 2 wk) before staining. For staining, the fixed cells were removed from the ethanol by centrifugation and resuspended in PBS with 50 μg/ml propidium iodide and 100 μg/ml RNase A. After 1 h of incubation at 37°C, the cells were either stored at 4°C or analyzed immediately by flow cytometry with an EPICS elite enhanced sorting performance (ESP) (Coulter Corporation, Hialeah, FL). The percentage of cells in each phase of the cell cycle was determined by the Multicycle software program (distributed by Phoenix Flow Systems, San Diego, CA).

The percentage of cellular debris was determined from ungated histograms with the Multicycle program. This is a measurement of total debris in the histogram. The percentage of background and debris (% BAD) was also measured. The % BAD was defined by the DNA Cytometry Consensus Conference as the proportion of the histogram events between the leftmost G1 and the rightmost G2 that is modeled as debris or aggregates. The % BAD is unaffected by histogram end points. For the samples shown in Figure 3, % BAD was as follows: phytohemagglutinin (PHA), 5.5%; PHA+IL-2, 2.7%; PHA+IL-2 depletion, 3.4%; restimulation, 7.1%.

Quantitation of Human Telomerase RNA

Total RNA was prepared from leukocytes cultured for 2 d in PHA or media alone. The cells were lysed in 0.4 ml of lysis buffer (1.7 M guanidinium thiocyanate, 10 mM sodium citrate, 90 mM sodium acetate, 40 mM β-mercaptoethanol, 0.4% *N*-lauroylsarcosine, and 50% water-saturated phenol, pH 4.5) per 10^6 cells with vortexing. Two-tenths (0.2) volume of chloroform was added, and the samples were incubated on ice for 15 min. After centrifugation, the aqueous phase was removed. The RNA was precipitated by adding an equal volume of isopropanol. The yield of RNA was 113 and 6 mg from 1.7×10^7 PHA-stimulated and 3.1×10^7 unstimulated cells, respectively.

Total RNA (1 or 5 μg) was loaded per lane on a 6% acrylamide/7 M urea/0.6× TBE gel. After electrophoresis, the RNA was transferred to nylon membrane (Hybond N+; Amersham, Arlington Heights, IL) by electroblotting in 1× TBE. The RNA was cross-linked to the filter with 120 mJ UV light in a Stratalinker (Stratagene, La Jolla, CA). The filter was prehybridized in 200 mM sodium phosphate, pH 7.2, 15% formamide, 1 mM EDTA, 7% SDS, and 10 mg/ml bovine serum albumin (BSA). Probes for the human telomerase RNA and 5S RNA were added simultaneously to the prehybridization mixture. The human telomerase RNA (hTR) antisense riboprobe was generated by in vitro transcription of the hTR gene cloned behind the SP6 promoter in the pGEM-5Zf vector. The specific activity of the hTR probe was 1.6×10^5 cpm/fmol. The 5S RNA probe was generated by random hexamer labeling of a 170 bp PCR fragment encompassing the 5S gene (PCR primers 5'GCACGGCCGGCCGGGCTG3' and 5'AAGCCTACAGCACCCGG3'). The specific activity of the 5S RNA probe was 5×10^4 cpm/fmol.

Immunoprecipitation, Kinase Assay, and Western

For immunoprecipitations and immunoblotting, the cells were lysed in NP40 buffer (50 mM HEPES, pH 7.0, 50 mM NaCl, 0.1% NP40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 5 mM dithiothreitol), and insoluble debris was removed by centrifugation at 14,000 × *g* for 10 min. For immunoprecipitation and kinase assays, the lysates were used immediately and were not frozen, because this results in decreased kinase activity. Before immunoblotting, lysates were stored at −80°C. Immunoprecipitations and immunoblotting were performed as described in Buchkovich and Ziff (1994). For immunoprecipitations, extracts containing 70 μg of total protein, as determined by Bio-Rad Protein Concentration Assay, were used. Anti-Cdk2 polyclonal antibody (5 μg; Upstate Biotechnology, Lake Placid, NY) or polyclonal rabbit anti-mouse antibody (Upstate Biotechnology) was added to each sample. For immunoblotting, extracts containing 50 μg of protein were loaded. The same blot was probed with 2 μg/ml anti-cdk2 polyclonal antibody (Upstate Biotechnology), 2 μg/ml anti-cyclin E (clone HE12; Pharmingen), and 1:1000 dilution of anti-$p27^{Kip1}$ antisera (kind gift of Hui Zhang, Cold Spring Harbor Laboratory, NY), followed by HRP-conjugated goat anti-rabbit (Sigma) or HRP-conjugated rabbit anti-mouse (Cappel). The HRP activity was detected by an enhanced chemiluminescent substrate system (SuperSignal; Pierce Chemical, Rockford, IL). The blot was stripped of antibodies for sequential probing by incubation in 2% SDS, 62.5 mM Tris, pH 6.8, and 100 mM β-mercaptoethanol for 20 min at 65°C.

For kinase assays, immune complexes were washed with kinase buffer containing (in mM) 20 Tris, pH 7.5, 30 NaCl, 10 $MgCl_2$, and 1.0 dithiothreitol and then incubated in a 50 μl reaction containing kinase buffer (1×), 20 μM ATP, 5 μCi γ-^{32}P-ATP (6000 Ci per mmol), and 50 μg/ml histone H1 (Boehringer Mannheim). The reactions were stopped by the addition of 2× Laemmli sample buffer. Histone H1 was separated from free γ-^{32}P-ATP on 10% polyacrylamide gel.

RESULTS

Human peripheral blood leukocytes were isolated from whole blood by density centrifugation in Ficoll. To determine the percentage of T cells and B cells, the leukocytes were analyzed for cell surface markers by antibody staining and flow cytometry. In a typical experiment 65% of the leukocytes were T cells ($CD2^+$), and 5% were B cells ($CD20^+$). A large percentage of the remaining cells were monocytes, based on the scattering properties of the unstained cells during flow cytometry.

The leukocytes were treated with PHA, which binds to the TCR/CD3 complex on T cells (Kanellopoulos *et al.*, 1985). In a mixed leukocyte population from peripheral blood, PHA stimulation is sufficient to cause T cell proliferation (Meuer and Meyer zum Bueschenfeld, 1986). After 2 d of PHA treatment, >30% of the leukocytes left the G0 phase and entered S phase (Figure 1). The levels of telomerase activity in extracts from the untreated and PHA-treated cells were compared (Figure 1). Telomerase activity was measured with a PCR-enhanced telomerase assay in which the enzyme was assayed under optimal conditions (Morin, 1989), and reaction products were amplified by PCR. A minimal number of PCR amplification cycles was used to minimize the possibility of PCR-generated artifacts and to insure quantitative results (see MATERIALS AND METHODS). In extracts from the untreated leukocytes, telomerase activity was not detected (Figure 1). In extracts from cells treated for 1 d with PHA, a low level of activity just above the lower limit of detection of the assay was observed. From day 1 to day 2 there was a >10-fold increase in activity as quantified by phosphorimaging (see MATERIALS AND METHODS), and the level of activity remained high in extracts from cells treated for 3 d with PHA. The timing of the increase in telomerase activity correlated with the increase in the percentage of cells in S phase.

To verify that the telomerase activity assayed in the heterogeneous population of leukocytes after PHA treatment resulted from T cells, we tested the ability of antibodies specific for the TCR/CD3 complex to upregulate telomerase activity in a T cell-enriched fraction of leukocytes. In the absence of antigen-presenting cells, maximal stimulation of interleukin 2 (IL-2) expression and T cell proliferation requires TCR/CD3 signaling along with a costimulatory signaling pathway (Schwartz, 1992; Shahinian *et al.*, 1993). Thus, we expected that, if the increase in telomerase activity results from T cells and is dependent on IL-2 expression, both TCR/CD3 and CD28 signaling would be required for maximal telomerase expression.

We isolated a T cell-enriched fraction of leukocytes (Firpo *et al.*, 1994) and cultured the cells in the presence of anti-TCR/CD3 and/or anti-CD28 antibodies. After 2 d of culturing in the presence of one or both antibodies, the cells were harvested and analyzed for telomerase activity (Figure 2). Telomerase activity was not observed in the control (media alone), anti-TCR/CD3, or anti-CD28 samples. However, telomerase activity was detected in the sample treated with both

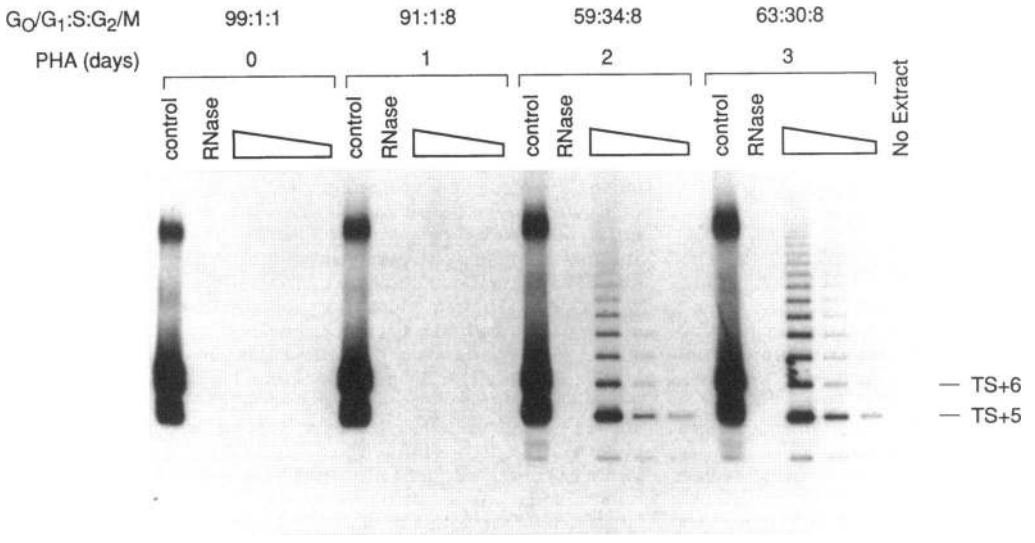


Figure 1. PHA treatment of peripheral blood leukocytes led to an increase in telomerase activity and an increase in the percentage of cells in S phase. Freshly isolated leukocytes (day 0) or leukocytes cultured in the presence of phytohemagglutinin (PHA) for 1, 2, or 3 d were assayed for cell-cycle phase distribution and telomerase activity. Cell-cycle phase distribution (the percentage of cells in G0/G1 phase, S phase, and G2/M phase) was determined by propidium iodide staining and flow cytometry. Telomerase activity was analyzed with a PCR-enhanced telomerase assay at three protein concentrations: 5.0, 2.5, and 1.3 μg of total protein per reaction. RNase treatment of each extract (at the highest protein concentration) before the telomerase assay verified that the assay measured an RNase-sensitive activity. The addition of a synthetic telomerase product, the oligonucleotide TS+5, to each RNase-treated extract before PCR amplification served as a control for PCR efficiency (control). Extract was omitted from one reaction sample to control for extract-independent activity (no extract). A ^{32}P-labeled synthetic telomerase product (TS+5) equivalent in base composition and sequence to the TS oligonucleotide plus $[TTAGGG]_5$, served as a molecular weight marker and comigrated with the smallest product observed in the TRAP assay.

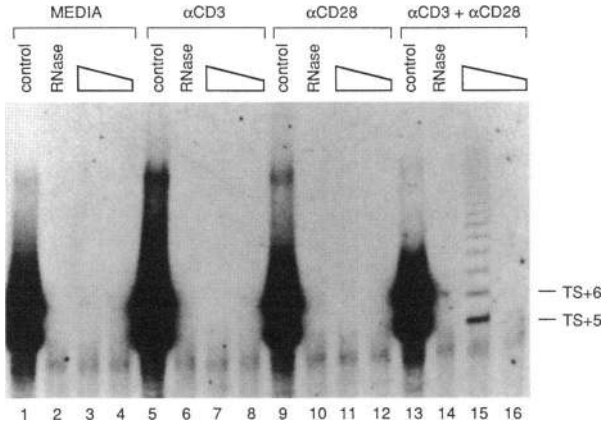


Figure 2. Treatment of leukocytes with antibodies specific for CD3 and CD28 resulted in an increase in telomerase activity. A T cell-enriched fraction of leukocytes was cultured for 2 d in the presence of only CD3 antibodies, only CD28 antibodies, both CD3 and CD28 antibodies, or media alone. The cells were lysed and assayed for telomerase activity at two protein concentrations (2.0 and 0.4 μg per reaction). Assay controls for PCR efficiency (control) and RNase-sensitivity (RNase) were included.

anti-TCR/CD3 and anti-CD28. Thus, telomerase was activated by the T cell-specific TCR/CD3 and CD28 signaling pathways. In this experiment, telomerase activation required both TCR/CD3 signaling and CD28 signaling (Figure 2). In a second experiment, a low level of telomerase activity was detected with anti-TCR/CD3 alone, no activity was detected with anti-CD28 alone, and a high level of activity was detected with anti-TCR/CD3 and anti-CD28 in combination (our unpublished results). In both experiments, maximal telomerase activation required both TCR/CD3 and CD28 signaling, as does maximal IL-2 expression and proliferation in T cells (Schwartz, 1992; Shahinian *et al.*, 1993). The requirement for both TCR/CD3 and CD28 signaling to achieve maximal telomerase activity is consistent with telomerase induction being downstream of IL-2 signaling. The timing of the increase in telomerase activity after PHA treatment of leukocytes (Figure 1) suggested that telomerase activation was a later event than IL-2 and IL-2Rα expression (Ullman *et al.*, 1990).

To further examine the correlation between cell cycling and telomerase activation, PHA-treated cells were treated with exogenous IL-2 at a concentration that would accelerate cell cycling, the IL-2 and PHA were then depleted to induce proliferative arrest, and the cells were restimulated with PHA. During this regimen of PHA and IL-2 stimulation, depletion, and restimulation (Figure 3A), telomerase activity and the percentage of cells in S phase were assayed. Cell number as an indicator of population doubling rates and

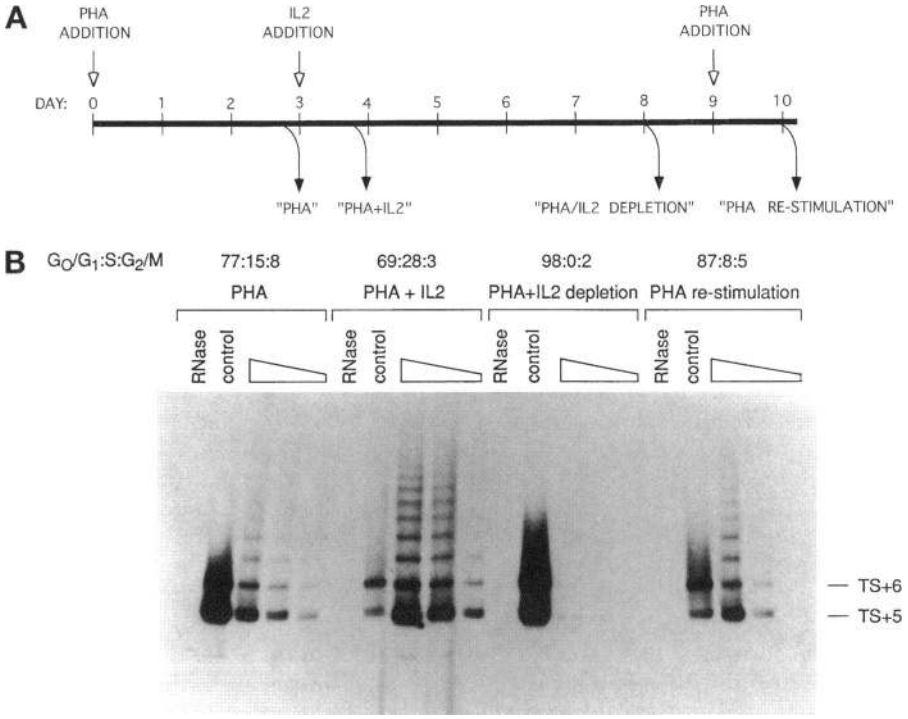


Figure 3. Telomerase activity varied with the proliferative state of leukocytes during PHA and IL-2 stimulation, depletion, and restimulation. (A) A timeline summarizing the culturing conditions and sampling time points. Leukocytes were cultured in the presence of PHA for 3 d (PHA) or in PHA for 3 d followed by IL-2 for 1 d (PHA+IL-2). The PHA was diluted 10-fold on day 3 at the time of IL-2 addition. The cell population doubled three times during the 24-h period after IL-2 addition. Neither PHA nor IL-2 was added to the media during days 4–8 (PHA/IL-2 depletion); the cell population doubled only once during this time period. PHA was added to the culture again on day 9 (PHA restimulation). Cellular debris as an indicator of cell death was monitored by flow cytometry. Percentage of cellular debris: PHA, 11%; PHA+IL-2, 6%; depletion, 19%; restimulation, 18%. (B) At each time point (days 3, 4, 8, and 10), a sample of leukocytes was removed from the culture and assayed for cell-cycle phase distribution and telomerase activity. Telomerase activity was analyzed with a PCR-enhanced telomerase assay at three protein concentrations: 2.5, 1.3, and 0.6 μg of total protein per reaction. Controls for RNase sensitivity (RNase) and PCR efficiency (control) were included.

cellular debris as an indicator of cell death were also monitored. Cells treated with PHA for 3 d had detectable telomerase activity (Figures 1 and 3B). After the initial 3 d of PHA treatment, cells were treated with IL-2. The cells divided rapidly after IL-2 addition, resulting in three population doublings in 1 d of treatment, an increase in the percentage of cells in S phase, and an increase in telomerase activity (Figure 3, A and B). During the next 4 d, IL-2 and PHA were not replenished in the culture, and the population doubling rate declined with only one doubling occurring from day 5 to day 8. By day 8 of the experiment, 98% of the cell population was in G0/G1 phase, and telomerase activity was no longer detected. Restimulation of the cells with PHA resulted in a modest increase in S phase cells and an increase in telomerase activity (Figure 3A). The magnitude of this increase cannot be compared directly with that seen after PHA stimulation of day 0 leukocytes, because the cellular composition of the population, the activation and differentiation state of the T cells, and the length of PHA treatment are different. This stimulation, depletion, and restimulation experiment demonstrated a correlation between cell-cycle entry and telomerase activation, as well as a correlation between cell-cycle exit and the down-regulation of telomerase activity. In addition, the superinduction of telomerase within 1 d of IL-2 treatment demonstrated telomerase regulation by IL-2 signaling pathways.

To determine whether the absence of telomerase activity in untreated leukocytes was due to the presence of an inhibitor of telomerase activity, telomerase-negative (day 0) and telomerase-positive (PHA, day 2) extracts were mixed at different concentrations (Figure 4). An equal amount or up to a ten-fold excess of telomerase-negative extract did not inhibit the activity in telomerase-positive extracts, although some decrease in the longest products was seen. We conclude that the telomerase-negative extracts did not contain a diffusible inhibitor of telomerase activity.

To investigate whether the level of the RNA component of telomerase (human telomerase RNA or hTR; Feng *et al.*, 1995) was regulated in leukocytes, we performed Northern analysis. RNA was isolated from leukocytes that were cultured in the absence or presence of PHA for 2 d. As a positive control, RNA was also isolated from a telomerase-positive (Prowse *et al.*, 1993), immortal T cell leukemia cell line, Jurkats. The steady-state levels of hTR relative to 5S RNA increased after 2 d of PHA stimulation (Figure 5), paralleling the increase in telomerase activity in these cells (Figure 1).

To investigate telomerase regulation in the pathway(s) downstream of IL-2 receptor and relative to cell-cycle progression, we blocked T cell proliferation with the immunosuppressive drug rapamycin. Rapamycin blocks antigen-induced T cell proliferation

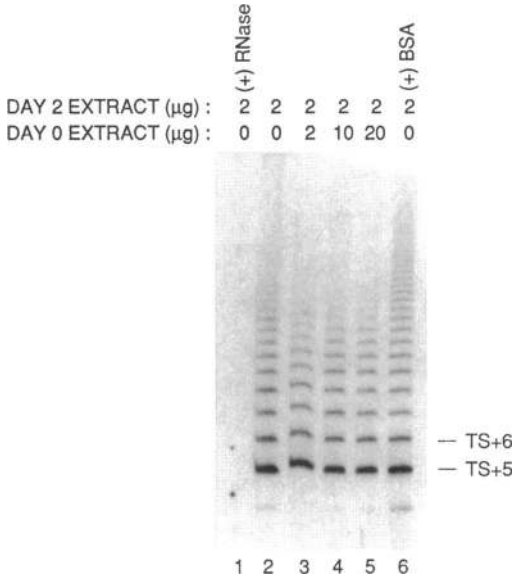


Figure 4. Extracts from quiescent leukocytes did not inhibit telomerase activity in extracts from PHA-treated leukocytes. A telomerase-negative extract (2, 10, or 20 μg, lanes 3–5) from untreated leukocytes (day 0, Figure 1) was mixed with 2 μg of a telomerase-positive extract (day 2, Figure 1) before assaying telomerase activity. The telomerase-positive extract was also assayed in the absence of the telomerase-negative extract (lane 2), in the presence of 10 μg of BSA (lane 6), or after RNase treatment (lane 1). The day 0 extract did not inhibit the telomerase activity in the day 2 extract, although some decrease in the longest products was observed.

downstream of IL-2 receptor and other growth factor receptors including IL-4 and IL-6 (reviewed in Sigal and Dumont, 1992; Morice *et al.*, 1993). Rapamycin inhibits the elimination of $p27^{Kip1}$, an inhibitor of cyclin-dependent kinases, and blocks the activation of the cyclin-dependent kinase 2 (cdk2), a kinase required for the G1-to-S phase transition (Nourse *et al.*, 1994). Rapamycin, however, does not block some other IL-2–mediated activities, such as the enhancement of cytolytic function in cytotoxic T cells (Morice *et al.*, 1993). We tested the ability of rapamycin to inhibit telomerase after PHA and IL-2 treatment. Freshly isolated quiescent leukocytes were treated with PHA and IL-2 in the presence of 5 or 50 nM rapamycin. The cells were harvested 2 d later and analyzed for cell-cycle phase distribution and telomerase activity (Figure 6). PHA and IL-2 treatment for 2 d resulted in an increase in S phase cells. Rapamycin treatment blocked both the increase in telomerase activity and the increase in S phase cells to control levels (Figure 6A). The effect of rapamycin over a broader concentration range, 0.05–500 nM, was determined (Figure 6B). Again, rapamycin inhibited both S phase and telomerase activity.

Rapamycin blocks cells in a G1-phase-like state (Firpo *et al.*, 1994; Nourse *et al.*, 1994). To determine

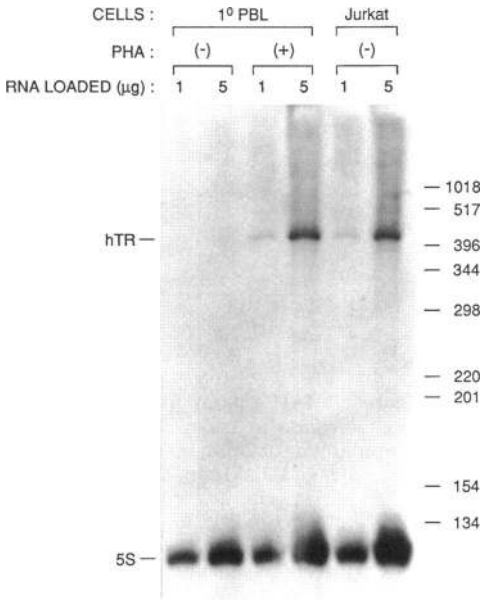


Figure 5. The steady-state level of the human telomerase RNA (hTR) increased after PHA treatment. Total RNA was isolated from leukocytes grown in the absence or presence of PHA for 2 d and from Jurkat cells, a human T cell leukemia line. Total RNA (1 and 5 μg) was run on a 6% acrylamide/7 M urea/0.6× TBE gel, transferred to nylon membrane, and probed with ^{32}P-labeled hTR antisense riboprobe and ^{32}P-labeled 5S RNA probe.

whether an inhibitor of DNA synthesis would have the same effect on telomerase activity, we treated leukocytes with hydroxyurea. Although hydroxyurea did inhibit S phase progression, the induction of telomerase activity was not blocked (Figure 6C). This demonstrated that telomerase induction occurred independently of DNA synthesis.

To rule out the possibility that rapamycin directly inhibited telomerase activity, rapamycin was added to a telomerase assay in vitro. Hydroxyurea was also tested for inhibition of telomerase in vitro. At concentrations equivalent to or 10-fold greater than those used in the leukocyte culture medium, neither rapamycin nor hydroxyurea inhibited telomerase activity in vitro (Figure 7). Thus, the effect of rapamycin on telomerase in vivo is likely to result from the inhibition of IL-2 signaling and of cell-cycle progression.

To examine the cell-cycle phase of the rapamycin- and hydroxyurea-blocked leukocytes, we used the cyclin-dependent kinase 2 (cdk2), cyclin E, and the $p27^{Kip1}$ cdk inhibitor as molecular indicators of cell-cycle phase progression. Cyclin E expression and cdk2 kinase activity increase gradually in G1 phase and are required for the G1-to-S phase transition (Pagano *et al.*, 1993; Tsai *et al.*, 1993; van den Heuvel and Harlow, 1993; Ohtsubo *et al.*, 1995); $p27^{Kip1}$ is expressed at high levels in quiescent T cells but at low levels in exponentially proliferating T cells (Nourse *et al.*, 1994).

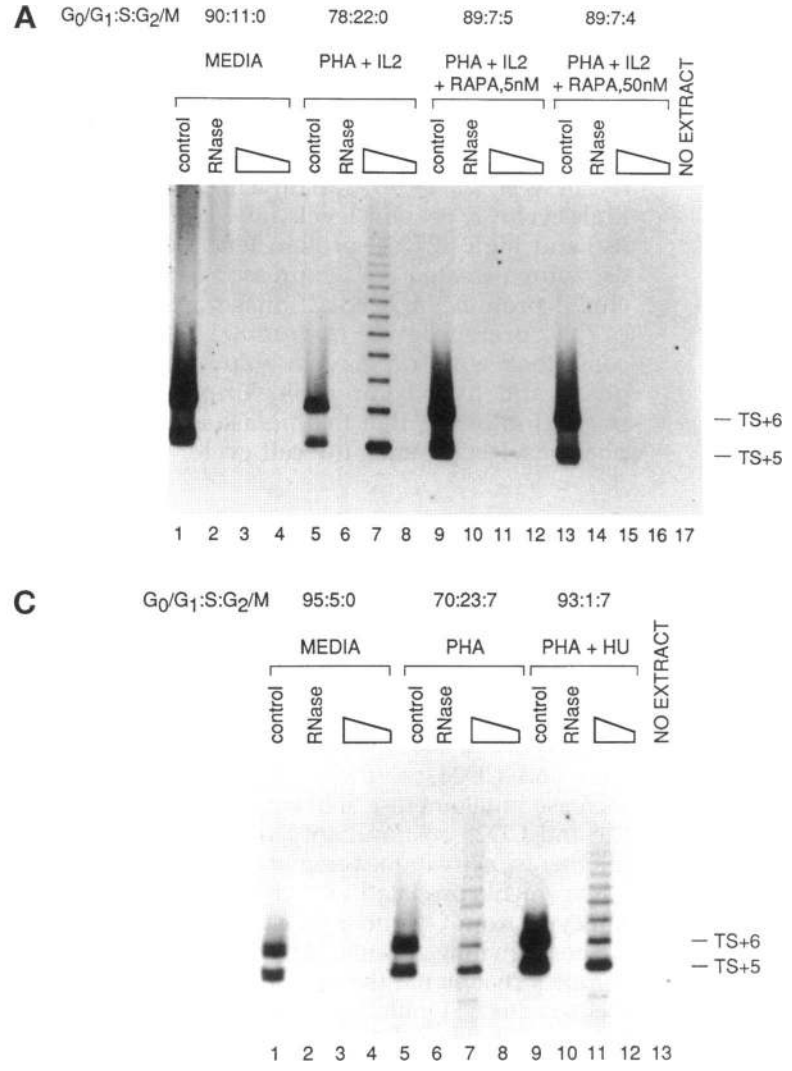
Leukocytes were treated for 2 d with PHA and IL-2 in the presence of rapamycin or hydroxyurea. The cells were lysed, and extracts were assayed for cdk2 kinase activity in vitro and for steady-state protein levels of cdk2, cyclin E, and $p27^{Kip1}$ by Western blotting. In agreement with published results (Nourse *et al.*, 1994), rapamycin-blocked cells had high cyclin E protein levels, low cdk2 kinase activity, and high $p27^{Kip1}$ protein levels (Figure 8). Hydroxyurea-treated cells expressed high levels of cyclin E protein, high cdk2 kinase activity, and low $p27^{Kip1}$ protein levels (Figure 8). These results are consistent with rapamycin inhibiting cells in G1 phase and hydroxyurea blocking cells in early S phase, indicating that telomerase is activated in G1 phase as T cells enter the cell cycle.

DISCUSSION

Although telomerase is not expressed in many somatic tissues, it is expressed in peripheral blood leukocytes (Broccoli *et al.*, 1995; Counter *et al.*, 1995; Hiyama *et al.*, 1995). These cells provided a normal human cell type in which to study telomerase regulation. PHA treatment of peripheral blood leukocytes (Figure 1; Hiyama *et al.*, 1995) or antibody activation of TCR/CD3 and CD28 receptors (Figure 2) resulted in an increase in telomerase activity. Thus, the T cell receptor and CD28 costimulatory pathways are linked to telomerase activation. Telomerase induction after TCR engagement correlated with the progression of T lymphocytes from G1 into S phase.

The signaling events downstream of TCR/CD3 complex that signal the entry into S phase have been well studied. Signals generated from the TCR establish an autocrine signaling pathway involving IL-2 and IL-2 receptor (IL-2R; Ullman *et al.*, 1990; Crabtree and Clipstone, 1994). Signals from the IL-2R commit the cell to a program of DNA synthesis by communicating with components of the cell-cycle machinery. The signaling pathway is blocked by the immunosuppressant rapamycin, which blocks the phosphorylation of the retinoblastoma protein (RB), the expression of cyclin A and PCNA, the down-regulation of $p27^{Kip1}$, and the activation of $p33^{cdk2}$ (reviewed in Chou and Blenis, 1995). We found that rapamycin blocked the induction of telomerase, whereas hydroxyurea did not, indicating that telomerase is regulated in G1 phase. The inability of hydroxyurea to block telomerase activation indicated that telomerase is induced before DNA synthesis and that DNA synthesis itself is not required for telomerase induction.

The increase in telomerase activity after PHA stimulation was accompanied by an increase in the level of the telomerase RNA component. Because the levels of the telomerase RNA component (Figure 4) and telomerase activity (Buchkovich and Greider,



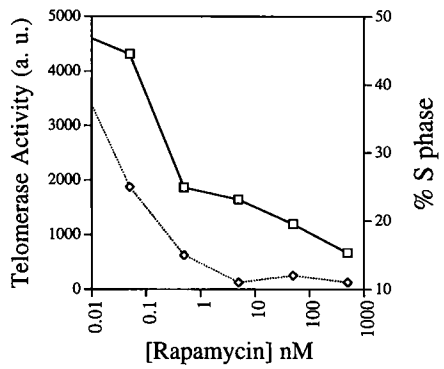


Figure 6. (A) Rapamycin inhibited the PHA-induced increase in telomerase activity. Leukocytes were cultured for 2 d in media alone; PHA and IL-2; PHA, IL-2, and 5 nM rapamycin; or PHA, IL-2, and 50 nM rapamycin. The cells were harvested and assayed for phase distribution by propidium iodide staining and flow cytometry and for telomerase activity with a PCR-enhanced telomerase assay at two protein concentrations (2.5 and 0.5 μg of total protein per reaction). Controls for RNase sensitivity (RNase) and PCR efficiency (control) were included. (B) Cells were cultured as in A, except that rapamycin was added at a final concentration of 0.05, 0.5, 5.0, 50, or 500 nM to the media. Telomerase activity was assayed at 2.0 μg of total protein extract per reaction (□). Telomerase activity in PHA and IL-2–treated cell extracts was quantitated on a BAS2000 and expressed as arbitrary units (a.u.). The percentage of cells in S phase (◇) was determined by propidium iodide staining and flow cytometry. (C) Hydroxyurea did not inhibit the PHA-induced increase in telomerase activity. Leukocytes were cultured for 2 d in media alone, PHA, or PHA and 10 mM hydroxyurea. The cells were harvested and assayed for cell-cycle phase distribution and telomerase activity. Telomerase activity was measured at two protein concentrations (5.0 and 0.5 μg of total protein per reaction). Controls for PCR efficiency (control; lanes 1, 5, and 9), RNase sensitivity (RNase; lanes 2, 6, and 10), and extract-independent activity (no extract; lane 13) were included.

unpublished results) were similar to those found in the homogeneous population of Jurkat cells. It is unlikely that only a small subset of the T cells in the leukocyte population was expressing telomerase. Telomerase induction during T cell activation could be regulated by mechanisms affecting the synthesis, processing, and assembly of telomerase components. Although mixing telomerase-negative extracts with telomerase-positive extracts did not inhibit telomerase, indicating there was not a diffusible telomerase inhibitor, regulation by a tightly-bound specific inhibitor is not excluded.

Although we observed a correlation between decreased telomerase activity and cell cycle exit, it is not yet clear to what extent these activities are linked in T cells and other cells. When immortal BALB/c3T3 cells were shifted from active growth to quiescence by either serum starvation or contact inhibition, the cells arrested in G0 after 24 h, yet telomerase activity remained constant relative to the levels in the cycling populations (Buchkovich and Greider, unpublished results). When similar immortal fibroblast populations were held quiescent only after 7–13 d, some decrease in telomerase activity was seen (Holt *et al.*, 1996). This suggests that telomerase activity is not tightly regulated with cell-cycle exit in immortal mouse fibroblast lines, or that the enzyme has a long half-life.

Differentiation in vitro of a variety of immortal cell types leads to low or undetectable telomerase activity (Sharma *et al.*, 1995; Holt *et al.*, 1996). The decline in telomerase activity was apparently slower than the decline of cell-cycle–associated activities in some cases. Whereas 2 d of retinoic acid treatment of HL60 cells is sufficient for the accumulation of

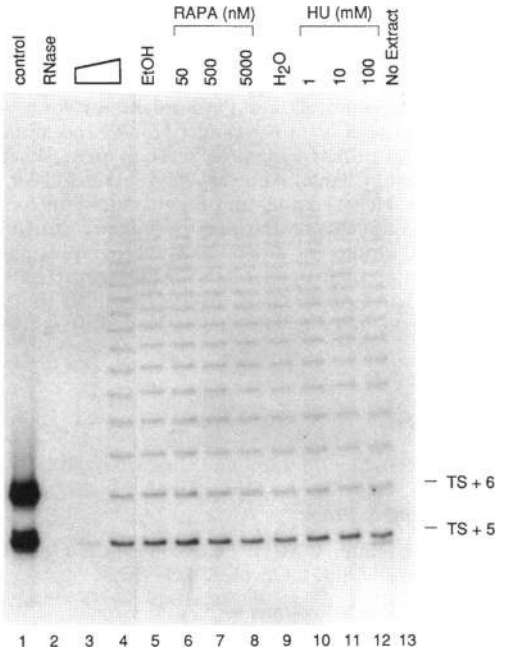


Figure 7. Neither rapamycin nor hydroxyurea inhibited telomerase activity in vitro. An extract from PHA-treated leukocytes was assayed for telomerase activity at two protein concentrations: 0.5 μg per reaction (lane 3) or 2.5 μg per reaction (lanes 1–2 and 4–12). Rapamycin (50, 500, or 5000 nM), hydroxyurea (1, 10, or 100 mM), or the solvents used to dissolve rapamycin and hydroxyurea (ethanol and water, respectively) were added to the leukocyte extract before the telomerase assay. Assay controls for PCR efficiency (control; lane 1), RNase-sensitivity (RNase; lane 2), and extract-independent activity (no extract; lane 13) were included.

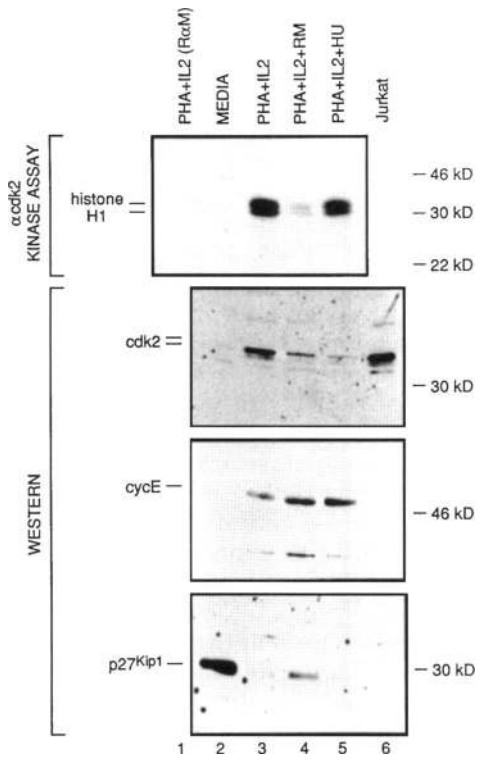


Figure 8. Rapamycin blocked cell cycling in G1 phase, whereas hydroxyurea blocked the cells at the start of S phase. Leukocytes were cultured for 2 d in media alone (lane 2); PHA and IL-2 (lanes 1 and 3); PHA, IL-2, and 50 nM rapamycin (lane 4); or PHA, IL-2, and 10 mM hydroxyurea (lane 5). The cells were lysed in NP40 buffer, assayed for cyclin-dependent kinase-2 (cdk2) activity in vitro, and the steady-state protein level of cdk2, cyclin E, and the cdk inhibitor $p27^{Kip1}$ by Western blotting. The immunoprecipitations before the kinase assay were performed with a polyclonal cdk2 antibody (top panel, lanes 2–5) or, as a control, a polyclonal rabbit anti-mouse IgG (top panel, lane 1). Histone H1 served as the substrate in the in vitro kinase reaction.

dephosphorylated retinoblastoma protein, a marker of G0/G1 phase cells (Mihara *et al.*, 1989), 3 d of retinoic acid treatment is insufficient for the down-regulation of telomerase activity (Sharma *et al.*, 1995). Whereas changes in E2F complexes are obvious by 5 d of retinoic acid treatment in F9 cells (La Thangue and Rigby, 1987), telomerase activity did not decrease even after 6 d (Sharma *et al.*, 1995). The long periods of time required to see reduction in telomerase in these systems again suggests that either telomerase activity has a long half-life or that the enzyme is not tightly regulated with cell-cycle exit in these cells.

The T cell experiments described in this paper addressed only the decisions to enter and exit the cell cycle, not the continued progression of cycling cells into each stage of the cell cycle. Phase-specific cell-cycle regulation of telomerase activity was not detected in HeLa (Avilion, 1995) or in two other immortal cell types, HT1080 or HL60 cells (Holt *et al.*, 1996). In all three cases, similar levels of activity were detected in cells in the G1, S, and G2/M phases of the cell cycle. Thus, the evidence to date suggests that telomerase activity is not regulated in a phase-dependent manner. Whether this lack of regulation is due to a relaxed control in immortal cell clones or is also found in normal human cells remains to be demonstrated.

The identification of telomerase regulation in normal human T cells by the TCR pathway demonstrates that mature T cells have telomerase activity. Previous experiments with unstimulated leukocytes showed low levels of telomerase activity in both mixed cell population (Counter *et al.*, 1995) and in fractionated samples containing specific cell types (Broccoli *et al.*, 1995). This activity may be attributed to the presence of progenitor stem cells in the population (Counter *et al.*, 1995; Harley, 1995b; Shay and Wright, 1996). However, peripheral blood T cells responding to TCR agonists are not progenitor hematopoietic cells but, rather, mature T cells that have survived thymic selection (Crabtree, 1989). The finding that telomerase is expressed in these cells is significant, because earlier data suggested that only

germline tissues, stem cells, and fetal tissues expressed telomerase.

The identification of telomerase activation in mature T cells raises questions as to the role of telomerase in these cells. Telomere shortening with aging has been observed in total leukocyte populations, in hematopoietic progenitor cells, and in $CD4^+$ naive and memory cells (Hastie *et al.*, 1990; Lindsey *et al.*, 1991; Vaziri *et al.*, 1993, 1994; Weng *et al.*, 1995). Although telomere shortening in many primary cell types in vitro has been interpreted as an indicator of the absence of telomerase (Harley, 1990; reviewed in Harley, 1991; Hastie *et al.*, 1990; Lindsey *et al.*, 1991), this may not always be the case. Telomeres shorten with age in hematopoietic progenitor cells ($CD34^+$ $CD38^{-/lo}$), yet telomerase activity is present (Vaziri *et al.*, 1994; Hiyama *et al.*, 1995). These data and our finding of telomerase activity in mature T cells suggest that the ability to express telomerase may not always lead to telomere length maintenance. There are large numbers of factors that are involved in maintaining telomere length, including telomerase, telomerase regulators, telomere binding proteins, and possibly telomere processing activities (reviewed in Greider, 1996). If some of these other factors are limiting in human T cells, the expression of telomerase will not be sufficient to maintain telomere length. An example of this is seen in the yeast *est1* mutant, which fails to maintain telomere length although the cells have levels of telomerase activity indistinguishable from wild-type cells (Lundblad and Szostak, 1989; Cohn and Blackburn, 1995). To understand the role of telomerase in mammalian somatic cells, it is essential to know whether or not telomerase is required in normal cells, such as T cells, that express it.

Normal peripheral blood T cells represent a highly specialized cell type that is poised in response to external signals to multiply or halt proliferation. The activation of telomerase seems to be keyed into the proliferative response pathways in this specialized cell type. The regulation of telomerase activity during the cycling of other human cells must be investigated more carefully to determine whether the regulation of telomerase with cell cycle entrance and exit is a common occurrence or unique to lymphocytes. Telomerase induction by TCR is the first example of a signaling pathway in normal cells that regulates telomerase. This opens the way for experimental analysis of "upstream" regulators. Mutations in signaling molecules that regulate telomerase in normal cells may be responsible for deregulated telomerase expression in tumor cells.

Note added in proof. In recent publications, Igarashi and Sakaguchi (1996) and Weng *et al.* (1996) also observed telomerase induction after stimulation of the T cell antigen receptor.

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