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Therapeutic Discovery

Molecular Cancer Therapeutics

JAK–STAT and JAK–PI3K–mTORC1 Pathways Regulate Telomerase Transcriptionally and Posttranslationally in ATL Cells

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Abstract

Adult T-cell leukemia (ATL) is a heterogeneous tumor that is resistant to chemotherapy. Telomerase activity plays a critical role in tumorigenesis and is associated with the prognosis of ATL patients. Interleukin (IL)-2 commonly promotes tumor growth in chronic ATL cells. The signaling pathways involved in IL-2–regulated telomerase activation were studied in ATL cells derived from chronic ATL patients. IL-2 challenge enhanced tyrosine phosphorylation of Janus-activated kinase (JAK)1–3 and STAT5, and induced JAK1 and JAK2 to associate with STAT5 in IL-2–dependent ATL cells. Chromatin immunoprecipitation assays revealed that STAT5 directly bound to the human telomerase reverse transcriptase (*hTERT*) promoter. STAT5 *short interfering RNA* inhibited *hTERT* transcription in IL-2–stimulated ATL cells. Inhibitors of PI3K, HSP90, and mTOR reduced IL-2–induced *hTERT* mRNA, protein expression, and telomerase activity. AKT, HSP90, mTOR, S6 kinase, and hTERT immunoprecipitate from IL-2–stimulated cells contained telomerase activity, suggesting that hTERT directly interacts with, and is regulated by, these proteins. Binding of the p85 regulatory subunit of PI3K to JAK2 was enhanced in an IL-2–dependent manner, indicating that JAK2 propagates activation signals from the IL-2 receptor and links hTERT activation to both the STAT5 and PI3K pathways. Finally, IL-2–induced activation of telomerase and STAT5 was observed in primary leukemic cells. These results indicate that IL-2 stimulation induces hTERT activation through the JAK/STAT pathway and the JAK/PI3K/AKT/HSP90/mTORC1 pathway in IL-2–responsive ATL cells. These signaling proteins represent novel and promising molecular therapeutic targets for IL-2–dependent ATL. *Mol Cancer Ther; 11(5); 1112–21. ©2012 AACR.*

Introduction

Adult T-cell leukemia (ATL) is an aggressive lymphoproliferative disorder that occurs in individuals infected with human T-cell leukemia virus type 1 (HTLV-I; refs. 1–3). ATL is classified into 4 subtypes, including smoldering, chronic, lymphoma, and acute types, according to clinical manifestations with distinct molecular mechanisms (4). Treating aggressive ATL is very difficult. In addition, effective therapies have yet to be developed for indolent types of ATL such as smoldering or chronic ATL. More than 50% of such ATL subtypes have been reported to progress to acute ATL and prognosis is poor once this occurs (5). Thus, novel therapy targeting ATL tumor cells during smoldering/chronic phases is mandatory to improve the prognosis of this incurable and debilitating disease. This is reminiscent of chronic myeloid leukemia in which inhibitors of the BCR-ABL tyrosine kinase have been successfully used as a specific therapy for the chronic phase of the disease (6).

The mechanism of leukemogenesis or tumor progression is distinct in each phases of ATL. In the early phase of HTLV-1 infection in T cells, several lines of evidence indicate that the HTLV-1 Tax protein plays a central role in leukemogenesis. For example, Tax activates critical signaling pathways such as NF-kB, PI3K, and AP1. Tax also induces the expression of cytokines, such as interleukin (IL)-2, and their receptors, leading to cell proliferation and transformation. In addition, HTLV-1–infected cells contain elevated activity of human telomerase, an RNA-dependent DNA polymerase composed of a catalytic subunit termed human telomerase reverse transcriptase (hTERT), which elongates telomeres shortened by successive replication cycles. Tax has been shown to upregulate the transcriptional activity of hTERT via the NF-kB pathway, although contradicting results have also been reported (7, 8). Although Tax is required for the IL-2–dependent expansion of HTLV-1–immortalized cells in the early phases of viral infection, T-cells expressing Tax

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can be eliminated by host immune surveillance due to Tax immunogenicity. Indeed, most ATL cells do not express Tax and tend to become independent of IL-2 (9), in particular in acute ATL. Nevertheless, IL-2 is often required for ATL cell proliferation and survival, suggesting the importance of IL-2 in tumor growth in some patients, such as those suffering from chronic ATL, regardless of Tax protein expression (10, 11).

IL-2 signaling has recently been shown to be associated with the upregulation of the *hTERT* promoter in Tax-negative HTLV-1–transformed cells (12). These findings suggest that telomerase is an attractive target for anticancer drug discovery in ATL; however, the mechanism of IL-2–dependent telomerase activation in Tax-negative ATL tumor cells has not been completely elucidated. The Janus-activated kinase (JAK)–STAT pathway is involved in IL-2 signaling in normal T cells and is activated in IL-2–independent HTLV-1–transformed T cells and ATL cells (13, 14). We previously reported that STAT5 controls telomerase transcription in chronic myeloid leukemia cells and is implicated in resistance to imatinib (15, 16). Thus, the JAK–STAT pathway could be a critical component of telomerase activity regulation in ATL cells. The aim of the current study is to assess the role of the JAK–STAT and the related pathways in regulating IL-2–induced telomerase activity, which promotes cell proliferation in ATL cells derived from chronic ATL patients. Results may lead to the development of new molecularly targeted therapies in chronic/smoldering ATL.

Materials and Methods

Cells

This study used IL-2–dependent ILT-Hod cells derived from chronic ATL patient (17). HUT102 cell line, which has the capacity for IL-2–independent cell growth, was also used (1). ILT-Hod and HUT102 were kind gifts from Dr. Mari Kannagi (Tokyo Medical and Dental University, Tokyo, Japan) and Dr. Masahiro Fujii (Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan) in 2008, who published the manuscripts using these cell lines (18, 19). Cell identities were not authenticated by the authors other than confirming the cell lines are ATL cells by Southern blot analysis for viral gene integration and status of Tax expression in addition to characteristic T-cell phenotypic markers. For baseline growth, the ILT-Hod cells require the constant addition of IL-2 at a concentration of at least 5 U/mL. For telomerase induction experiments, cells were exposed to a concentration of 100 U/mL. To stimulate the T-cell antigen receptor, ILT-Hod cells were grown on either anti-CD3, anti-CD28, or anti-CD3/CD28 coated plates for 2 days, and the cells were subsequently harvested. Plates were coated at 37°C for 90 minutes before the cells were seeded.

Primary leukemic cells were obtained from 6 patients with ATL. All patients were newly diagnosed (untreated), chronic ATL according to the diagnostic criteria by Shimoyama (4). Leukemic cells were isolated from blood samples using a Ficoll–Conray density gradient, washed twice, and their viability was determined by trypan blue dye exclusion. Cells showing more than 80% viability were used in experiments to exclude unreliable data derived from cell death and protein degradation. Four of 6 samples were found to be suitable for use. All samples were collected after obtaining informed consent, and the study protocol was approved by the Human Investigation Committee of our institution.

Chemicals and antibodies

LY294002, PD98059, geldanamycin, radicicol, and rapamycin were all purchased from Calbiochem. IL-2 was kindly provided by Takeda Pharmaceutical and Shionogi Pharmaceutical. Polyclonal rabbit antibodies against PI3K, AKT, phospho-AKT(Ser473), p70S6K, mTOR, YB-1, hTERT, HSP90, JAK1, JAK2, JAK3, STAT3, STAT5, phospho-STAT5 (Tyr694), ß-actin, and the anti-CD3 mouse monoclonal antibody (OKT3), anti-CD28 (9.3), STAT5, phospho-tyrosine (4G10), and α-tubulin were used as already reported (16). A monoclonal antibody against Tax1 protein was generated previously (20).

Reverse transcription PCR

Total RNA was isolated using Isogen (Nippongene), and cDNA was synthesized using the Advantage RT-for-PCR Kit (Clontech) as reported previously (21). The resulting cDNA (25 ng) was subjected to PCR amplification. The relative concentrations of the PCR products were determined by comparing the ratio of the product in each lane to *β-actin*. The PCR primers used were already reported (15).

Telomerase assay and quantification of enzyme activity

Telomerase activity was measured using the telomere repeat amplification protocol (TRAP) as previously described (22). To quantify telomerase activity in each sample, enzyme activity was expressed in arbitrary units as reported previously (15). Internal telomerase assay standard (ITAS) was used as the internal control (23).

RNA interference

Separate aliquots of 2×10^6 cells were transfected with a double-stranded *short interfering RNA (siRNA)* targeting *STAT5A* mRNA or a control nonsilencing *siRNA* (purchased from Dharmacon) using the Amaxa Nucleofector electroporation technique (Amaxa) according to the manufacturer's guidelines. The final concentration of each *siRNA* was 0.5 μg/mL, and the *siRNA* sequence targeting *STAT5A* was designed using *siRNA*-design software (Dharmacon). One hour after transfection with siRNA, 100 U/mL of IL-2 or solvent alone was added to the cell cultures. After 2 days of culture, the cells were harvested for immunoblot analysis and telomerase activity assays.

Immunoprecipitation and immunoblotting

Cells (2×10^7) were lysed in radioimmunoprecipitation assay (RIPA) buffer, and precleared samples were

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incubated with the appropriate antibodies for 2 hours or overnight. Immune complexes were resolved by 5% linear or 5% to 20% gradient SDS-PAGE and transferred to polyvinylidene difluoride membranes (BioRad). Blots were incubated with primary antibodies and processed as already reported (15).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were conducted as described previously (24). Cells were fixed in formaldehyde for 10 minutes at 25°C and then were resuspended in lysis buffer, followed by preclearing with a salmon sperm DNA/protein G-Agarose slurry at 4°C. Each precleared solution was then incubated with STAT5 antibody or normal rabbit serum overnight at 4°C. Immune complexes were separated by incubation with salmon sperm DNA/protein G-Agarose slurry at 4°C. After washing the pellets, DNA elution buffer was added and the samples were heated to 65°C, followed by treatment with 4 mg of RNase at 37°C and 10 mg of proteinase K at 55°C. The DNA was then purified and subjected to PCR as reported before (24).

Immunoprecipitation-TRAP assay

Equal amounts of cell lysates were precleared with purified preimmune mouse or rabbit IgG and protein G-agarose for 1 hour at 4°C. Cell lysates were incubated with rabbit antisera against hTERT, AKT, HSP90, mTOR, or S6K for 1 hour or overnight at 4°C and then incubated with protein G-agarose for 1 hour at 4°C. Agarose beads were washed 5 times in wash buffer and 2 μL of each immunoprecipitate was subjected to TRAP assay analysis as described before.

Statistical analysis

Data are expressed as the mean ± SD of 3 or more independent experiments. Statistical analysis was carried out using the 2-tailed Student *t* test for paired data. $P < 0.05$ was considered statistically significant.

Results

IL-2 induces telomerase activity and hTERT expression in ATL cells

ILT-Hod cells, an IL-2–dependent T-cell line derived from chronic ATL patients, were IL-2 starved for 6 days and then stimulated with either anti-CD3/CD28 or IL-2 for 48 hours. Stimulation with IL-2 alone was sufficient to induce telomerase activity, whereas nonmitogenic stimulation with anti-CD28 and/or anti-CD3 alone (T-cell receptor stimulation) did not induce telomerase expression (Fig. 1A). For cell starvation and restimulation, ILT-Hod cells cultured with the constant addition of IL-2 were starved of IL-2 and then stimulated with 100 U/mL IL-2 for various time periods. As shown in Fig. 1B, telomerase activity decreased to less than 20% after 6 days and readdition of IL-2 induced telomerase activity by 24 hours and higher levels were observed at 48 hours. Cells that had more than 80% viability were recovered during the experiments. *hTERT* mRNA was detectable by 24 hours, and higher levels were detected 48 hours after IL-2 stimulation (Fig. 1C). Consistent with the *hTERT* mRNA results, hTERT protein was detectable by immunoblotting 24 hours after IL-2 stimulation (Fig. 1D). In addition, during IL-2 stimulation, the number of cells in S phase increased together with the induction of telomerase activity (Fig. 1E).

Activation of JAK–STAT signaling molecules and interactions between these proteins

The pleiotropic functions of the HTLV-I Tax protein are thought to cooperate in promoting the proliferation of infected T cells (25). We thus examined whether IL-2 induces Tax expression in ILT-Hod cells. IL-2 did not upregulate Tax expression in the IL-2–dependent ATL cell lines. In contrast, Tax is constitutively expressed in IL-2–independent HUT102 cells (Fig. 2A). Next, IL-2–induced signal transduction pathways downstream of IL-2R activation were examined. Upon binding to receptors, cytokines induce phosphorylation and activation of members of the JAK family, which leads to the recruitment and phosphorylation of STATs. To identify which JAKs are phosphorylated and associated with STAT proteins in cells stimulated with IL-2, cell extracts were immunoprecipitated with antibodies against JAK1, JAK2, JAK3, STAT3, or STAT5 and then immunoblotted with the 4G10 antiphosphotyrosine antibody. Immunoblotting with 4G10 revealed increased phosphorylation of JAK1, JAK2, JAK3, and STAT5 in IL-2–treated ILT-Hod cells and constitutive activation of all of these signaling proteins in HUT102 cells (Fig. 2B). Coimmunoprecipitation assays revealed that JAK1 and/or JAK2 but not JAK3 are involved in the IL-2 signaling events that activate STAT5 in ILT-Hod cells, whereas JAK2 and/or JAK3 are involved in both STAT3 and STAT5 activation in the IL-2–independent growth of HUT102 cells (Fig. 2C).

STAT5 is a telomerase transcription factor in IL-2–responsive ATL cells

Because our data indicated that IL-2 could induce tyrosine phosphorylation of STAT5, we then studied whether the activation of STAT5 correlated with its potential to transcriptionally activate telomerase using DNA-binding experiments (Fig. 3A and B). As shown in Fig. 3B, STAT5 became associated with the promoter following activation by IL-2, suggesting that STAT5 is one of the transcription factors that regulates telomerase during IL-2 activation of ILT-Hod cells. To confirm the role of STAT5 in telomerase expression, we analyzed the relationship between STAT5 activation and telomerase activity. ILT-Hod cells were incubated with *siSTAT5* or nonsilencing *siRNA* for 2 days, and lysates were then examined by immunoblot analysis with anti-STAT5. Telomerase activity, together with STAT5, was clearly downregulated within 48 hours of transfection with *siSTAT5* (Fig. 3C).

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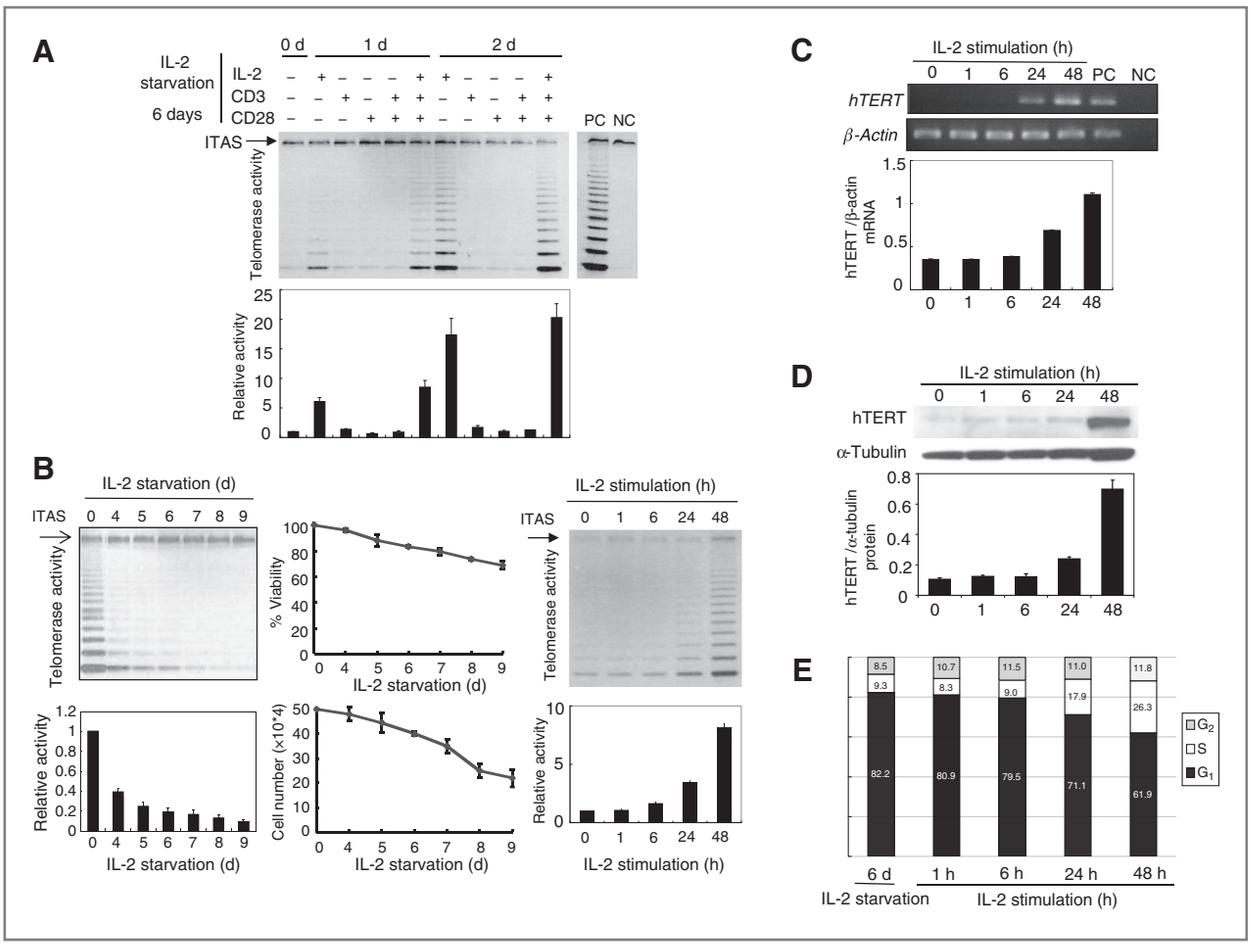

Figure 1. IL-2 induces telomerase activity and increases the levels of *hTERT* mRNA and protein in ATL cells. A, after a 6 day starvation period, ILT-Hod cells were stimulated either with IL-2 (100 U/mL) or anti-CD3 or anti-CD28 antibodies, or a combination of these stimulators, for 2 days. Telomerase activity was assessed by the TRAP assay at the indicated time points. PC, positive control (Namalva cells); NC, negative control; ITAS, internal standard. Telomerase activity is expressed relative to the activity observed in IL-2–starved cells (0 day). Data represent the mean ± SD of at least 3 separate experiments. B, IL-2–induced telomerase activity was measured at the indicated times. ILT-Hod cells cultured with the constant addition of IL-2 were starved of IL-2 for 6 days and then stimulated with 100 U/mL IL-2 for various time periods. Telomerase activity is expressed relative to that of IL-2–starved cells (0 hour or 6 days), whose activity was normalized to 1.0. Cells showing more than 80% viability were used in experiments to exclude unreliable data from dead cells. Data are the mean ± SD of at least 3 separate experiments. C, expression of *hTERT* mRNA as assessed by reverse transcription PCR (RT-PCR; top). *β-Actin* mRNA was analyzed as a positive internal control (middle). The levels of *hTERT* mRNA were then normalized to the levels of *β-actin* (bottom). The data represent the mean ± SD of at least 3 separate experiments. D, hTERT protein levels as measured by immunoblotting with an hTERT-specific antibody (top). α-Tubulin was examined as an internal control (middle). The levels of hTERT were normalized to the levels of α-tubulin (bottom). The data represent the mean ± SD of at least 3 separate experiments. E, IL-2–starved ILT-Hod cells were stimulated with 100 U/mL IL-2 for 1 to 48 hours, and the DNA content was analyzed by flow cytometry. The proportion of cells in different cell-cycle phases (percent) was determined using WinCycle analysis software.

Inhibition of IL-2–induced telomerase activity, *hTERT* mRNA expression, and hTERT protein by various pharmacologic inhibitors

To further clarify the mechanism by which IL-2 increases telomerase activity, we treated cells with various reagents that block critical signaling pathways involved in cell proliferation and cell survival. The viability of ILT-Hod cells cultured in the presence of the inhibitors was not reduced significantly after 48 hours of treatment (Fig. 4A, lower panel). IL-2–induced telomerase activity was abolished in a dose–dependent manner by treatment with LY294002, a specific inhibitor of PI3K; radicicol and geldanamycin, inhibitors of HSP90; and rapamycin, an inhibitor of mTOR, which is a downstream effector of AKT (Fig. 4A, top and middle panels). In contrast, PD98059, a specific inhibitor of MEK1/2 that blocks extracellular signal-regulated kinase (ERK)1/2, did not affect IL-2–induced telomerase activity. We next examined whether IL-2–induced expression of *hTERT* mRNA or protein was affected by these inhibitors. When cells were treated with 20 μmol/L LY294002, IL-2–induced expression of *hTERT* mRNA and protein was significantly blocked (Fig. 4B and C). Treatment with 0.1 μmol/L radicicol, 0.1 μmol/L geldanamycin, and 5 μmol/L rapamycin also blocked increased *hTERT* mRNA and protein levels, but 50 μmol/L PD98059 had no effect on

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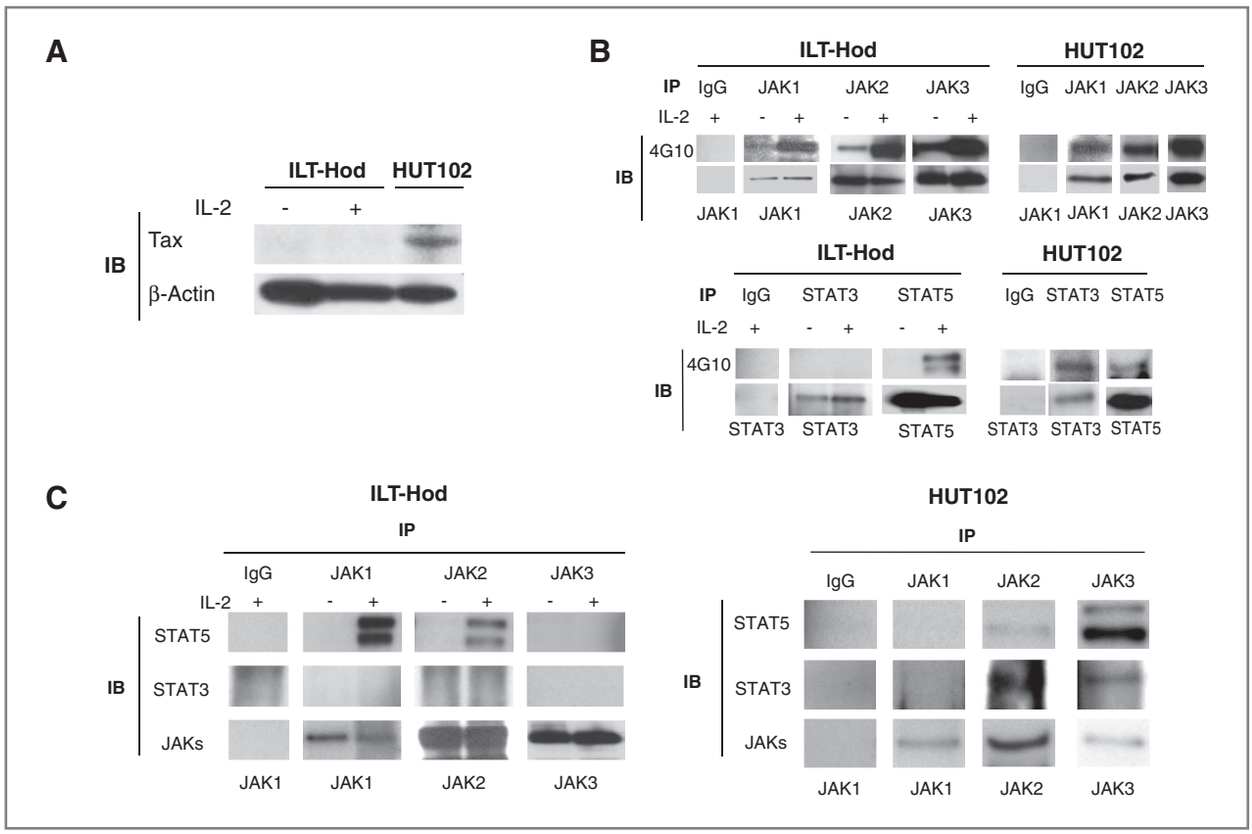

Figure 2. Activation of JAK–STAT signaling molecules and the interaction between these proteins in response to IL-2 stimulation. A, Tax protein levels were measured in IL-2–dependent ILT-Hod cells or in IL-2–independent HUT102 ATL cells by immunoblotting. B, tyrosine phosphorylation of STAT and JAK proteins in ATL cells either unstimulated or stimulated with IL-2 for 2 days. Cell extracts were immunoprecipitated with either rabbit IgG (control) or antibodies against JAK1, JAK2, JAK3, STAT3, or STAT5 followed by immunoblotting with 4G10 (anti-phosphotyrosine), JAK1, JAK2, JAK3, STAT3, or STAT5 antibodies. C, interaction between JAK and STAT proteins in ATL cells either unstimulated or stimulated with IL-2. Cell extracts were immunoprecipitated with either IgG (control) or antibodies against JAK1, JAK2, JAK3, STAT3, and STAT5 followed by immunoblotting with each antibody. Each panel is representative of more than 3 separate experiments.

either IL-2–induced *hTERT* mRNA expression or protein levels, suggesting that the PI3K/AKT/HSP90/mTOR pathway is involved in transcriptional regulation of telomerase activity (Fig. 4B and C).

Activation of the JAK2/PI3K/AKT/mTORC1 pathway is associated with hTERT activity in ATL cells

As shown in Fig. 5A, the low level of constitutive AKT phosphorylation was increased by exposure to IL-2 following IL-2 deprivation. Similarly, 2 other downstream components of the AKT signaling pathway, mTOR and p70S6K, also showed increased phosphorylation (Fig. 5A).

We showed that JAK1, JAK2, and JAK3 were tyrosine phosphorylated in response to IL-2 (Fig. 2B). Although these molecules have been reported to bind PI3K in other cell systems (26, 27), its interaction has not been investigated in ATL cells. To determine whether PI3K associates with JAKs in response to IL-2, coimmunoprecipitation assays were conducted. As shown in Fig. 5B, IL-2 enhanced the association of the PI3K p85 subunit with JAK2, indicating that IL-2 induces a physical interaction between JAK2 and PI3K, as well as between JAK2 and STAT5. To confirm the association of these PI3K signaling pathway molecules with telomerase, we carried out TRAP assays using immunoprecipitates (IP-TRAP) generated with specific antibodies against hTERT, mTOR, S6K, AKT, and HSP90. These immunoprecipitates contained telomerase activity that was reduced by treatment with the corresponding inhibitors of these proteins (Fig. 5C). In contrast, immunoprecipitates using antibodies against either IgG or ERK did not show telomerase activity, being consistent with the results that IL-2–induced telomerase activity was not sensitive to PD98059, a MEK/ERK inhibitor (Fig. 5C). These data indicate that IL-2–induced telomerase activity is also regulated posttranslationally through the JAK2/PI3K/AKT/HSP90/mTORC1 signaling pathway.

Telomerase activity and immunoblotting in primary ATL cells

We next examined whether the components we identified were also involved in the regulation of telomerase

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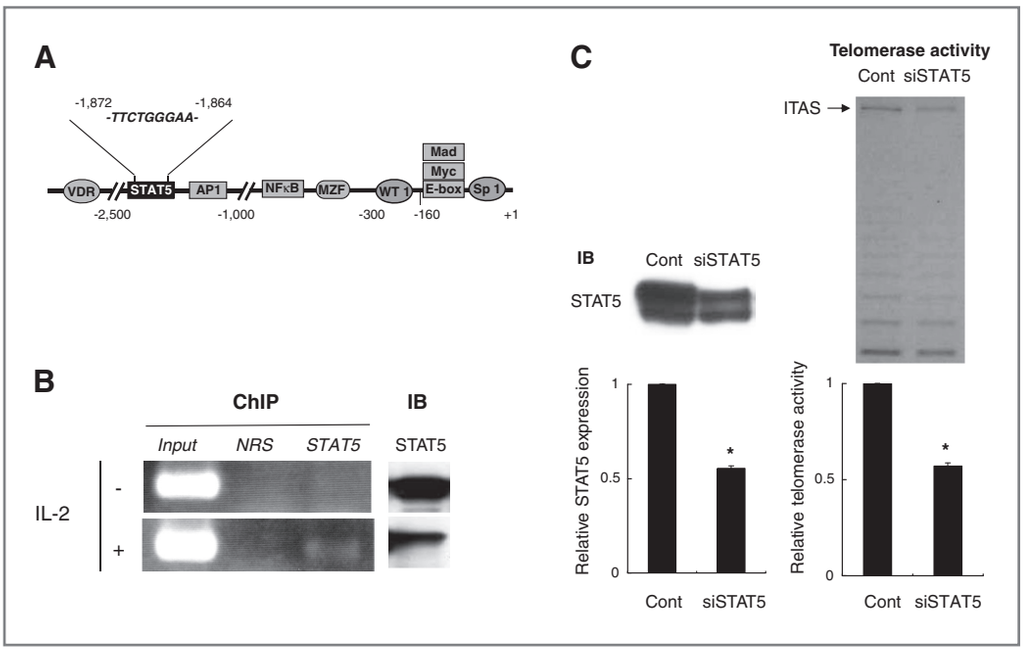

Figure 3. STAT5 is a telomerase transcription factor in IL-2–responsive ATL cells. A, a schematic representation of the hTERT promoter containing the STAT5 consensus sequence. B, ChIP assay of the endogenous telomerase promoter. ILT-Hod cells were either left unstimulated or stimulated with IL-2 for 2 days and then examined by ChIP assay. DNA was extracted and then immunoprecipitated using an anti-STAT5 antibody followed by PCR amplification with primers directed against the putative STAT5 binding site in the hTERT promoter. C, STAT5 siRNA (*siSTAT5*) inhibits telomerase activity in IL-2–stimulated ATL cells. ATL cells were transfected with *siSTAT5* and then examined either by immunoblotting with an anti-STAT5 antibody (left) or by the TRAP assay (right). The levels of STAT5 were densitometrically quantified. The telomerase activity of *siSTAT5*-transfected cells is expressed relative to the activity observed in cells transfected with a negative control. Data represent the means ± SD of 3 separate experiments. *, $P < 0.05$.

activity in primary leukemic cells. Using 4 primary chronic phase leukemic samples, we conducted TRAP assays and immunoblotting before and after stimulation with 100 U/mL of IL-2 for 2 days. Increased telomerase activity was detected in 3 of 4 samples after IL-2 stimulation (Fig. 6A). Interestingly, an increased level of telomerase protein was observed concomitant with activated STAT5 (Fig. 6B), suggesting that the same components involved in upregulation of telomerase activity in ILT-Hod cells are also involved in the regulation of telomerase activity in primary ATL cells.

Discussion

In this study, we showed that stimulation of a T-cell line established from a patient with chronic ATL through the IL-2 receptor but not the T-cell receptor induces cell-cycle entry and telomerase activity within 24 hours. Tax was detected in the IL-2–independent HUT102 cells but not in the IL-2–dependent ILT-Hod cells. Leukemic cells generally do not express Tax *in vivo* due to several regulatory mechanisms (28). Thus, it is unlikely that Tax is involved in the induction or activation of telomerase in this system. Alternative mechanisms independent of the Tax protein may be involved in hTERT expression and telomerase activity in IL-2–dependent chronic ATL cells.

Binding of IL-2 to the IL-2R β and γ chains results in the activation and recruitment of JAK1, JAK2, and JAK3 and the phosphorylation and nuclear translocation of the STATs (13). JAK and STAT activation is associated with the proliferation and survival of IL-2–independent ATL cell lines and primary ATL cells (14). However, the mechanism by which IL-2 signaling is involved in the activation of the JAK–STAT pathway in IL-2–dependent ATL cells is not clear. To ensure that any basal phosphorylation of JAK/STAT proteins was not due to prior exposure to IL-2, the ILT-Hod ATL cells were cultured for 6 days without IL-2. Subsequent stimulation of ILT-Hod cells with IL-2 caused tyrosine phosphorylation of JAK1, JAK2, JAK3, and STAT5. In contrast, the constitutive activation of each of these signaling proteins was observed in IL-2–independent HUT102 cells. Coimmunoprecipitation assays revealed that JAK1 and/or JAK2 but not JAK3 are involved in the IL-2 signaling events that activate STAT5 in ILT-Hod ATL cells, whereas JAK2 and/or JAK3 are involved in both STAT3 and STAT5 activation in the IL-2–independent growth of HUT102 cells. These findings indicate that JAK1/JAK2/STAT5 phosphorylation is triggered by IL-2 stimulation in ILT-Hod ATL cells and that STAT5 is functional and presumably required for IL-2–mediated proliferation and telomerase induction. Intriguingly, IL-2 activated both STAT5 and telomerase in primary samples, suggesting that this signaling pathway is active in ATL tumors *in vivo*. To further investigate the transcriptional regulation of telomerase by STAT5, ChIP assays were carried out to examine whether STAT5 could

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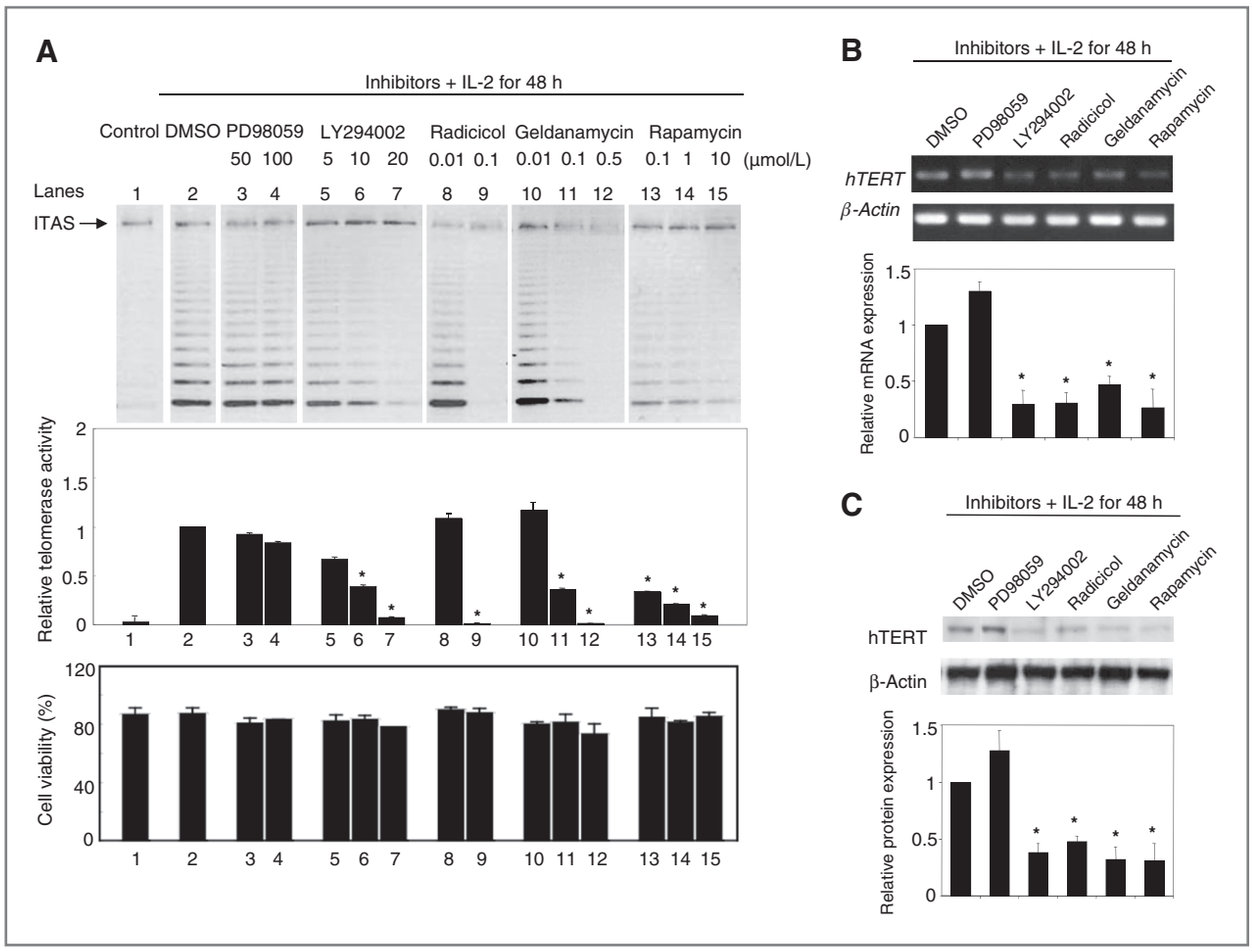

Figure 4. Inhibition of IL-2–induced telomerase activity, *hTERT* mRNA expression, and the induction of hTERT protein by various pharmacologic inhibitors in ATL cells. A, IL-2–starved ILT-Hod cells were stimulated with IL-2 for 48 hours in the presence or absence of each inhibitor. A representative example of telomerase activity is shown (top). Telomerase activity is expressed relative to the activity observed following IL-2 stimulation + DMSO (middle). The viability of ATL cells cultured in the presence of inhibitors was evaluated by trypan blue dye exclusion (bottom). Lane 1, IL-2 starvation; lane 2, IL-2+DMSO; lanes 3 and 4, IL-2 + PD98059; lanes 5–7, IL-2 + LY294002; lanes 8 and 9, IL-2 + radicicol; lanes 10–12, IL-2 + geldanamycin; lanes 13–15, IL-2 + rapamycin. B, effect of inhibitors on *hTERT* mRNA expression as measured by RT-PCR (top). The cells were incubated with different concentrations of each inhibitor (50 μmol/L of PD98059, 20 μmol/L of LY294002, 0.1 μmol/L of radicicol, 0.1 μmol/L of geldanamycin, and 5 μmol/L of rapamycin). *hTERT* mRNA expression was quantified by normalizing the levels *hTERT* to the levels of *β-actin* (bottom). Values represent the means ± SD of 3 separate experiments. C, effect of inhibitors on hTERT protein levels. The concentration of each inhibitor is same as in B. The levels of hTERT were quantified by normalizing to the levels of β-actin (bottom). Values represent the means ± SD of 3 separate experiments. *, $P < 0.05$.

directly bind to the promoter region of *hTERT*. STAT5 was associated with the telomerase promoter after exposure to IL-2, indicating that STAT5 is one of the transcription factors that regulate hTERT expression in IL-2–stimulated ILT-Hod cells. *siRNA*-mediated STAT5 knockdown resulted in the functional silencing of telomerase activity, confirming the role of STAT5 in this pathway. We have previously observed the direct binding of STAT5 to the promoter region of *hTERT* in chronic myeloid leukemia (15, 16). To the best of our knowledge, this is the first report of STAT5 as a direct regulator of *hTERT* transcription in lymphoid cells.

Because IL-2 promotes the entry of ILT-Hod cells into the cell cycle, the activation of telomerase by IL-2 should be associated with cell proliferation. IL-2 activates the ERK and AKT signaling pathways, which are associated with normal T-cell proliferation (29). Therefore, we hypothesized that these pathways might participate in the regulation of IL-2–induced telomerase activity in ATL cells. PD98059, a specific inhibitor of MEK1/2, did not block the IL-2–induced increase in telomerase activity or the expression of *hTERT* mRNA. In contrast, LY294002, a specific inhibitor of PI3K, blocked IL-2–induced telomerase activity and the expression of *hTERT* mRNA. Rapamycin, an inhibitor of mTOR, also blocked both telomerase activity and the increase in *hTERT* mRNA and protein observed in IL-2–stimulated cells. These results suggest that the PI3K/AKT/mTOR pathway transcriptionally regulates telomerase activity in response to IL-2. Moreover, antibodies against AKT,

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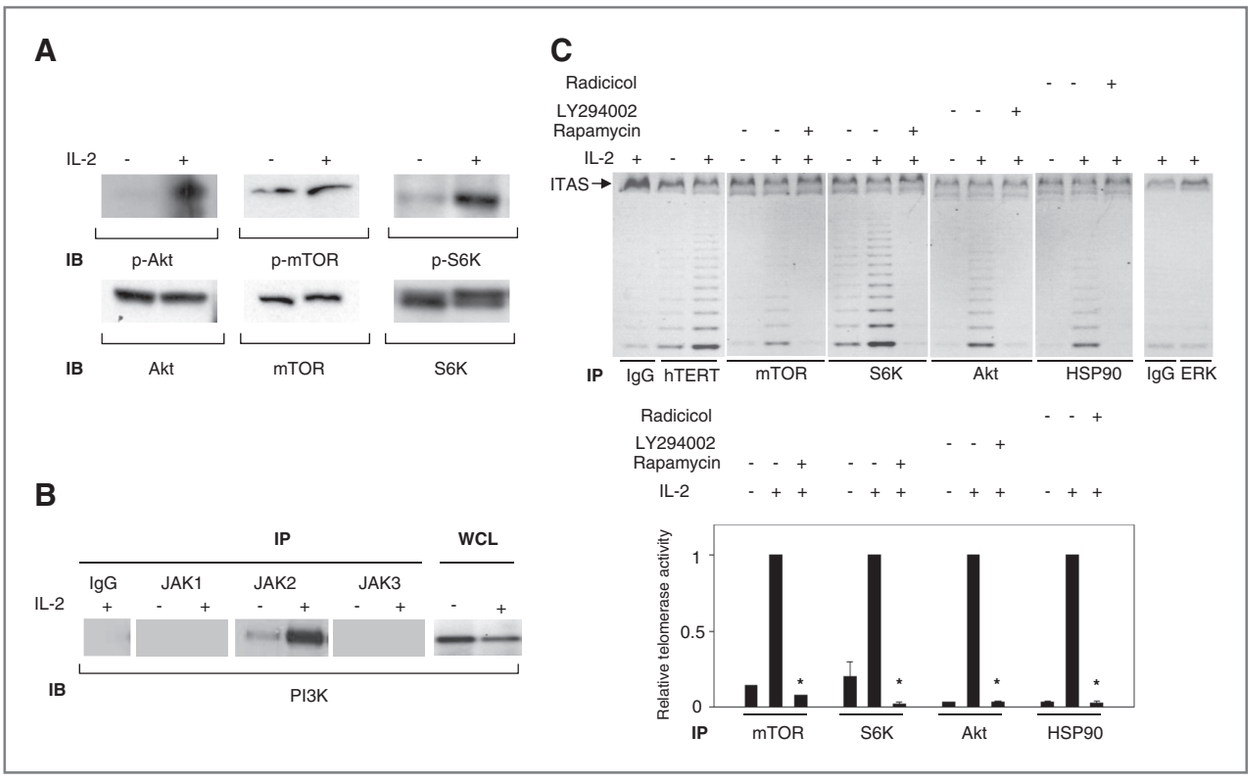

Figure 5. IL-2 stimulates JAK2/PI3K/AKT/mTOR pathway activation associated with hTERT activity in ATL cells. A, the AKT/mTOR pathway is activated in ATL cells by IL-2 challenge. IL-2–starved ILT-Hod cells were stimulated with 100 U/mL of IL-2 for 2 days. Whole-cell lysates (WCL) were subjected to immunoblotting with antibodies against AKT, phospho-AKT (Ser473), mTOR, phospho-mTOR (Ser2448), S6K, and phospho-S6K (Thr389). Each panel represents more than 3 separate experiments. B, an association between JAK2 and PI3K is induced by IL-2 stimulation. IL-2–starved ATL cells were stimulated as in A. Cell lysates were immunoprecipitated with antibodies against rabbit IgG (control), JAK1, JAK2, or JAK3 and then immunoblotted with an antibody against the p85 subunit of PI3K. The presence of PI3K was confirmed by immunoblotting of WCL. Each panel is representative of the results of more than 3 separate experiments. C, telomerase activity in IgG, hTERT, mTOR, S6K, AKT, HSP90, or ERK immunoprecipitates of IL-2–stimulated ATL cell lysates in the presence or absence of LY294005 (20 μmol/L), radicicol (0.1 μmol/L), and rapamycin (5 μmol/L; top). The quantification of telomerase activity in immunoprecipitates with or without inhibitors is shown in the bottom panel. Cell lysates were immunoprecipitated with antibodies and then subjected to the TRAP assay as described in the Materials and Methods. The relative telomerase activity of IL-2–stimulated cells in the absence of each inhibitor (defined as 1.0) was compared with the activity observed in the presence of the inhibitors. Values represent the means ± SD for at least 3 separate experiments. *, $P < 0.05$.

mTOR, and S6K immunoprecipitated the telomerase activity, indicating that AKT, mTOR, and S6K interact directly with active hTERT protein in an IL-2–dependent manner. Thus, the PI3K/AKT/mTORC1 signaling pathway may be implicated in the posttranslational regulation of hTERT.

Our studies also revealed a possible role for HSP90 in regulating telomerase activity. HSP90 is an abundant cytosolic protein that acts in concert with cochaperones, such as HSP70 and p23, to prevent aberrant protein folding, which can lead to protein inactivation and aggregation (30). HSP90 has been shown to be required for efficient telomerase assembly (31). In ATL cells, IL-2–induced telomerase activity was blocked by geldanamycin and radicicol, which are inhibitors of HSP90. These compounds also inhibited *hTERT* mRNA expression and the corresponding increase in hTERT protein, indicating that HSP90 is involved in the transcriptional modulation of *hTERT*. Furthermore, HSP90 immunoprecipitates of IL-2–stimulated ATL cells contained active telomerase. Thus, HSP90 may act as an hTERT chaperone. These findings indicate that PI3K, AKT, mTORC1, and HSP90 upregulate *hTERT* in both a transcriptional and posttranslational manner in IL-2–dependent ATL cells. This mechanism is different from what was observed in NK cell tumors, in which mTOR and HSP90 are only implicated in the posttranslational regulation of hTERT (32). Recently, Bellon and colleagues reported that IL-2 signaling is associated with PI3K-dependent transcriptional upregulation of *hTERT* through the suppression of WT-1 in Tax-negative HTLV-1–transformed cells (12). This mechanism might also contribute to the IL-2–induced telomerase activity observed in this ATL system, even in the absence of Tax protein.

Upon IL-2 stimulation, PI3K has been shown to localize to the IL-2R in a murine T-cell line (33, 34). We observed that IL-2 stimulation of ATL cells leads to the phosphorylation of downstream effectors of PI3K, such as mTOR, p70S6K, and AKT, and found that JAK2 coimmunoprecipitated with the p85 subunit of PI3K in an IL-2–

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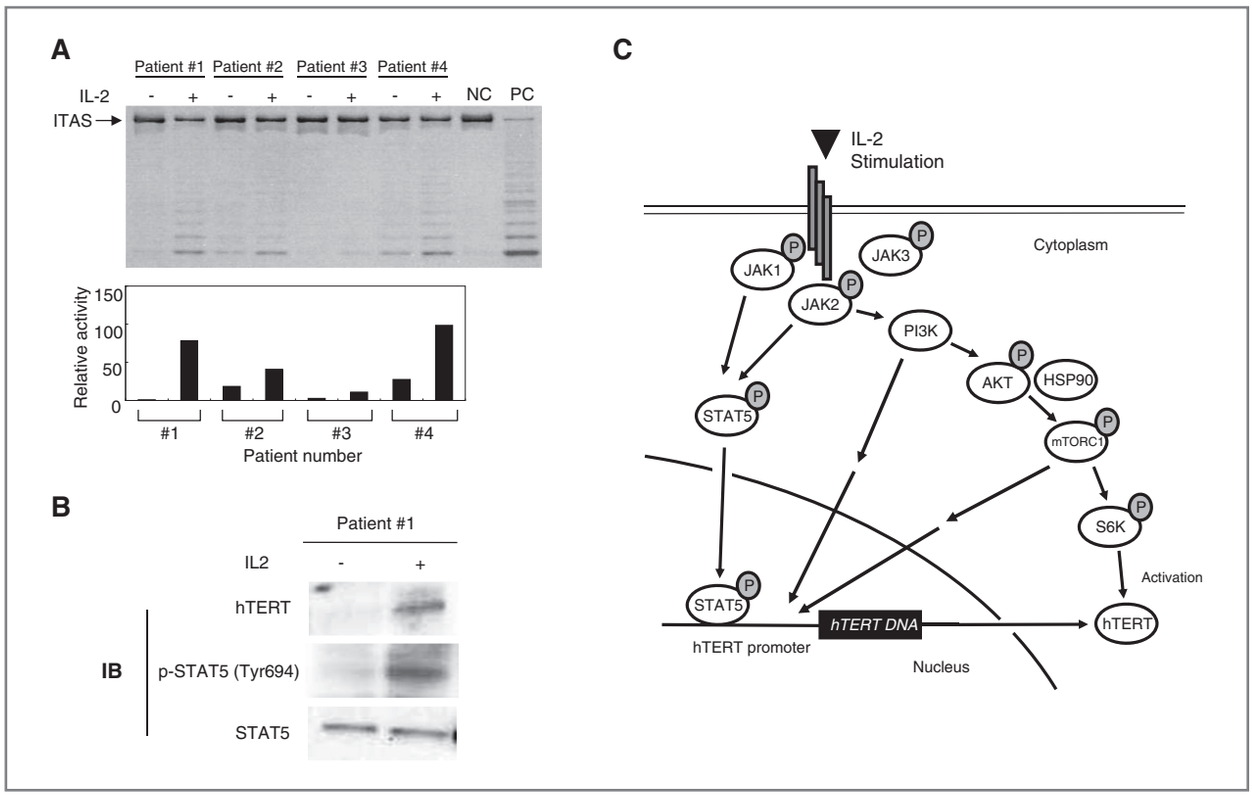

Figure 6. Telomerase activity in primary ATL samples and schematic representation of the activation pathway driven by IL-2 stimulation in ATL cells. A, the telomerase activity in primary leukemic cells (patients #1, #2, #3, and #4) was measured by the TRAP assay before and after stimulation with 100 U/mL of IL-2 for 2 days. PC, positive control; NC, negative control. The telomerase activity in the cells from each patient is expressed relative to the activity of the PC, which was set at 100, as described in the Materials and Methods. B, cell lysates of the primary tumor cells described in A were subjected to immunoblotting with antibodies against STAT5, p-STAT5, and hTERT. Representative results from patient #1 are shown. C, in ATL cells, IL-2 binding to its receptor induces tyrosine phosphorylation (activation) of JAKs, and in turn STAT5 phosphorylation, leading to the transcriptional activation of hTERT. Simultaneously, IL-2–driven JAK2 activation may contribute to the activation of the PI3K/AKT/mTORC1/S6K pathway, leading to transcriptional and posttranslational upregulation of hTERT activity. Thus, IL-2–induced hTERT activity can be regulated transcriptionally or posttranslationally through both the JAK–STAT and the JAK–PI3K pathway in ATL cells.

dependent manner, consistent with previous evidence that JAK2 can bind to the N-terminal SH2 domain of the PI3K p85 subunit in other hematopoietic systems (26, 27). The current data indicate that IL-2–induced activation of JAK2 occurs upstream not only of STAT5 but also of the PI3K pathway. Therefore, JAK2 may be one of the key molecules in the regulation of IL-2–induced telomerase activity in ATL cells.

In conclusion, this study shows that IL-2 stimulation induces hTERT activation in IL-2–dependent T-cell line established from a patient with chronic ATL and in primary chronic ATL cells. IL-2 is required for tumor growth in smoldering/chronic ATL and even in some acute ATL patients (11, 35). Therefore, the dissection of the mechanism by which IL-2 promotes tumor formation in such ATL cells is important to develop effective therapies for ATL, particularly before progression to aggressive ATL. We showed that IL-2–induced telomerase activation involves transcriptional and posttranslational regulation through dual signaling pathways, JAK/STAT and JAK/PI3K/AKT/HSP90/mTORC1 (Fig. 6C). These results shed light on the mechanism of ATL cell proliferation in response to IL-2 and uncovered novel therapeutic targets in ATL (36).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: O. Yamada, K. Ozaki, M. Akiyama, K. Kawauchi
Development of methodology: O. Yamada, K. Ozaki, K. Kawauchi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): O. Yamada, K. Kawauchi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): O. Yamada, K. Ozaki, K. Kawauchi
Writing, review, and/or revision of the manuscript: O. Yamada, K. Kawauchi
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): O. Yamada, K. Ozaki, K. Kawauchi
Study supervision: O. Yamada, K. Kawauchi

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