The Upregulation of p27\textsuperscript{Kip1} by Rapamycin Results in G1 Arrest in Exponentially Growing T-Cell Lines

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RAPAMYCIN (Rap) EXERTS antiproliferative effect by forming the complex with an intracellular protein FKBP12. Rap-FKBP12 interacts with FRAP (also known as RAFT), a human homologue of the target molecule of Rap (TOR) in yeast and inhibits FRAP activity that is required for the activation of p70 S6 kinase.\textsuperscript{12} Although FRAP itself is not a mitogen-activated p70 S6 kinase, the Rap-FKBP12–FRAP complex selectively inhibits the mitogen-induced p70 S6 kinase activation without disrupting other known pathways.\textsuperscript{8-13}

The mitogen-stimulated signaling pathways also involve a sequential regulation of the cell cycle-related molecules such as the elimination of cdks inhibitor p27\textsuperscript{Kip1}, the increment of p21\textsuperscript{Waf1} and the activation of kinase activity of the G1 cyclin/ cdks complexes that may phosphorylate Rb protein when quiescent cells enter into the cell cycling.\textsuperscript{14-17} Rap has also been reported to exert its antiproliferative effect by affecting these molecules in G1 phase. Rap blocks the elimination of cdks inhibitor p27\textsuperscript{Kip1} and inactivates the kinase activity of the G1 cyclin/cdks complex by facilitating the formation of the G1 cyclin/cdks-p27\textsuperscript{Kip1} complex.\textsuperscript{15-25} The continuous elimination of p27\textsuperscript{Kip1}, which is reported to be mediated by the ubiquitin-dependent degradation\textsuperscript{26,27} is observed throughout G1 phase when quiescent cells enter into the cell cycling in response to mitogenic stimuli. However, the mechanism of how Rap blocks the elimination of p27\textsuperscript{Kip1} remains to be elucidated. Furthermore, with our limited knowledge about the mechanism of G1 cell cycle progression, we cannot even tell whether the inhibition of p70 S6 kinase and the inhibition of G1 cyclin/cdks kinase activities occur serially or independently after the treatment of Rap.

Most of our knowledge about the effects of Rap has been provided by previous studies that focused on the events in G1 phase when quiescent cells were incubated with Rap and mitogens, while the mechanism of the antiproliferative effect of Rap on exponentially growing cells has been poorly investigated. The G1 cell cycle events of exponentially growing cells that skip a putative G0/G1 transition may differ from that of the cells leaving the quiescent state and entering into the cell cycling in response to mitogenic stimuli. It is, therefore, possible that Rap may use different mechanisms to exert its antiproliferative effect depending on the cell cycling status. In addition, Rap does not always exert an antiproliferative effect on exponentially growing cells, which provides us with the ground to postulate the factor(s) that may determine the sensitivity of exponentially growing cells to Rap.

To address these issues, we examined the antiproliferative effect of Rap on various T-cell lines that were growing exponentially, comparing with the previous findings on the cells leaving the quiescent state in response to mitogenic stimuli. We found that Rap upregulated p27\textsuperscript{Kip1} at both mRNA and protein levels and caused G1 arrest in Rap-sensitive cells. Based on the correlation between the intracellular protein level of p27\textsuperscript{Kip1} and the sensitivity to Rap, a possible mechanism of the antiproliferative effect of Rap on exponentially growing T cells will be discussed.

MATERIALS AND METHODS

Cells and culture conditions. Kit225, a human interleukin (IL)-2 dependent CD4-positive T-cell line,\textsuperscript{28} was cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (Summit, Monfort, Ft. Collins, CO), 60 mmol/L tobramycin, 2 mmol/L L-glutamine, and 0.5 mmol/L recombinant human IL-2 (rIL-2, a gift of Shionogi Research Laboratories, Osaka, Japan). Kit225 cells were synchronized in quiescent state by rIL-2 depletion for 48 hours. The cells entered into the cell cycling in response to 2 mmol/L IL-2. ED-40515 (–) is a human T-cell leukemia virus (HTLV-I)–infected IL-2 independent T-cell line established from a patient with adult T-cell leukemia (ATL).\textsuperscript{29} TL-Oml,\textsuperscript{30} MJ,\textsuperscript{31} MT-2\textsuperscript{32} are HTLV-I–infected T-cell lines previously described. ED-40515 (–), TL-Oml, MJ, Mo74, HSB-2, HPB-ALL, and Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 60 mmol/L tobramycin and 2 mmol/L L-glutamine.

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Cell proliferation assay. Cell proliferation was measured by trium-thymidine ([\(^3\)H]thymidine) incorporation into DNA. After 48 hours depletion of IL-2, Kit225 cells were plated to a 96 well flat-bottomed plate at 1 × 10^5 cells/100 µL/well and cultured with 2 mmol/L IL-2 at 37°C in 5% CO\(_2\) for 48 hours. A total of 10 mmol/L Rap was added at the indicated time and 0.5 µCi of [\(^3\)H]thymidine (DuPont/NEN Research Products, Boston, MA) was added to each well 6 hours before the harvest of the culture. Incorporation of [\(^3\)H]thymidine was measured by liquid scintillation counting. For cell proliferation assay of exponen-tially growing T-cell lines, cells were kept in exponentially growing phase by changing the medium (RPMI 1640 plus 10% FCS) every 2 to 3 days and 8 hours before the assay. Cell proliferation with or without 10 mmol/L Rap was examined as described above.

Cell cycle analysis. Cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS) containing 0.1% glucose. Cells were then fixed with 70% ethanol for 1 hour and resuspended in acridine orange solution (50 mmol/L acridine orange in PBS/0.1% glucose) for 1 hour at room temperature.\(^{34}\) DNA content analysis was performed by a FACScan with LYSIS II and Cell Quest softwares (Becton Dickinson, San Jose, CA).

Immunoprecipitation. Cell lysates from 5 × 10\(^6\) cells were prepared by lysing the cells in 100 µL of lysis buffer (25 mmol/L HEPES [pH 7.4], 0.15 mol/L NaCl, 0.5% Triton-X, 1 mmol/L phenylmethylsulfonyl fluoride [PMSF], 1 mmol/L MgCl\(_2\), 10% glycerol, 1 mmol/L EDTA, 20 µg/mL TPCK, 20 µg/mL soybeans trypsin-inhibitor, 10 µg/mL leupeptin). The cell extracts were incubated with polyclonal anti-cyclin E antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), anti-cyclin D\(_2\) antibody (Santa Cruz), or anti-cdk4 antibody (Santa Cruz), and 20 µL of Protein A Sepharose 4 fast flow beads (Pharmacia Biotech, Uppsala, Sweden) for 2 hours at 4°C. The immunoprecipitates were subjected to immunoblotting with anti-p27\(^{kip1}\) antibody (Santa Cruz).

Immunoblotting. Cell lysates containing 40 µg of soluble protein or immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% or 12.5% gel (ATTO, Tokyo, Japan). The samples were transferred to 0.45 µm polyvinylidene difluoride (PVDF) filters (Millipore Corp, Bedford, MA) with a semidy with apparatus. The filters were incubated with 10% bovine serum albumin (BSA) blocking buffer for 5 hours followed by diluted antibody (anti-p27\(^{kip1}\) antibody [Santa Cruz], anti-p21\(^{kip1}\) antibody [Santa Cruz], anti-cyclin E antibody [Santa Cruz], or anti-cyclin A antibody [Upstate Biotechnology Incorporated, Lake Placid, NY]) for 1 hour. The immunoblots were visualized by ECL detection system (Amersham Life Science, Arlington Heights, IL).

Time course study of [\(^35\)S]methionine-labeled p27\(^{kip1}\). Exponen-tially growing ED-40515(−) cells were harvested and cultured with methionine-free RPMI 1640 medium (GIBCO) for 30 minutes and labeled with 150 µCi/mL of [\(^35\)S]methionine (Dupont/NEN) for 45 minutes at the indicated points (0, 24, 48 hours of the culture). The cells were harvested again for lysis in hypotonic buffer (1 mol/L Tris-HCl [pH 7.5], 1 mol/L KCl, 1 mol/L MgCl\(_2\), 1 mmol/L dithiothreitol [DTT], 1 mmol/L PMSF). The half-life of p27\(^{kip1}\) at 28 hours of the culture was evaluated by labeling the cells with [\(^35\)S]methionine as stated above. The cells were placed in full medium to chase the metabolic labeling and harvested at the indicated times. The lysates were incubated with anti-p27\(^{kip1}\) antibody (Santa Cruz) and Protein A Sepharose 4 fast flow beads (Pharmacia). The immunoprecipitates were separated by SDS-PAGE with 12.5% gel. The radioactive signals associated with the p27\(^{kip1}\) probe were obtained by autoradiography. Kinase assay of the immunocomplex was performed by suspending the beads in 40 µL of kinase buffer (20 mmol/L Tris-HCl [pH 7.5], 10 mmol/L MgCl\(_2\), 2 mol/L glycerol, 0.1 mg/mL BSA, 0.4 mmol/L DTT) containing 50 µmol/L ATP, 10 µCi of \([\(^32\)P]ATP, 125 µmol/L S6 peptide. After a 20-minute incubation at 30°C, phosphorylation reaction was terminated by adding 20 µL of 250 mmol/L ice-cold EDTA (pH 8.0). The triplicate aliquots of each sample were spotted onto phosphocellulose paper. The radioactivity was measured by liquid scintillation counting.

rrb phosphorylation assay was determined by \(^{32}\)P incorporation into glutathione S-transferase-pkB (GST-pkB; corresponding to amino acid 769-921 [Santa Cruz]).\(^{35}\) Cell extracts prepared as above were incubated with anti-p70 S6 kinase antibody (UBI) and 20 µL of protein A Sepharose 4 fast flow beads. Kinase assay of the immunocomplex was performed by suspending the beads in 40 µL of kinase buffer (20 mmol/L Tris-HCl [pH 7.5], 10 mmol/L MgCl\(_2\), 2 mol/L glycerol, 0.1 mg/mL BSA, 0.4 mmol/L DTT) containing 50 µmol/L ATP, 10 µCi of \([\(^32\)P]ATP, 125 µmol/L S6 peptide. After a 20-minute incubation at 30°C, phosphorylation reaction was terminated by adding 20 µL of 250 mmol/L ice-cold EDTA (pH 8.0). The triplicate aliquots of each sample were spotted onto phosphocellulose paper. The radioactivity was measured by liquid scintillation counting.

Northern blotting. Total cellular RNA was isolated from 1 × 10\(^7\) cells by the acid-phenol chloroform method. A total of 10 µg of total RNA was fractionated by electrophoresis through 1% agarose/formaldehyde denaturing gel and transferred to Hybond-N membrane (Amersham). The membranes were hybridized with \(^{32}\)P-labeled probes. Human \(\beta\)-actin cDNA probe (Clontech, Palo Alto, CA) was used as control. For the detection of p27\(^{kip1}\) mRNA by Northern blotting, p27\(^{kip1}\) cDNA probe was generated by the reverse transcriptase-PCR (RT-PCR) method. BamHI site-tagged oligonucleotides covering the entire coding region of p27\(^{kip1}\) and cDNA from phytohemagglutinin (PHA) stimulated human peripheral blood mononuclear cells were used as the primers and the template, respectively. The PCR product was inserted into pT7Blue T-vector (Novagen, Madison, WI). The cDNA probe of p27\(^{kip1}\) was obtained by digesting with BamHI. For the detection of p27\(^{kip1}\), cDNA probe was also generated by the RT-PCR method.

Preparation of antisense or frame shifted-sense cDNA of p27\(^{kip1}\) and transfection to ED-40515 (−) cells. The BamHI site-tagged cDNA of p27\(^{kip1}\) was generated by the RT-PCR method as stated in Materials and Methods.
Methods for Northern blotting. The PCR product was inserted into pBluescript II SK(+) cloning vector (STRATEGENE, La Jolla, CA). The construct was digested with Xho I and Not I and ligated into expression vector pMKIT Neo (a gift from Dr. Maruyama of Tokyo Medical and Dental University) at Xho I and Not I sites (Xho I site—3′ side, Not I site—5′ side) to generate pMKIT Neo antisense p27Kip1. The frame shifted sense cDNA of p27Kip1 was generated by the PCR method. Xho I site-tagged frame shifted 5′ oligonucleotide (CCTCGAG-GATGTCGACAAGTTCGAGTGC) and Not I site-tagged 3′ oligonucleotide (TGGCGGCGAATACGTTTGACGCTTCTTG) and SK(+)-p27Kip1 were used as the primers and the template, respectively. The PCR products were ligated into pMKIT Neo. The pMKIT Neo-p27Kip1 constructs were transfected into ED-40515(-) cells by the electroporation method. The total amount of transfected vector was adjusted to 10 µg by adding a empty vector. The transfected cells were cultured in medium containing 1 µg/mL G418 (Sigma Chemical Co) for 3 weeks (bulk culture) and subjected to immunoblotting and proliferation assay in the presence or absence of 10 nmol/L Rap.

RESULTS
Inactivation of p70 S6 kinase by Rap is observed at any cell cycle phase, while the antiproliferative effect of Rap is cell cycle phase-dependent in an IL-2-dependent T-cell line Kit225. Kit225 cells were synchronized in a quiescent state by IL-2 depletion for 48 hours. The cells entered into the cell cycle and passed the G1/S transition 18 hours after the addition of 2 nmol/L rIL-2.

Activation of p70 S6 kinase activity was observed within 1 hour after the addition of 2 nmol/L rIL-2 to the cells synchronized in a quiescent state by IL-2 depletion. Rap inhibited and reduced p70 S6 kinase activity within 1 hour to the basal level when added at any of the time points of 0 (concomitantly with IL-2), 6, 18, or 36 hours after the addition of 2 nmol/L rIL-2 (Fig 1A). However, Rap failed to exhibit an antiproliferative effect when added 18 hours (more than 60% of the cells pass through G1/S transition) or 36 hours (the cells were growing exponentially) after the addition of 2 nmol/L rIL-2 (Fig 1B).

Antiproliferative effect of Rap on exponentially growing T-cell lines is cell type-specific. Molt4, HSB-2, HPB-ALL, Jurkat, TL-Oml, MJ, MT-2, ED-40515(-) cells were kept in exponentially growing phase by changing the condition medium every 2 to 3 days and 8 hours before the assay. The antiproliferative effect of Rap was evaluated by tritium-thymidine incorporation into DNA. As shown in Fig 2, Rap did not always exert an antiproliferative effect on the growth of the exponentially growing cell lines examined. No obvious correlation between HTLV-I infection of the cell lines and their sensitivity to Rap was observed. Among the cell lines we tested, the HTLV-I-infected T-cell line, ED-40515(-) showed a relatively high sensitivity (67% to 82% suppression in cell proliferation) to Rap.

Rap inhibits cdk2 kinase activity, cyclin E-dependent kinase activity, and cyclin A-dependent kinase activity and causes G1 arrest in exponentially growing ED-40515(-) cells. ED-40515(-) cells in exponentially growing phase were incubated with 10 nmol/L Rap. Rap caused cell cycle arrest in G1 phase at 28 hours of the culture (Fig 3A). Rap inhibited cdk2 kinase activity, cyclin E-dependent kinase activity, and cyclin A-dependent kinase activity and markedly inhibited p70 S6 kinase activity to the basal level (Fig 3B). There was no obvious correlation between the inhibition of p70 S6 kinase and the sensitivity to Rap, as the inhibition of p70 S6 kinase was also observed in Rap-insensitive exponentially growing cell lines such as Kit225 (Fig 1A), HPB-ALL, and HSB-2 (data not shown). Rap slightly suppressed cdk4 kinase

Fig 1. Inhibition of p70 S6 kinase (A) and suppression of cell proliferation (B) by Rap in Kit225 cells entering into the cell cycling in response to IL-2. Kit225 cells deprived of IL-2 for 48 hours were cultured with or without Rap in the presence of 2 nmol/L IL-2. (A) A total of 10 nmol/L Rap was added 0 (●), 6 (▲), 18 (■), or 36 hours (○) after the initiation of the culture with IL-2. As a control, Kit225 cells were cultured with IL-2 only (●). The cell lysates prepared from 4 × 10⁶ cells harvested at 0, 1, 6, 7, 18, 19, 36, and 37 hours of the culture were subjected to immunoprecipitation with anti-p70 S6 kinase antibody. The kinase activity of the immunoprecipitates was assayed with S6 peptide as the substrate and was shown as [γ-32P]ATP incorporation into S6 peptide. Each point represents the average of duplicate samples. (B) IL-2-depleted Kit225 cells were cultured with 10 nmol/L Rap (columns 2 to 4) or without Rap (column 1) in the presence of 2 nmol/L IL-2 for 48 hours. Column 2, Rap was added concomitantly with IL-2; column 3, at 6 hours; column 4, at 18 hours after the initiation of the culture. The exponentially growing Kit225 cells were cultured in the absence of Rap (column 5) or the presence of 10 nmol/L Rap (column 6) for 48 hours. The incorporation of [3H]thymidine by cultured cells for the last 6 hours was measured. Each column represents the means ± standard deviation (SD) of the triplicate cultures.
activity and cyclin D1-dependent kinase activity (Fig 3C). However, Rap inhibited neither cyclin D1-dependent kinase activity nor cdk6 kinase activity (Fig 3C). The expression of cyclin D1 was not detected by immunoblotting (data not shown).

Rap induces p27Kip1 expression and facilitates the formation of the cyclin E/cdk2-p27Kip1 complex in exponentially growing ED-40515(−) cells. The induction of p27Kip1 mRNA, but not p21Waf1 mRNA, was detected by Northern blotting from 24 hours up to 48 hours of the culture in the presence of 10 nmol/L Rap. Protein levels of p27Kip1 also increased gradually with the treatment of Rap. In contrast, upregulation of p21Waf1 mRNA, but not p27Kip1 mRNA, was detected by Northern blotting in serum-stimulated ED-40515(−) cells, which ceased to proliferate due to overgrowth without Rap (Fig 4A). Rap suppressed expression of cyclin A, but not cyclin E protein levels (Fig 4B). Rap increased the amount of p27Kip1 that was immunoprecipitated with anti-cyclin E antibody, but not markedly with anti-cyclin D1 antibody or with anti-cdk4 antibody (Fig 4B). To evaluate de novo synthesis of p27Kip1 protein, metabolic labeling of \[^{35}S\]methionine was performed at 0, 24, and 48 hours of the culture. Rap upregulated the amount of \[^{35}S\]methionine pulse-labeled p27Kip1 markedly at 24 and 48 hours of the culture (Fig 4C). The half life of p27Kip1 evaluated by pulse chase of \[^{35}S\]methionine-labeled p27Kip1 at 28 hours of the culture was over 9 hours in the presence of 10 nmol/L Rap and about 5.5 hours in the absence of Rap, respectively, indicating that elevated protein level of p27Kip1 by Rap at 28 hours and after 28 hours of the culture was ascribed to both upregulation of de novo synthesis and retarded degradation process of p27Kip1 (Fig 4D).

Expression level of p27Kip1 correlates well to the sensitivity to Rap. As shown in Fig 2, sensitivity to Rap differed among various T-cell lines. To elucidate the factor(s) that determine(s) the sensitivity to Rap, we examined the expression levels of p27Kip1 in a Rap-sensitive cell line, ED-40515(−), and in a Rap-insensitive cell line, HPB-ALL, and found that the basal levels of p27Kip1 were much higher in ED-40515(−) than in HPB-ALL (Fig 5A). Based on this result, we postulated that Rap might show potent antiproliferative effects preferentially on the cells with elevated p27Kip1 levels and induce G1 arrest by increasing the amount of p27Kip1 beyond a putative threshold. To test this hypothesis, we examined the antiproliferative effect of Rap on ED-40515(−) cells into which antisense cDNA of p27Kip1 was introduced. As shown in Fig 5B, the introduction of antisense cDNA of p27Kip1 into the cells markedly reduced the antiproliferative effect of Rap, as well as the amount of p27Kip1 detected by immunoblotting.

**DISCUSSION**

In the present study, we analyzed the antiproliferative effect of Rap on exponentially growing cells comparing with that on cells entering into the cell cycling in response to mitogenic stimuli. Rap exerted its antiproliferative effect well when it was added to quiescent cells together with mitogenic agent. Indeed, the addition of 10 nmol/L Rap and 2 nmol/L rIL-2 to Kt225 cells synchronized in a quiescent state by IL-2 depletion caused G1 arrest as previously reported. However, as shown in Fig 1, Rap failed to exert its antiproliferative effect when it was added to the culture 18 hours after the IL-2 stimulation or later. In addition to this, Rap did not always exert its effect on exponentially growing cells as shown in Fig 2. These facts suggest that the antiproliferative effect of Rap might be both cell cycle phase- and cell type-dependent.

It was reported that the microinjection of the neutralizing antibody against p70 S6 kinase into the quiescent cells blocked the cell cycle entry in response to serum. In accordance with this, we confirmed the inhibition of both p70 S6 kinase activity and the cell proliferation when quiescent cells were incubated with Rap and IL-2. However, Rap failed to inhibit the proliferation of the cells that already pass G1/S transition or growing exponentially, although the inhibition of p70 S6 kinase was always observed within 1 hour at any cell cycling phase. Our results and reported data suggest one possibility that p70 S6 kinase or one(s) of the downstream molecule(s) of p70 S6 kinase plays a pivotal role in the cell cycle progression at the G0/G1 transition when the cells leave a quiescent state in response to mitogenic stimuli. Rap may cause cell cycle arrest by inactivating p70 S6 kinase at the early stage of G1 phase, but not once the cells enter into the cell cycling.

In addition to the inactivation of p70 S6 kinase, Rap has also been reported to inactivate cdk2 kinase by forming the cyclin E/cdk2-p27Kip1 complex and cause cell cycle arrest in late G1 phase. The antiproliferative effect of Rap does not seem to be potent enough to disrupt all of the cell cycling mechanisms instantly and simultaneously. For example, Rap did not affect the expression of cdk2 or cyclin E, while it kept the p27Kip1 protein levels as high as the initial level throughout the culture period. It is, therefore, possible that Rap facilitates the transient formation of the cyclin E/cdk2-p27Kip1 complex even if cell cycle progression down stream of p70 S6 kinase is disrupted by Rap at the early G1 phase. We need to accumulate further evidence to determine whether the formation of the cyclin E/cdk2-p27Kip1 complex indeed acts as the negative regulator of the cell cycle progression or merely represents the state of Rap-induced G1 arrest when Rap is added concomitantly with mitogens to quiescent cells.

Extending the cell cycle studies of quiescent cells entering...
into the cell cycling, we examined the effect of Rap in exponentially growing T cells. Rap did not always exert its antiproliferative effect on various exponentially growing T-cell lines. Among various T-cell lines, we selectively examined an HTLV-I–infected IL-2 independent T-cell line, ED-40515(2) that was found to be highly sensitive to Rap and obtained the following results.

1. Rap caused cell cycle arrest in G1 phase.
2. Rap inhibited p70 S6 kinase activity.
3. Rap upregulated p27 Kip1 at both mRNA and protein levels.
4. Rap inhibited cyclin A-dependent kinase activity.
5. Rap inhibited cyclin E/cdk2 kinase activity and increased the amount of p27 Kip1 coimmunoprecipitated with cyclin E.
6. Rap slightly inhibited cyclin D1/cdk4 kinase activity and did not markedly increase the amount of p27 Kip1 coimmunoprecipitated with cyclin D1 or cdk4.

As the inhibition of p70 S6 kinase by Rap was observed in both Rap-sensitive and -insensitive cell lines, it is unlikely that it plays a major role in Rap-induced G1 arrest of exponentially growing cells. The [3S]methionine pulse study of p27 Kip1 showed that the marked increase of the protein by Rap was ascribed to both increased de novo synthesis rate and retarded degradation process, while moderate increase of the protein without Rap at 48 hours of culture might be ascribed to retarded degradation process due to overgrowth of the cells. As Rap suppressed the expression of cyclin A, but not cyclin E, inhibition of cyclin A-dependent kinase activity by Rap might...
be explained by the decreased amount of cyclin A. The cdk2 exerts its kinase activity by forming the complex with its cyclin counter partners, cyclin E and cyclin A, while cdk4 with cyclin Ds. As shown in Figs 3 and 4, Rap inhibited cyclin E/ckd2 kinase activity clearly, but slightly cyclin D/ckd4 kinase activity in the cells. One possible interpretation of these results is that the substantial proportion of p27Kip1 might preferentially associate with and be sequestered with cyclin D/cdk4 complex rather than with cyclin E/ckd2 complex and the final regulations to enter S phase might be conducted by the periodical and spiky activation of the cyclin E/ckd2 complex in normal G1 cell cycle progression. The upregulated p27Kip1 by
the treatment of Rap might selectively bind to cyclin E/cdk2 complex after being completely titrated with cyclin D3/cdk4 complex and inhibited both kinase activities.

Recently, Zerfass-Thome et al. reported that p27Kip1 blocked cyclin E-dependent transactivation of cyclin A by forming cyclinE/cdk2-p27Kip1 complex, which might explain the Rap-induced suppression of cyclin A. Therefore, the formation of the cyclin E/cdk2-p27Kip1 complex by upregulated p27Kip1 and subsequent suppression of cyclin A expression by this complex might be the major cause of Rap-induced G1 arrest in ED-40515(−) cells. Although the mechanism that leads to the upregulation of de novo synthesis rate of p27Kip1 by Rap remains to be elucidated, the cell cycle arrest caused by increased de novo synthesis of p27Kip1 consequently retarded the degradation process of p27Kip1. These two factors might work cooperatively to maintain the state of Rap-induced G1 arrest.

We then explored the factor(s) that might determine or correlate with the sensitivity to Rap in exponentially growing T cells and found that the cells with high expression levels of p27Kip1 tended to be more sensitive to Rap than the cells with low levels. To elucidate the correlation between the basal level of p27Kip1 and the sensitivity to Rap, we introduced antisense cDNA of p27Kip1 into ED-40515(−) cells and showed the loss of the sensitivity to Rap when antisense cDNA of p27Kip1 was
INTRODUCTION

It has been suggested that the p27Kip1 level might play a pivotal role in driving the cell cycle at late G1 phase of the human cell cycle. **E/cdk2-p27Kip1 complex, which might play a pivotal role in inactivating cyclin E/cdk2 kinase activity by forming the cyclin D1-cdk association in early G1 of an osteosarcoma. J Biol Chem 268:25385, 1993**

In other words, the cells with lower levels of p27Kip1 may pass the G1/S boundary, while the cells with higher levels may fail to pass and are arrested in the late G1 phase. Rap may exert its antiproliferative effect on the growth of exponentially growing T cells by increasing the amount of p27Kip1 that exceeds a putative threshold, which results in the cell cycle arrest at the late G1 phase. The basal levels of p27Kip1 may be, therefore, one of the limiting factors to determine the sensitivity to Rap in exponentially growing T cells. Luo et al. reported that the cells selected for resistance to Rap exhibited constitutively low p27Kip1 levels, and p27Kip1 cells derived from p27Kip1 mice exhibited a significant resistance to growth inhibition effect of Rap when growing exponentially. Their report referred to the correlation between expression of p27Kip1 and sensitivity to Rap from a different approach and may support our experimental results.

Studies with p27Kip1 knock out mice by Nakayama et al. showed that Rap exerted its antiproliferative effect in both wild-type and p27Kip1-/- thymocytes. They activated T cells with anti-CD3 and anti-CD28 monoclonal antibodies and examined the effect of Rap on day 6. If, however, wild and p27Kip1-/- cells are no longer in an exponential phase, but rather causes G1 arrest regardless of the p27Kip1 level as we discussed in the previous paragraph.

In summary, our current understanding of the G1 cell cycle progression and the effect of Rap is as follows. In cells leaving a quiescent state and entering into the cell cycling, p70 S6 kinase might play a pivotal role in driving the cell cycle at the G0/G1 transition. Rap inactivates p70 S6 kinase, which might result in Rap-induced G1 arrest. On the other hand, in exponentially growing cells that skip a putative G0/G1 transition, the level of p27Kip1 is able to serve as one of the limiting factors in driving the cell cycle at late G1 phase. Rap exerts its antiproliferative effect by upregulating p27Kip1 in Rap-sensitive T cells. Rap inactivates cyclin E/cdk2 kinase activity by forming the cyclin E/cdk2-p27Kip1 complex, which might play a pivotal role in causing late G1 arrest in Rap-sensitive T cells. Rap also inhibits p70 S6 kinase, which, however, does not seem to be a major cause of Rap-induced G1 arrest once the cells enter into the cell cycle.

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The Upregulation of p27<sup>Kip1</sup> by Rapamycin Results in G1 Arrest in Exponentially Growing T-Cell Lines

Shin Kawamata, Hitoshi Sakaida, Toshiyuki Hori, Michiyuki Maeda and Takashi Uchiyama