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

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

An immunosuppressant Rapamycin (Rap) has been reported to cause G1 arrest by inhibiting p70 S6 kinase and G1 cyclin/cdks kinase activities when added to quiescent cells with mitogens. However, antiproliferative effects of Rap on exponentially growing cells have been poorly investigated. We examined the intracellular events after the treatment of Rap in exponentially growing T cells and found that Rap upregulated a cdks inhibitor, p27^{Kip1} at both mRNA and protein levels in Rap-sensitive cells. Antiproliferative effect of Rap was mainly ascribed to the inhibition of cyclin E/cdk2 kinase activity through the formation of cyclin E/cdk2-p27^{Kip1} complex rather than inhibition of p70 S6 kinase activity. Furthermore, we showed that Rap-sensitive cells with elevated p27^{Kip1} expression lost sensitivity to Rap when antisense p27^{Kip1} was introduced, which indicates that the basal level of p27^{Kip1} is one of the limiting factors that determine the sensitivity to Rap in already cycling cells. These data suggest the presence of a putative threshold level of p27^{Kip1} at late G1 phase in already cycling cells. Rap may cause G1 arrest by upregulating the amount of p27^{Kip1} beyond the threshold in some Rap-sensitive cells that are exponentially growing.

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.[1-7] Although FRAP itself is not a mitogen-activated p70 S6 kinase kinase, the Rap-FKBP12–FRAP complex selectively inhibits the mitogen-induced p70 S6 kinase activation without disrupting other known pathways.[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^{Kip1}, the increment of p21^{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.[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^{Kip1},[14] and inactivates the kinase activity of the G1cyclin/cdks complex by facilitating the formation of the G1cyclin/cdks-p27^{Kip1} complex.[15-25] The continuous elimination of p27^{Kip1}, which is reported to be mediated by the ubiquitin-dependent degradation[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^{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^{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^{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,[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 nmol/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 nmol/L rIL-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).[29,30] TL-Oml,[31] MJ,[32] and MT-2[33] are HTLV-I–infected T-cell lines previously described. ED-40515(−), TL-Oml, MJ, Molt4, 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 tritium-thymidine ([^{3}H]thymidine) incorporation into DNA. After 48 hours depletion of IL-2, Kit225 cells were plated into a 96 well-flat-bottomed plate at 1×10^4 cells/100 μL/well and cultured with 2 nmol/L IL-2 at 37°C in 5% CO_2 for 48 hours. A total of 10 nmol/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 exponentially 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 nmol/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 μmol/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 lysing 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_3 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 semidry 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^{Waf1} 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}. Exponentially 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 7.9], 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 (Pharmacia). The immunoprecipitates were separated by SDS-PAGE with 12.5% gel. The radioactive signals associated with the p27^{Kip1} band were measured by a phosphorimager, BIO-IMAGE ANALYZER BAS 2000 (FujiX, Minamiashigara, Kanagawa, Japan).

Kinase assay. Histone H1 kinase assay was performed as described previously.[23] Cell lysates for histone H1 kinase assay were prepared by lysing 4×10^6 cells in 100 μL of H1 lysing buffer (50 mmol/L Tris-HCl [pH 7.5], 0.25 mol/L NaCl, 0.5% Nonidet P-40, 1 mmol/L PMSF, 1 mmol/L Na_3VO_4, 20 μg/mL N-Tosyl-phenylalanine chloromethyl ketone [TPCK], 20 μg/mL soybeans trypsin-chymotrypsin inhibitor, 10 μg/mL leupeptin). The cell extracts were incubated with anti-cdk2 antibody (Santa Cruz), anti-cyclin E antibody (Santa Cruz), or anti-cyclin A antibody (UBI) and 20 μL of Protein A-Sepharose 4 fast flow beads for 2 hours at 4°C. Immunoprecipitates were then washed twice with buffer containing 50 mmol/L Tris-HCl (pH7.5), 30 mmol/L $Na_4P_2O_7$, 50 mmol/L NaF, 5 mmol/L EDTA (pH7.0), 5% wt/vol glycerol, 0.5% Triton X-100, 1 mmol/L DTT, 10 μg/mL leupeptin, 5 μg/mL aprotinin, and 10 mmol/L β-glycerophosphate, followed by washes two times with kinase buffer (50 mmol/L HEPES [pH 7.0], 10 mmol/L β-glycerphosphate, 5 mmol/L $MgCl_2$, 10 μg/mL leupeptin, and 1 mmol/L DTT). Histone phosphrylation was initiated by the addition of 40 μL kinase buffer containing 0.6 mg/mL calf thymus histone type III-S (Sigma Chemical Co, St Louis, MO) and 10 μCi of [γ-^{32}P] adenosine triphosphate (ATP) (Amersham). After a 30-minute incubation at 37°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 (Whatman International Ltd, Maidstone, UK). The paper was immersed briefly in 1% H_3PO_4 solution containing 10 mmol/L $Na_4P_2O_7$. After three additional 15-minute washes in H_3PO_4, the radioactivity incorporated into the filter-bound histone was measured by liquid scintillation counting.

Specific activity of p70 S6 kinase was determined by ^{32}P incorporation into S6 synthetic peptide (AKRRRLSSLRA, [UBI]). 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 suspending the beads in 40 μL of kinase buffer (20 mmol/L Tris-HCl [pH 7.5], 10 mmol/L $MgCl_2$, 10 mmol/L $MgCl_2$, 0.1 mg/mL BSA, 0.4 mmol/L DTT) containing 50 μmol/L ATP, 10 μCi of [γ-^{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.

pRB phosphorylation assay was determined by ^{32}P incorporation into glutathione S-transferase-pRb (GST-pRb; corresponding to amino acid 769-921 [Santa Cruz]).[35] Cell extracts prepared as above were incubated with anti-cdk4 antibody, anti-cdk6 antibody, anti-cyclin D_1 antibody, anti-cyclin D_2 antibody, or anti-cyclin D_3 antibody (Santa Cruz). Kinase assay of the immunocomplex was performed by suspending the beads in 40 μL of kinase buffer containing 0.2 μg of GST-Rb, 2.5 mmol/L EGTA, 20 μmol/L ATP, and 10 μCi of [γ-^{32}P]ATP. The triplicate aliquots of each sample were incubated for 20 minutes at 30°C, denatured in SDS sample buffer, and analyzed by SDS-PAGE with 10% gel. The radioactive signals associated with GST-pRb protein were measured by liquid scintillation of the excised bands.

Northern blotting. Total cellular RNA was isolated from 1×10^7 cells by the acid-guanidium thiocyanate-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 β-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. BamH I 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, MI). The cDNA probe of p27^{Kip1} was obtained by digesting with BamH I. For the detection of p21^{Waf1}, 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 BamH I site-tagged cDNA of p27^{Kip1} was generated by the RT-PCR method as stated in Materials and

Methods for Northern blotting. The PCR product was inserted into pBluescript II, SK(+) cloning vector (STRATAGENE, 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 $p27^{Kip1}$. The frame shifted-sense cDNA of $p27^{Kip1}$ was generated by the PCR method. Xho I site-tagged frame shifted 5′ oligonucleotide (CCTCGAG-GATGTGTCAAACGTGCGAGTGTCT), Not I site-tagged 3′ oligonucleotide (TTGCGGCCGCAATTACGTTTGACGTCTTCTG) and SK(+)-$p27^{Kip1}$ were used as the primers and the template, respectively. The PCR products were ligated into pMKIT Neo. The pMKIT Neo-$p27^{Kip1}$ 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 mg/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, cyclin A-dependent kinase activity, and p70 S6 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 cells lines such as Kit225 (Fig 1A), HPB-ALL, and HSB-2 (data not shown). Rap slightly suppressed cdk4 kinase

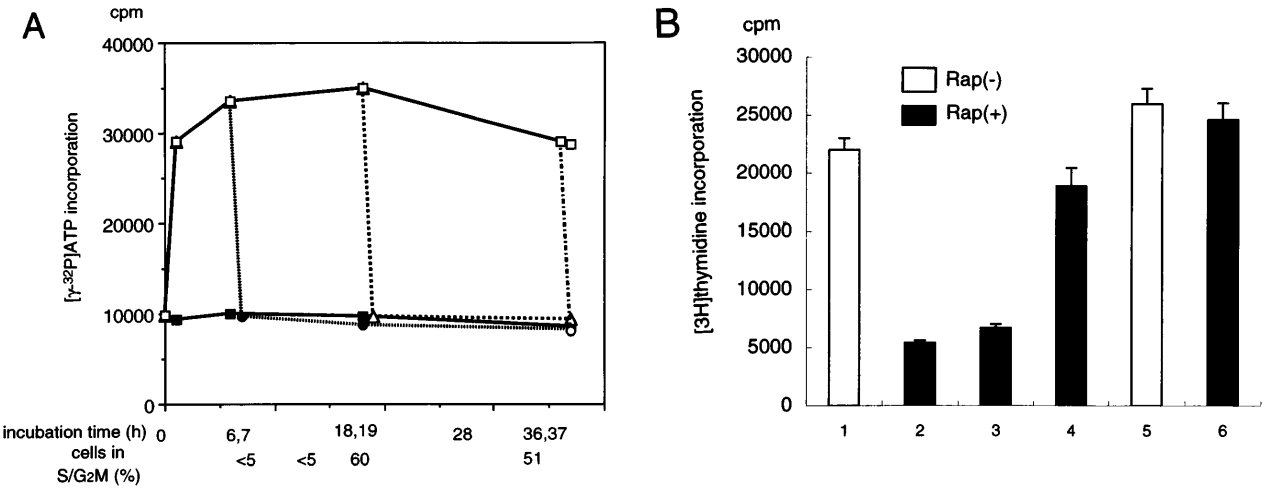

Fig 1. Inhibition of p70 S6 kinase (A) and suppression of cell proliferation (B) by Rap in Kit225 cells entering into the cell cycling in response to IL-2. Kit225 cells deprived of IL-2 for 48 hours were cultured with or without Rap in the presence of 2 nmol/L IL-2. (A) A total of 10 nmol/L Rap was added 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 4×10^6 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.

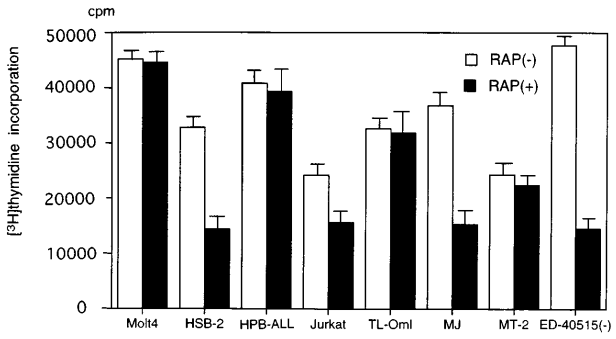

Fig 2. Effect of Rap on the [³H]thymidine incorporation by various T-cell lines that are exponentially growing. HTLV-I–infected (TL-Oml, MJ, MT-2, ED-40515[−]) or -uninfected (Molt4, HPB-ALL, HSB-2, Jurkat) T-cell line cells kept in an exponentially growing phase were cultured in fresh RPMI 1640 medium containing 10% FCS at low concentrations of cells with or without 10 nmol/L Rap for 48 hours. The [³H]thymidine uptake by cultured cells for the last 6 hours was measured. Each column represents the means ± SD of triplicate samples. Three independent experiments gave similar results.

activity and cyclin D_3-dependent kinase activity (Fig 3C). However, Rap inhibited neither cyclin D_2-dependent kinase activity nor cdk6 kinase activity (Fig 3C). The expression of cyclin D1 was not detected by immunoblotting (data not shown).

Rap induces p27^{Kip1} expression and facilitates the formation of the cyclin E/cdk2-p27^{Kip1} complex in exponentially growing ED-40515(−) cells. The induction of p27^{Kip1} mRNA, but not p21^{Waf1} 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 p27^{Kip1} also increased gradually with the treatment of Rap. In contrast, upregulation of p21^{Waf1} mRNA, but not p27^{Kip1} 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 p27^{Kip1} that was immunoprecipitated with anti-cyclin E antibody, but not markedly with anti-cyclin D_3 antibody or with anti-cdk4 antibody (Fig 4B). To evaluate de novo synthesis of p27^{Kip1} protein, metabolic labeling with [^{35}S]methionine was performed at 0, 24, and 48 hours of the culture. Rap upregulated the amount of [^{35}S]methionine pulse-labeled p27^{Kip1} markedly at 24 and 48 hours of the culture (Fig 4C). The half life of p27^{Kip1} evaluated by pulse chase of [^{35}S]methionine-labeled p27^{Kip1} at 28 hours of the cuture 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 p27^{Kip1} by Rap at 28 hours and after 28 hours of the cuture was ascribed to both upregulation of de novo synthesis and retarded degradation process of p27^{Kip1} (Fig 4D).

Expression level of p27^{Kip1} 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 p27^{Kip1} in a Rap-sensitive cell line, ED-40515(−), and in a Rap-insensitive cell line, HPB-ALL, and found that the basal levels of p27^{Kip1} 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 p27^{Kip1} levels and induce G1 arrest by increasing the amount of p27^{Kip1} beyond a putative threshold. To test this hypothesis, we examined the antiproliferative effect of Rap on ED-40515(−) cells into which antisense cDNA of p27^{Kip1} was introduced. As shown in Fig 5B, the introduction of antisense cDNA of p27^{Kip1} into the cells markedly reduced the antiproliferative effect of Rap, as well as the amount of p27^{Kip1} 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 Kit225 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.[36,37] 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-p27^{Kip1} complex and cause cell cycle arrest in late G1 phase.[14,22,23] 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,[23] while it kept the p27^{Kip1} 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-p27^{Kip1} 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-p27^{Kip1} 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

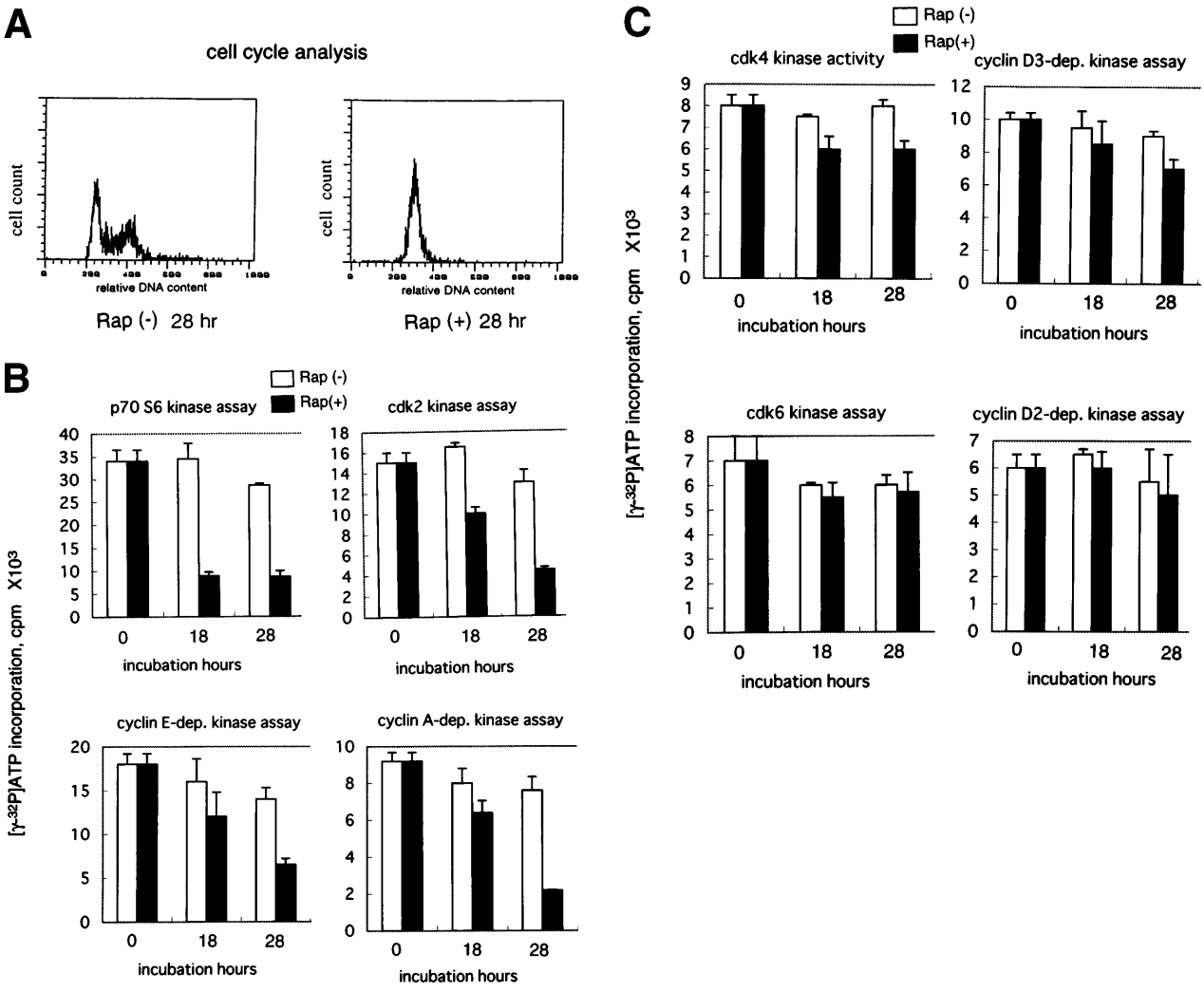

Fig 3. Effect of Rap on the cell growth (A), cdk2 kinase activity, p70 S6 kinase activity, cyclin E- and A-dependent kinase activity (B), cdk4 kinase activity, cdk6 kinase activity. cyclin D_2- and D_3-dependent kinase activity (C) in exponentially growing ED-40515(−) cells. (A) ED-40515(−) cells were incubated with or without 10 nmol/L Rap for 28 hours, then fixed with 70% ethanol for 1 hour and stained with 50 μmol/L acridine orange in PBS/0.1% glucose for 1 hour. The relative DNA content was determined by a flow cytometric analysis. (B) ED-40515(−) cells (4×10^6 cells) cultured with or without 10 nmol/L Rap for the indicated times were harvested and immunoprecipitated with anti-cdk2 antibody, anti-p70 S6 kinase antibody, anti-cyclin E antibody, or anti-cyclin A antibody. The kinase activity of the immunoprecipitates was assayed with histone H1 or S6 peptide as a substrate. Each column represents the average of triplicate samples with ± SD. (C) ED-40515(−) cells (4×10^6 cells) cultured with or without 10 nmol/L Rap for the indicated times were harvested and immunoprecipitated with anti-cdk4 antibody, anti-cdk6 antibody, anti-cyclin D_2 antibody, or anti-cyclin D_3 antibody. The kinase activity of the immunoprecipitates was assayed with GST-Rb as a substrate. Each column represents the average of triplicate samples ± SD.

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(−) 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 D_3/cdk4 kinase activity and did not markedly increase the amount of $p27^{Kip1}$ coimmunoprecipitated with cyclin D_3 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 [^{35}S]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

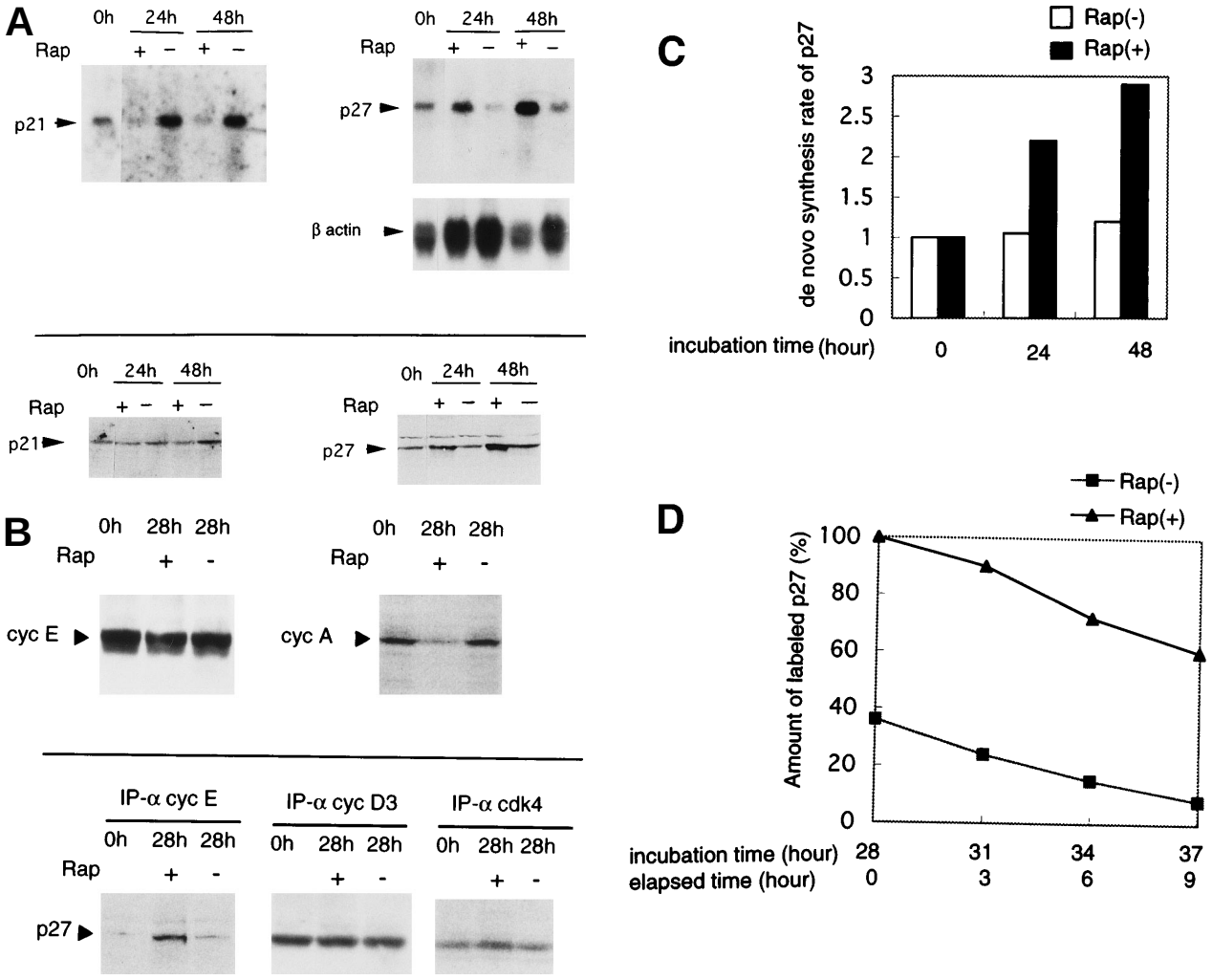

Fig 4. Effect of Rap on the expression of cdks inhibitors (A), the formation of the G1 cyclin/cdks-p27^{Kip1} complex (B) and [^{35}S] methionine-labeled p27^{Kip1} (C and D) in ED-40515(−) cells. Exponentially growing ED-40515(−) cells incubated with or without Rap were harvested 0, 24, 28, or 48 hours after the initiation of the culture. (A) Upper: the expression of p21^{Waf1} and p27^{Kip1} at mRNA level. A total of 10 μg of total RNA was fractionated by electrophoresis and subjected to Northern blotting with ^{32}P labeled p21^{Waf1} or p27^{Kip1} probes. The same filter membrane was used for p21^{Waf1} and p27^{Kip1} mRNA hybridization. Bottom: the expression of p21^{Waf1} and p27^{Kip1} at protein level. The cell lysates containing 40 μg of the protein were subjected to immunoblotting with anti-p27^{Kip1} antibody and anti-p21^{Waf1} antibody. (B) Upper: immunoblotting of cyclin A and cyclin E. The cell lysate containing 40 μg of protein from indicated samples was separated with 10% gel, followed by immunoblotting with anticyclin A or E antibody. Bottom: the immunoprecipitation (IP)-immunoblotting of p27^{Kip1}. The cell lysates prepared from 5 × 10^6 cells of the indicated samples were immunoprecipitated with anti-cyclin E antibody, anti-cdk4 antibody, or anti-cyclin D$_3$ antibody followed by SDS-PAGE separation with 12.5% gel and immunoblotting with anti-p27^{Kip1} antibody. (C) The de novo synthesis rate of p27^{Kip1} evaluated by [^{35}S]methionine metabolic labeling. ED-40515(−) cells cultured in RPMI 1640 plus 10% FCS were incubated with or without 10 nmol/L Rap for 24 or 48 hours. The cells were harvested at the indicated points and labeled with 150 μCi/mL of [^{35}S]methionine for 45 minutes. The cells were harvested again for lysis in hypotonic buffer. The cell lysates were immunoprecipitated with anti-p27^{Kip1} antibody. The radioactive signals associated with the p27^{Kip1} bands, which were measured by a phosphorimager, were plotted as the relative signal intensity. (D) The pulse chase study of [^{35}S]methionine-labeled p27^{Kip1}. ED-40515g(−) cells cultured with or without 10 nmol/L Rap for 28 hours were harvested and labeled with [^{35}S]methionine by the same method as in (C). The cells were placed in full medium to chase the metabolic labeling with or without 10 nmol/L Rap and harvested at the indicated points (0, 3, 6, and 9 hours after [^{35}S]methionine labeling) for lysis in hypotonic buffer. The radioactive signals associated with the p27^{Kip1} bands, which were measured by a phosphorimager, were plotted as the percent of the signal at the start of the chase of Rap-treated cells.

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/cdk2 kinase activity clearly, but slightly cyclin D$_3$/cdk4 kinase activity in the cells. One possible interpretation of these results is that the substantial proportion of p27^{Kip1} might preferentially associate with and be sequestered with cyclin D$_3$/cdk4 complex rather than with cyclin E/cdk2 complex[20,25] and the final regulations to enter S phase might be conducted by the periodical and spiky activation of the cyclin E/cdk2 complex in normal G1 cell cycle progression. The upregulated p27^{Kip1} by

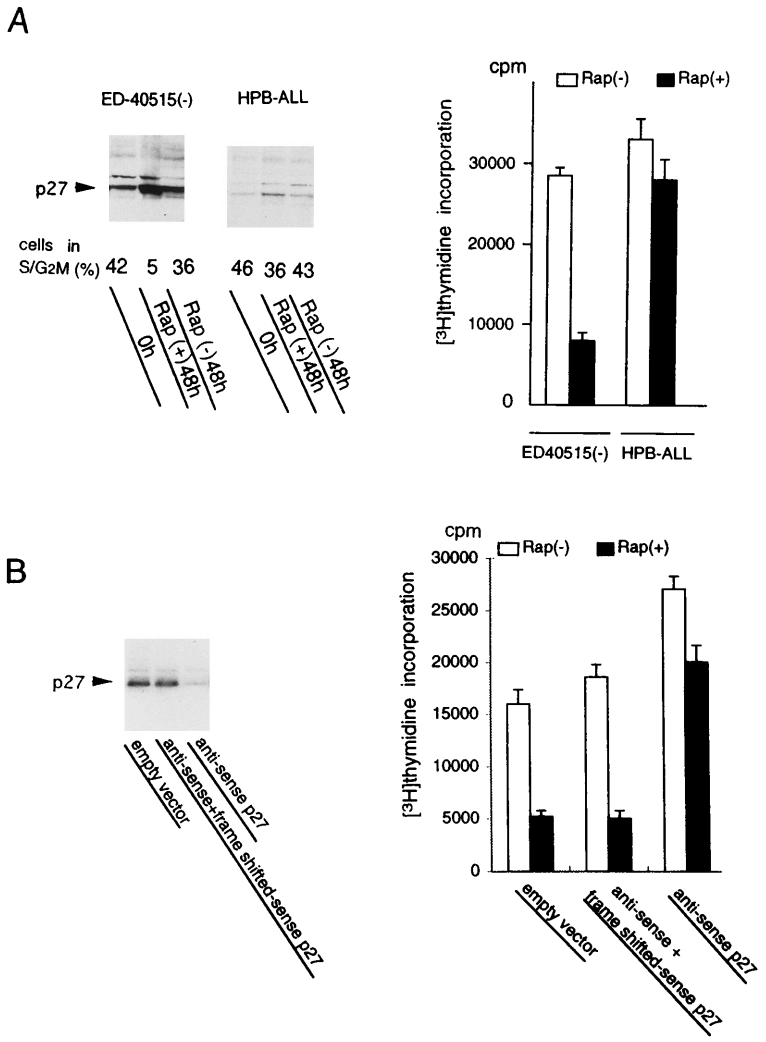

Fig 5. Correlation between Rap sensitivity and p27Kip1 levels (A) and the abrogation of antiproliferative effect of Rap by introduction of antisense cDNA into ED-40515(−) cells (B). (A) Left, the expression of p27Kip1 after 48 hours culture with or without 10 nmol/L Rap in ED-40515(−) cells and HPB-ALL cells were examined. A total of 40 μg of whole cell lysates were subjected to immunoblotting with anti-p27Kip1 antibody. The relative proportion (%) of cells at S/G2M was also determined by a flow cytometric analysis after staining with acridine orange. Right: the antiproliferative effect of Rap on ED-40515(−) cells and HPB-ALL cells were examined by [3H]thymidine uptake for the last 6 hours of the 48-hour culture with or without 10 nmol/L Rap. Each column represents the means ± SD of triplicate samples. (B) Left: 5 μg of antisense p27Kip1 cDNA, 5 μg of frame shifted-sense together with 5 μg of antisense p27Kip1 cDNA, or 10 μg of empty vector were transfected into ED-40515(−) cells by electroporation method. The total amount of transfected plasmids was adjusted to 10 μg by adding a empty vector. The transfected cells were cultured in RPMI 1640 plus 10% FCS with 1 mg/mL G418 for 3 weeks. A total of 20 μg of protein from the transfected cells was subjected to immunoblotting with anti-p27Kip1 antibody. Right: the transfected cells were cultured with or without 10 nmol/L Rap for 48 hours. The [3H]thymidine uptake by the cells for the last 6 hours was assayed. Each column represents the means ± SD of triplicate samples.

the treatment of Rap might selectively bind to cyclin E/cdk2 complex after being completely titrated with cyclin D_3/cdk4 complex and inhibited both kinase activities.

Recently, Zerfass-Thome et al[38] 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 degadation 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

introduced into the cells. These results suggest the presence of a putative $p27^{Kip1}$ threshold level at the late G1 phase in already cycling cells. In other words, the cells with lower levels of $p27^{Kip1}$ 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 $p27^{Kip1}$ that exceeds a putative threshold, which results in the cell cycle arrest at the late G1 phase. The basal levels of $p27^{Kip1}$ may be, therefore, one of the limiting factors to determine the sensitivity to Rap in exponentially growing T cells. Luo et al[39] reported that the cell lines selected for resistance to Rap exhibited constitutively low $p27^{Kip1}$ levels, and $p27^{-/-}$ cells derived from $p27^{-/-}$ mice exhibited a significant resistance to growth inhibition effect of Rap when growing exponentially. Their report referred to the correlation between expression of $p27^{Kip1}$ and sensitivity to Rap from a different approach and may support our experimental results.

Studies with $p27^{Kip1}$ knock out mice by Nakayama et al[40] showed that Rap exerted its antiproliferative effect in both wild-type and $p27^{Kip1-/-}$ thymocytes.[41,42] They activated T cells with anti-CD3 and anti-CD28 monoclonal antibodies and examined the effect of Rap on day 6.[39] If, however, wild and $p27^{Kip1-/-}$ cells are no longer in an exponential phase, but rather in a resting state on day 6, Rap inactivates p70 S6 kinase and causes G1 arrest regardless of the $p27^{Kip1}$ 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 $p27^{Kip1}$ 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 $p27^{Kip1}$ in Rap-sensitive T cells. Rap inactivates cyclin E/cdk2 kinase activity by forming the cyclin E/cdk2-$p27^{Kip1}$ 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 cycling.

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