Telomerase Activity is Constitutively Expressed in the Murine CD8$^+$ T Cells and Controlled Transcriptionally and Post-Translationally

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ABSTRACT

Background: Telomerase, a ribonucleoprotein enzyme capable of synthesizing telomeric repeats, attracts attention for its possible role in determining the replicative capacity of normal somatic cells, transformed cells, and cells of the germline lineage. Differently from normal somatic cells with no telomerase activity, normal lymphocytes has been reported to have telomerase activity comparable to that found in transformed cells during development and activation, which substantiate a role in supporting the capacity of lymphocytes for extensive clonal expansion. **Methods:** Here, in order to define the telomerase regulation in murine T lymphocytes, telomerase activity in cloned murine CD8$^+$ T cells and naïve CD8$^+$ T cells isolated from C57BL/6 mice was examined. Next, the regulatory mechanism of telomerase activity at transcriptional and post- translational levels was investigated by determining the expression level of the TERT protein, a key component for telomerase activity. **Results:** It was demonstrated that telomerase activity was expressed in an inactivated state as well as in an activated state in the murine CD8$^+$ T lymphocytes by using TRAP assay. The increase of telomerase activity was partially dependent on the net increase of TERT expression. Also, telomerase activity was decreased after treatment with protein kinase inhibitors, indicating that telomerase activation was prevented by inhibition of phosphorylation. **Conclusion:** Therefore, these results suggest that telomerase activity is constitutively expressed in the murine resting T lymphocytes and controlled by both transcriptional regulation and post-translational modifications. **(Immune Network 2004;4(3):166-175)**

Key Words: Telomerase, CD8$^+$ T cells

Introduction

The telomeric ends of chromosomes have been identified as a candidate for the 'replicative clock' that monitors cell division and accounts for cessation of replication. The template priming requirement of DNA polymerase results in the loss of terminal bases during lagging strand chromosomal replication, leading to the shortening of telomeres with each cell division. Shortening of the telomeres beyond a certain length triggers cell cycle arrest and other changes characteristic of senescent cells (1-3). In deed, telomere shortening has been identified *in vivo* in normal somatic tissues as a consequence of aging as well as *in vitro* cultured human fibroblasts (4).

Transformed cells and germline cells appear to have an unlimited capacity to divide and proliferate, suggesting that a compensatory mechanism capable of avoiding the consequences of telomere shortening must exist. Indeed, one such mechanism is mediated by the ribonucleoprotein enzyme telomerase (1). Telomerase holoenzyme consists of an RNA component, telomerase RNA (TER), and a protein catalytic component, the telomerase reverse transcriptase (TERT), which mediates RNA template-dependent synthesis of telomeric DNA, and telomerase-associated proteins (TEP). The cellular activity of telomerase is determined by the presence or absence of TERT, whereas all somatic cells constitutively express TER. TEP is a protein that binds to both TER and TERT and maintains the tertiary and/or quaternary structure of the telomerase holoenzyme.

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Telomerase is reversibly regulated by several factors involved in regulating telomerase gene expression, structure and function (5-7). Both *in vitro* and *in vivo* studies have shown that TERT transcription is the major determinant of telomerase activity. TERT transcripts are low or undetectable in most somatic cells but readily detectable in transformed counterparts and ectopic expression of TERT in telomerase-negative cells is sufficient to reconstitute telomerase activity, to elongate telomeres, and to ex tend cellular life span (8-11). Posttranslational modification and protein-protein interaction also seem to modulate telomerase activity. Telomerase is slowly metabolized with a half-life of more than 24 hours, because of posttranslational assembly, maintenance, and conformational changes required by the components of the holoenzyme. Protein phosphorylation, a key posttranslational mode of regulating protein structure and function, is involved in telomerase activity regulation in human breast cancer cells (12-14) and human peripheral blood lymphocytes (15). In spite of, however, several studies about telomerase regulation, the precise mechanisms of telomerase regulation remain to be elucidated.

The function of lymphocytes is dependent on a high degree of cell division during development, differentiation, and activation (16,17). Thus, the ability of lymphocytes to undergo repeated cell division is essential for effective immune function (18). T and B lymphocytes have adapted a mechanism, telomerase, otherwise used by malignant cells and germline cells to extend the replicative capacity necessary for lymphocyte function (19-21). Recent findings establish a clear relationship between telomere length and differentiation stages in both T and B cell lineage and demonstrate that there is stringent regulation of telomerase activity during T and B lymphocyte activation (22-24). It is consistent with a model in which maintenance of telomere length, mediated at least in part through the activity of telomerase, may function to support the capacity of lymphocytes for extensive clonal expansion. A correlationship between telomere shortening and life span has also been found in cells of the immune system. The average telomeric length and the replicative potential are higher in naive T cells as compared with memory T cells from same donor, in both $CD4^+$ cells and $CD8^+$ cells (25,26). The aim of the present study was to identify the telomerase regulation during activation in murine T lymphocytes. Telomerase activity in a resting state of cloned T cells and naive T cells isolated from C57BL/6 mice were compared with that in an activated state, and the TERT protein expression and an effect of phosphorylation were also analyzed. Telomerase activity was measured *in vitro* by a polymerase chain reaction (PCR)-based assay designated TRAP (for telomeric repeat amplification protocol) (27). The results from this study demonstrate that murine resting T lymphocytes express telomerase activity constitutively and the telomerase activity is controlled by both transcriptional regulation and post-translational modifications.

Materials and Methods

Establishment and maintenance of T lymphocyte clones. Cloned T lymphocytes used in the present study were $CD8^+$ cells specific for human adenovirus type 5 E1B-derived peptide, VNIRNCCYI. Cloned $CD8^+$ T cells were generated from splenocytes of C57BL/6 mice (Daehan Laboratory Animal Research Center Co. Ltd., Daejon, Korea) immunized with the peptide and maintained by weekly stimulation with the peptide-pulsed antigen presenting cells (APC) and concanavalin A-activated rat spleen culture supernatant (CAS).

Two or three mice were intraperitoneally injected with human adenovirus type 5. Rechallenge of the mice was performed at 25-35 days after primary challenge. For generation of CTL clones, the spleens were removed from the immunized mice two or three weeks after primary challenge and were generated into single cell suspensions in HBSS. After washing with HBSS, RBC were lysed by incubating with RBC lysis buffer at 37°C for 5 min. The resulting splenocytes were cultured with the E1B-derived peptide (E1Bp) to induce polyclonal E1Bp-specific CTL population. To generate CTL clones, they were further restimulated weekly with syngeneic splenocytes treated with 50μg/mL mitomycin C (Sigma Chem. Co., St. Louis, MO, U.S.A.) in the presence of the peptide and 2.5% CAS as a source of IL-2. The resulting polyclonal CTLs were plated in 96-well round bottom plates containing mitomycin C-treated syngeneic splenocytes (2×10^5 cells), peptide, and 2.5% CAS at 0.3-5 cells per well. When the frequency of the microwells containing growing cells at a given cell dilution was less than 20%, the proliferating cells of the culture plate were chosen for further expansion and tested for their antigenic specificity using a standard cytotoxicity assay. The CTL clones obtained were maintained by weekly restimulation.

APCs were prepared from spleens of C57BL/6 mice. When necessary, the cells were treated with mitomycin C (Sigma) to prevent from proliferation by incubating with 50μg/mL mitomycin C for 30 min at 37°C in a concentration of 5×10^7 cells/mL. The cells were washed three times with HBSS before used as APCs. The APCs were suspended in a 'complete' RPMI-1640 tissue culture medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum

(FBS; Life Technologies, Detroit, MI, U.S.A.), 2 mM L-glutamine, 10 units/mL penicillin G, 100μg/mL streptomycin, 250 ng/mL amphotericin B, 10 mM Hepes, 24 mM $NaHCO_3$ and 5×10^{-5}M 2-mercaptoethanol. 5×10^6 cells of these APCs were incubated with an appropriate peptide at the final concentration of 5μM for 2～4 h at 37°C, 5% CO_2 and added to 5×10^5 cloned T cells plated in a 24-well plate (Falcon, Lincoln Park, NJ, U.S.A.) with 2.75% CAS. These cells were cultured for 7～8 days in a humidified atmosphere containing 5% CO_2 at 37°C.

Preparation of Concanavalin A-activated rat spleen culture supernatant (CAS). Spleens of Sprague-Dawley rat (Daehan) were placed in a petri dish, and grounded into a single cell suspension with a glass pestle onto stainless steel mesh in complete RPMI 1640 medium. The cells were washed with HBSS, and red blood cells were lysed by incubating with the RBC lysis buffer at 37°C for 5 min. The resulting cells were washed twice with HBSS and suspended in complete RPMI 1640 medium supplemented with concanavalin A (Con A) at 10μg/mL. After incubation at 37°C in a 5% CO_2 incubator for 24 h, the supernatant from the cultured cells was collected by centrifugation at 330×g for 8 min, and the pelleted cells were resuspended in fresh complete RPMI 1640 medium containing equal volume of Con A as described previously, then cultured for additional 24 h before collecting another batch of supernatant. These collected supernatants were mixed and added with 500 μg/mL α-methylmannoside and filtered through 0.20μm-pored syringe filters (Corning Glass Works, Corning, NY, U.S.A.).

Preparation of cell extracts and PCR-based telomerase assay. Cell extracts were prepared according to the procedure described previously (28). Cells were washed with PBS and resuspended in CHAPS (3-[3-cholamidopropyl-dimethylammonio]-1-propanesulfonate; Sigma) lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM benzamidine, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol) at a concentration of 10^5～10^6 cells per 200μL. After 30 min incubation on ice, the extracts were centrifuged at 12,000×g for 20 min at 4°C. The supernatant was then examined for protein concentration, and telomerase activity in the cell extracts was determined by a PCR-based TRAP (telomeric repeats amplification protocol) assay (27,28). The procedures are separated into two steps: telomerase synthesis and PCR amplification. In the first step of the reaction, telomerase synthesizes a number of telomeric repeats (GGTTAG) onto the 3' end of a substrate oligonucleotide (TS: 5'-AATCCGTCGAGCAGAGTT-3'). In the second step, the extended products were amplified by PCR using TS and RP (reverse) primers, generating a ladder of products with 6 base increments starting at 50 nucleotides. A 'master mix' containing 10x PCR reaction buffer (200 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 630 mM KCl, 0.5% Tween 20, 10 mM EGTA), 50x dNTP mix (2.5 mM each dATP, dTTP, dGTP and dCTP), TS primer and primer mix was added to cell extracts and incubated at 30°C for 30 min. During the incubation, telomerase of cell extracts synthesizes telomeric repeats (TTAGGG) onto an oligonucleotide primer and the extension products serve as templates for PCR amplification. PCR amplification was performed at 94°C, 30 seconds and 59°C, 30 seconds for 33 cycles in a GeneAmp PCR system (Perkin-Elmer Co., Norwalk, CT, U.S.A.). As a negative control, sample extract was incubated at 85°C for 10 min before telomerase synthesis step, and as a primer-dimer/PCR contamination control, CHAPS lysis buffer was used instead of cell extract. The amplified products were separated on a 12.5% non-denaturating polyacrylamide gel and stained SYBR® Green I (Biowhittacker Molecular Application, Rockland, ME, U.S.A.) for 1 h. The results were analyzed using an image analyzer (Alpha Innotech, San Leandro, CA, U.S.A.)

Naive $CD8^+$ T cell isolation from C57BL/6 mice. Spleens from C57BL/6 were ground on stainless steel mesh, homogenized in HBSS. After hemolysis, as described above, connective tissues of homogenized spleen were removed from single cell suspension, dropping suspension in buffer used for MACS® separation column (Miltenyi Biotec, Germany) from the pipet. This buffer is D-PBS at final pH 7.2, supplemented with 0.5% bovine serum albumin (BSA), and 2 mM EDTA. Buffer should be degassed at room temperature. After washing with buffer, cell pellet was resuspended in buffer and incubated with FITC-conjugated anti-CD8 Ab (Pharmingen, San Diego, CA, U.S.A.) on ice for 30 min. Stained cells were then resuspended in 90μL of cold buffer per 10^7 cells, added MACS Multisort Anti-FITC microbeads 10μL and incubated on ice for 45 min with protection from light. During washing cells, magnetic column was fixed in magnetic field and eluted with 500μL of buffer. Cell pellet was resuspended in 500μL of cold buffer, applied onto the column before the column was dry. The column was rinsed with 500μL of cold buffer three times. Magnetically labeled cells eluted from the column were incubated with 20μL MACS Multisort Release Reagent per mL cell suspension for 10 min at 6～12°C, followed by addition of MACS Multisort Stop Reagent 30μL per 10^7 cells. Cell suspension was then stained with anti-CD62L antibody, followed by goat anti-rat Ig microbeads and applied onto magnetic column as described above. Separated cell suspension was used for the experi-

ments after it was washed with HBSS twice. Separation efficacy was examined by flow cytometry.

Results

Telomerase activity of the cloned murine T cells. Telomerase activity in the T cells isolated from mice has been shown to be expressed when cells were activated with mitogenic stimuli (22,23,29). In order to study telomerase activity in murine T lymphocytes, a murine CD8$^+$ T cell clone, 5F13 that have been maintained *in vitro* culture by stimulating periodically with antigen-pulsed APCs and CAS were used. 5F13 is specific for a human adenovirus type 5 E1B-derived peptide (VNIRNCCYI) and derived from splenocytes of C57BL/6 mice immunized with the peptide. Telomerase activity of these T cells was examined by TRAP assay. The specificity of the TRAP assay was confirmed by the observation that no band was detected in positive control sample (RMA extract) preheated for 10 min at 85^{o}C to inactivate telomerase (data not shown). T cells were harvested before and after the stimulation with peptide-pulsed APCs and CAS. In consistence with the previous reports, telomerase activity was increased upon stimulation, reached a peak on day 3 (two-fold), and decreased thereafter (Fig. 1). However, it appeared that the T cells expressed telomerase activity before the antigenic stimulation as well. Since non-stimulated peripheral T cells have been reported to have little or no detectable telomerase activity in the previous reports (15), the activation state of these cloned T cells was examined by a cell surface staining of activation markers, CD25 and CD69 that have been reported to be expressed on activated T cells. The expression levels of CD25 and CD69 that were remained very low without antigenic stimulation were substantially increased on day 3 or 4 after stimulation suggesting that the non-stimulated 5F13 were in a resting state (data not shown). To exclude the effect of the contaminating APCs on the telomerase activity of the cloned T cells, the telomerase activity of the mitomycin C-treated APCs cultured with CAS was examined without detectable telomerase activity (data not shown).

In many previous studies with human peripheral T cells, telomerase activity was induced with mitogenic stimuli, such as anti-CD3 Ab/anti-CD28 Ab, anti-CD3 Ab/IL-2, PMA/ionomycin, and PHA/IL-2 (22). Also, in the murine T cells, telomerase activity was reportedly induced with such stimuli. Ogoshi et al. reported that the signals from cytokine receptors were required to induce telomerase activity in the murine splenic T cells (29), and Hathcock et al. suggested that both TCR mediated signals and costimulatory signals were required (30). To examine if the telomerase activity could be induced in the cloned murine T cells, 5F13 were treated with the following stimuli: peptide-pulsed APCs plus CAS, CAS alone, anti-CD3 Ab, IL-2, or PMA plus ionomycin. The telomerase activity was similarly increased in all cases (data not shown). These results indicated that the telomerase activity of the cloned T cells was also induced with mitogenic stimuli and the induction did not seem to require co-stimulatory signals in the cloned T cells.

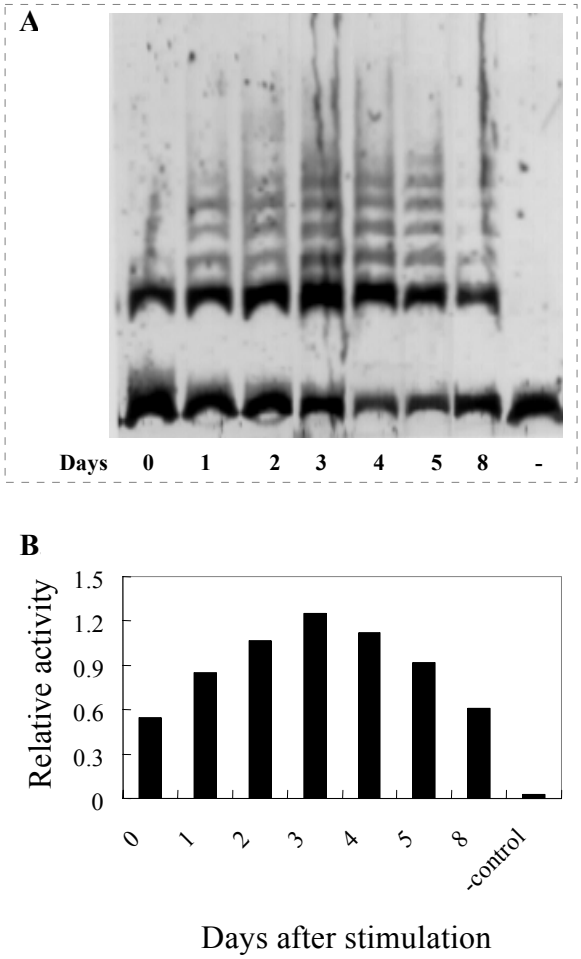

Figure 1. Telomerase activity in cloned T cells during culture with APCs and CAS. Cloned T cells were cultured with peptide-pulsed APCs and CAS for 11 days, and harvested at the indicated time points. Telomerase activity of the cloned CD8$^+$ T cells was measured by TRAP assay. The PCR products of telomeric repeats were separated on a 12.5% non-denaturating polyacrylamide gel. The gel was stained with SYBR® Green I, and the band was detected upon UV exposure (A). The band intensity was measured by using image analyzer, then plotted on a graph (B). Relative telomerase activity was calculated as a percentage of that from equivalent numbers of positive control cells (RMA). For the negative control, cell extract was replaced by lysis buffer.

Telomerase activity of naive T cells isolated from C57BL/6 mice. The cloned T cells might have the characteristics of memory cells because they have been cultured with repeated antigenic stimulations. As shown in Figure 2B, the cloned T cells expressed very low level of CD62L that is highly expressed in naive T cells. To assess the telomerase activity in naive T cells, naive $CD8^+$ T cells were purified from C57BL/6 mouse spleen by magnetic cell sorting. CD62L was used as a surface marker of the naive T cells. The purity of the isolated $CD8^+$ $CD62L^+$ T cells was $>$ 98% (Fig. 2A and B), and those cells expressed very low levels of both CD25 and CD69 (Fig. 2C and D). The telomerase activity was determined for the cells freshly isolated from mice and the naive T cells cultured with anti-CD3 mAb plus CAS for 6 days. As shown in Fig. 3, the freshly isolated T cells expressed telomerase activity comparable to the cloned T cells, and the telomerase activity was increased after *in vitro* stimulation. The kinetics of changes in the telomerase activity was also similar to that of cloned T cells (Fig. 1), increasing up to day 3 and decreasing thereafter. A previous study reported that naive and memory $CD4^+$ T cells of human peripheral blood expressed little telomerase activity (15). Although the purity of the isolated $CD8^+CD62L^+$ T cells in this study was $>$98%, it could not be ruled out the possibility that the cells in a $CD62L^-$ fraction from the isolation procedure might be responsible for the telomerase activity. However, the $CD8^+CD62L^-$ T cells turned out to express only a little higher level of telomerase activity (about 1.5 fold) than $CD8^+$ $CD62L^+$ T cells (Fig. 4). Since telomerase activity was assayed from the cell extract equivalent of 2×10^5 cells in this study, the number of contaminating cells should not be more than 4×10^3 cells. When telomerase activity was measured for the serially diluted cell extracts of $CD8^+CD62L^-$ T cells, no band was detectable in the cell extract equivalent of less than 1×10^4 cells (data not shown). Therefore, the small numbers of the contaminating $CD8^+$ $CD62L^-$ T cells would not be observable in the sample of $CD8^+CD62L^+$ T cells.

In order to examine the telomerase activity in a more clearly defined naive $CD8^+$ T cell population, T cells isolated from TCR transgenic (Tg) mice were employed. The TCR Tg mice have been established to produce cytotoxic T lymphocytes specific to glycoprotein residue 33-41 of LCMV (gp33) and most of $CD8^+$ T cells from the Tg expressed a TCR specific to gp33 (31). The $CD8^+$ $CD62L^+$ T cells purified from LCMV TCR Tg mice kept under specific pathogen-free condition could be regarded to be naive cells. As in T cells from normal C57BL/6 mice, telomerase activity was also detected in naive Tg T cells (Fig. 4). These results indicate non-activated or resting T cells expressed a significant level of telomerase activity.

The regulatory mechanisms of telomerase activity. The present study showed that the telomerase activity in non-activated T cells was regulated upon stimulation. The human telomerase catalytic subunit or telomerase reverse transcriptase (hTERT) has recently been identified to be a～130 kDa protein that is concomitantly expressed with the activation of telomerase during cellular immortalization and tumor pro-

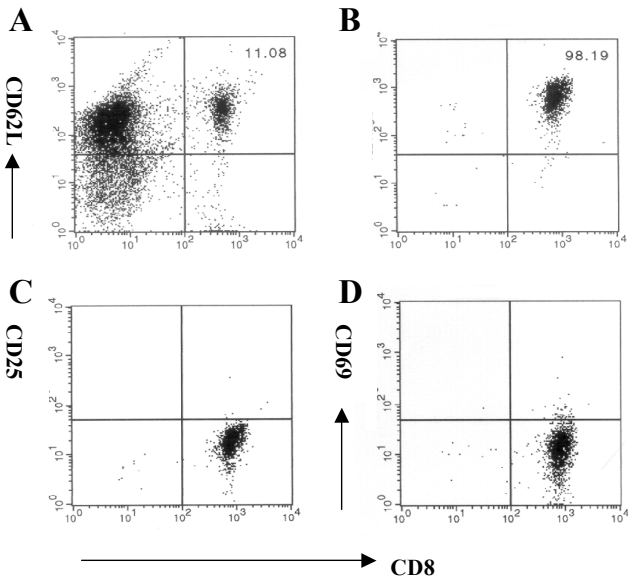

Figure 2. Isolation of naïve $CD8^+$ T cells from C57BL/6 mice. $CD8^+$ $CD62L^+$ T cells were isolated from spleens of C57BL/6 mice by positive selection using anti-FITC Multisort Kit, as described in Materials and Methods. Briefly, $CD8^+$ T cells were purified by incubating spleen cells with FITC- conjugated anti-CD8 Ab for 30 min on ice, followed by anti- FITC microbeads and fractionation on a column (Miltenyi- Biotec.). The positive fraction was incubated with goat anti-rat Ig microbeads after preincubating with rat anti-CD62L Ab, and then applied to a column. Unseparated (A) and separated (B) fractions were assesed by anti-CD62L mAb staining and analyzed by flow cytometry. The number in the panel indicates the percentage of $CD8^+CD62L^+$ cells. PE-conjugated antibodies to CD25 (C), CD69 (D) were used to analyze the expression of activation markers of purified T cells.

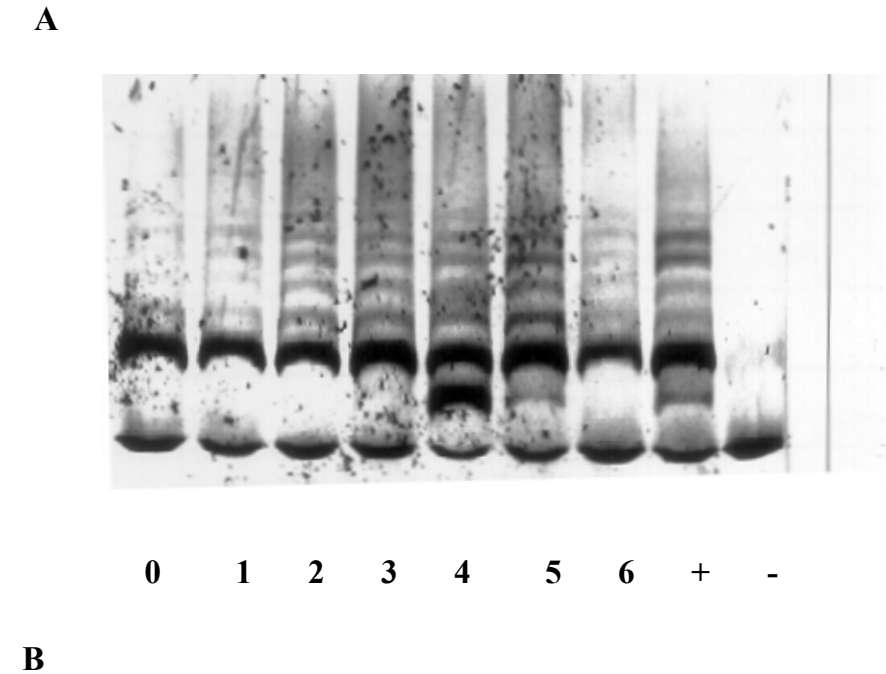

Figure 3. Telomerase activity upon activation of naive CD8$^+$ T cells. Naïve CD8$^+$ T cells (CD8$^+$CD62L$^+$) were isolated from C57BL/6 splenocytes as described above. Cells were cultured with immobilized anti-CD3 mAb (Pharmingen) plus IL-2. Samples were collected prior to stimulation, at day 1, 2, 3, 4, 5 and 6 after stimulation and analyzed for telomerase activity by TRAP assay. Lanes of (+) and (-) lane show the telomerase activity of positive control cell (RMA) and negative control with no cell extract added, respectively.

gression (8-11). To determine whether TERT expression is regulated in the activated murine CD8$^+$ T cells, freshly isolated naive T cells and 5F13 were treated with anti-CD3 Ab and CAS, and subsequently measured for the TERT expression by Western blot. TERT protein with a molecular mass of 136 kDa appeared to be present in both non-activated naive T cells and cloned T cells, and there was no significant difference in the quantity of TERT protein expressed between these two cell types (Fig. 5A). TERT protein was barely increased after stimulation, while telomerase activity increased approximately two-fold as shown in Fig. 1 and 3. Furthermore, TERT mRNA was also expressed constitutively in the non-activated T cells and the expression level did not significantly changed after stimulation (data not shown). This result is consistent with the previous report that hTERT protein was present in all subsets of lymphocytes isolated from thymus and peripheral blood regardless of the status of telomerase activity (9,15). When, however, 5F13 were treated with cycloheximide immediately after the stimulation, the telomerase activity decreased after 24 h, and disappeared after 48 h (Fig. 6B), indicating that

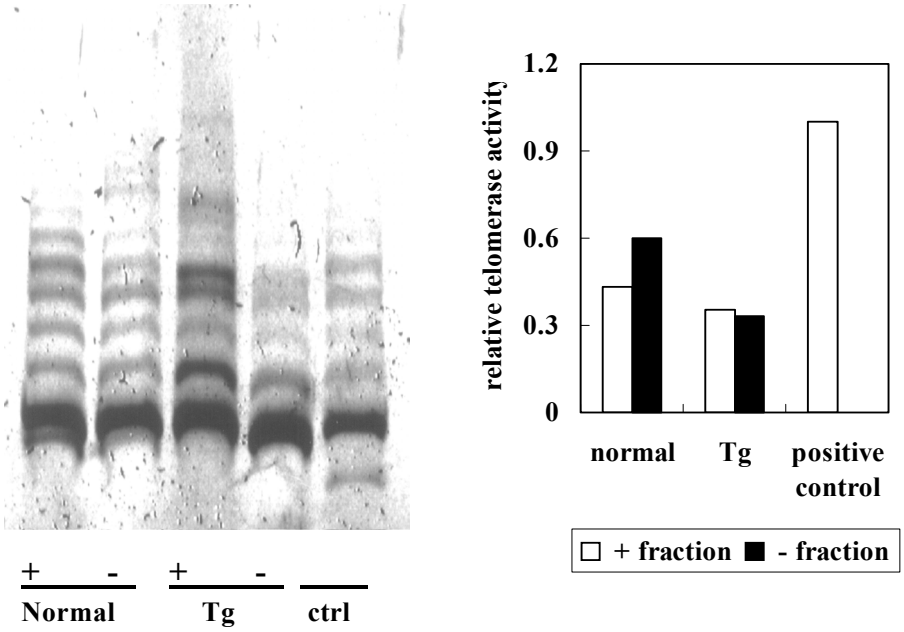

Figure 4. Telomerase activities of naïve TCR Tg T cells and normal mouse T cells in a negative fraction from isolation procedure. Naive $CD8^+$ T cells (open bar, $CD8^+CD62L^+$) were purified from C57BL/6 or LCMV TCR Tg mice by magnetic cell sorting as described above, and assayed for telomerase activity. The cells in the negative fraction from the isolation procedure (closed bar, $CD8^+$ CD62L$^-$) were also collected for TRAP assay.

protein synthesis was required in activity expression. Therefore, it seemed that telomerase activity required protein synthesis, but that induction of telomerase activity in the activated murine $CD8^+$ T cells did not require a net increase in TERT protein.

Little is known how telomerase is activated and maintained at the enzyme levels. One possible mechanism is that the tertiary and quaternary structures of the large telomerase complex are modulated by protein phosphorylation in such a way that the enzyme is activated. Based on this hypothesis, it has been reported that constitutive phosphorylation of hTERT was found in a tumor cell line (12) and that hTERT was phosphorylated during $CD4^+$ T cell activation (15). It was also reported that protein phosphatase 2A specifically inhibited telomerase activity in the nuclear lysates of human breast cancer cells (14), and that protein kinase C (PKC) was selectively eluted from the hTEP1 peptide affinity column and mediated phosphorylation of hTEP1 and hTERT in intact human breast cancer cells (12). To investigate the effect of protein kinase on the telomerase activity during murine $CD8^+$ T cell activation, 5F13 were pretreated with protein kinase inhibitors and telomerase activity was measured at 48 h after stimulation with anti-CD3 mAb and CAS. As shown in Figure 8, genistein, a protein tyrosine kinase inhibitor, affected little telomerase activity after stimulation. In contrast, pretreatment with bisindolylmaleimide or rottlerin, protein kinase C (PKC) inhibitors, resulted in a lowered telomerase activity after activation. Although, both bisindolylmaleimide and rottlerin are PKC inhibitors, only rottlerin dramatically decreased the telomerase activity of 5F13. Rottlerin has been observed to inhibit other protein kinases such as protein kinase A, src kinase or CaM kinase III as well as PKC at a concentration of 5mM, in contrast with bisindolylmaleimide to inhibit only PKC at a concentration of 0.1μM (14). The telomerase activity dramatically decreased by treatment with rottlerin, in part, might be due to an inhibitory action on other kinases of rottlerin. This result suggests that protein phosphorylation might be involved in the regulation of telomerase activity. Therefore, these results collectively suggest that telomerase activity in murine $CD8^+$ T cells was controlled by both regulated TERT transcription and phosphorylation of telomerase protein.

Discussion

During the course of an immune response, lymphocyte function is dependent on extensive cell proliferation and differentiation. Previous studies have demonstrated that one mechanism to regulate the replicative capacity of normal somatic cells was the control of telomere length through the activity of tel-

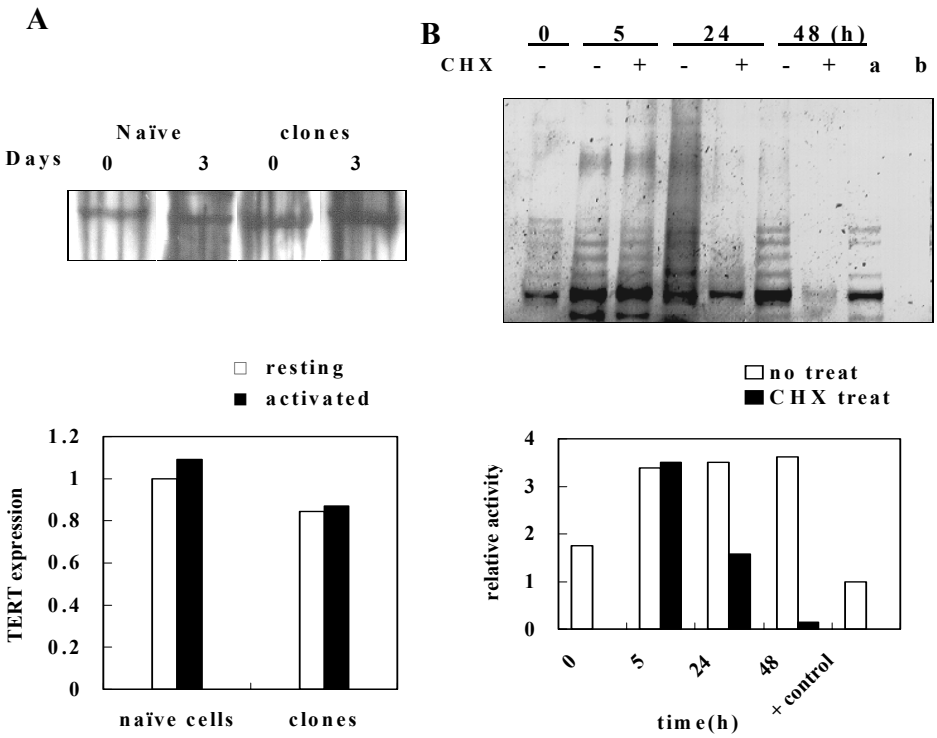

Figure 5. The expression of TERT protein in resting and activated T cells and the effect of cycloheximide on telomerase activity. (A) Naive T cells from C57BL/6 mice and cloned T cells were stimulated with anti-CD3 Ab and CAS for 72 h. They were then lysed and separated by 8% SDS-PAGE, transferred onto nitrocellulose paper, and probed with anti-TERT antibody. The band intensity was measured by an image analyzer, and the relative TERT expression level was shown. Three independent experiments exhibited similar results and one typical result was shown here. (B) Cloned T cells were cultured with peptide-pulsed APCs and CAS. Cycloheximide (CHX) was added at a concentration of 10μg/mL. The telomerase activities of these cells were measured by using TRAP assay at 5, 24, and 48 h after cycloheximide addition. Relative telomerase activity to the positive control (a) was calculated. RNase A (Sigma) was added to the reaction mixture (b) to confirm the specificity of the reaction products.

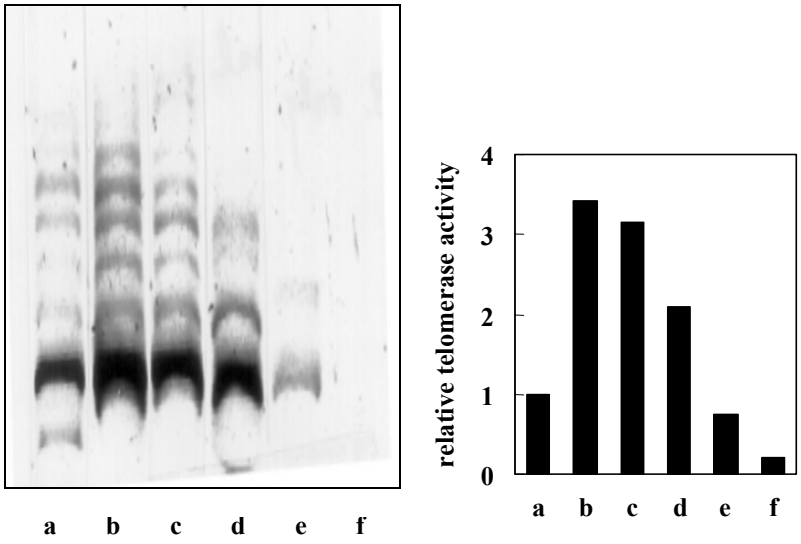

Figure 6. The effect of protein kinase inhibitors on telomerase activity. Cloned T cells were pretreated with genistein 3μM (c), or bisindolylmaleimide 0.1μM (d), or rottlerin (Sigma) 5 mM (e) for 3 h, and stimulated with APCs plus CAS. After 48 h, cells were harvested and telomerase activities were measured. Telomerase activity of untreated cells (b) was compared with those. Lane (a) shows telomerase activity in cells before stimulation, and in lane (f) no cell extract was added.

omerase (1,2). Although telomerase regulation in the murine T lymphocytes has not been as well characterized as in humans, several experiments have shown that the normal murine T lymphocytes expressed telomerase activity only after stimulation (9,15,22,29, 30). In this study, however, telomerase activity was expressed in non-activated T cells as well as in activated T cells of murine origin. The T cell clone 5F13 used in the present study was in a resting state before stimulation as CD25 and CD69, the activation-induced cell surface molecules, were expressed at a very low level.

Since, in fact, cloned T cells have been maintained by periodical stimulation *in vitro*, non-activated clones may be not exactly same as the resting cells *in vivo*. To investigate this possibility, telomerase activity of naive $CD8^+CD62L^+$ T cells freshly isolated from normal C57BL/6 mice was examined. Isolated $CD8^+$ $CD62L^+$ T cells were of resting, naive cell phenotype, as they express a high level of CD62L, and low levels of CD25, CD69 and CD44 (data not shown). The resting naive T cells, however, also expressed telomerase activity comparable to that of cloned T cells, and expressed increased activity when stimulated, although there is a little difference in the kinetics that the telomerase activity of cloned T cells was increased rapidly and decreased slowly than that of naïve T cells. The possibility could also be ruled out that the telomerase activity resulted from the contaminating $CD8^+CD62L^-$ cells, when a quantitative TRAP assay with serially diluted $CD8^+CD62L^-$ cell extracts indicated that telomerase activity from such a small numbers of contaminating $CD8^+CD62L^-$ cells could not detected in the present system, although they were expressed a little higher telomerase activity than $CD8^+CD62L^+$ T cells. Moreover, non-stimulated $CD8^+CD62L^+$ T cells freshly isolated from LCMV TCR Tg mice, which were believed to be naive cells, also expressed telomerase activity. These results collectively suggest that murine non-activated T lymphocytes expressed telomerase activity. The regulation of telomerase activity has known to involve several factors including telomerase gene expression, post-translational modification and protein-protein interactions. Many studies with human peripheral blood T lymphocytes have reported that telomerase activity was controlled at the level of hTERT transcription (8,10,11). However, the recent studies have proposed that human lymphocytes expressed hTERT protein independently of the presence of detectable telomerase activity (9,15). It was demonstrated, in the present study, that there was no strict quantitative relationship between telomerase activity and TERT transcription level in the murine T lymphocyte population. Both TERT protein level and TERT mRNA level were up-regulated after stimulation, but the increased levels were not exactly proportional to that of enzymatic activity. When, however, 5F13 were treated with cycloheximide, a protein synthesis inhibitor, telomerase activity began to decrease at 24 h after the stimulation, reflecting that a half-life of telomerase was more than 24 hours. This suggested that protein synthesis might play a key role in the telomerase regulation.

Phosphorylation of hTERT has also been proposed as a regulatory mechanism because constitutive phosphorylation of hTERT was found in a tumor cell line (12). In this study, it was observed that telomerase activity of the activated 5F13 was decreased by the treatment with protein kinase inhibitors such as bisindolylmaleimide and rottlerin. Telomerase might be activated by phosphorylation, especially phosphorylation of serine or threonine residues in the enzyme, since genistein, a protein tyrosine kinase inhibitor, had little effect on telomerase activity in contrast with the serine or threonine protein kinase inhibitors (bisindolylmaleimide and rottlerin). This result was not inconsistent with the previous reports that telomerase activity was controlled by protein kinase C and inhibited by protein phosphatase 2A in human breast cancer cells (12,14). Therefore, these results suggest that regulation of telomerase activity is controlled at multiple levels, both transcriptional and post-translational events.

The correlation between telomerase activity and cellular activation as observed in this study is consistent with the hypothesis that telomere length and telomerase enzymatic activity play an important role in sustaining the capacity for extensive clonal expansion in antigen-specific lymphocytes (19). It is reasonable to assume that the conditions that induce proliferation of T cells also increase the expression of telomerase activity, which may serve to slow the rate of telomere shortening. Maintenance of telomere length may in turn conserve the residual capacity for further cell division.

In addition to providing insights into basic immune function, manipulation of telomere length has potential therapeutic application as well. For example, the ability to extend the replicative capacity of cells such as hematopoietic stem cells or mature lymphocytes through telomerase induction could be critical to therapeutic approaches to adoptive cell transfer or reconstitution. The information obtained from the study of telomerase regulation in murine T lymphocytes will provide the basis for the better understanding of human lymphocyte replication.

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