FEBS Letters 584 (2010) 2779–2785

FEBS Letters

journal homepage: www.FEBSLetters.org

Mammalian target of rapamycin complex 1 signaling opposes the effects of anchorage loss, leading to activation of Cdk4 and Cdc6 stabilization

Shiho Arakawa-Takeuchi, Kohei Kobayashi, Jung-ha Park, Baasanjav Uranbileg, Hanako Yamamoto, Shigeki Jinno, Hiroto Okayama *

Department of Biochemistry and Molecular Biology, Graduate School and Faculty of Medicine, The University of Tokyo, Tokyo 113-0033, Japan

ARTICLE INFO

Article history:
Received 22 January 2010
Revised 29 April 2010
Accepted 1 May 2010
Available online 11 May 2010

Edited by Angel Nebreda

Keywords:
Anchorage
Mammalian target of rapamycin complex 1
Eker
Rheb
Cdc6
Cdk4
Emi1
APC/C^{Cdh1}

ABSTRACT

When deprived of an anchorage to the extracellular matrix, fibroblasts arrest in the G_1 phase with inactivation of Cdk4/6 and Cdk2 and destruction of Cdc6, the assembler of prereplicative complexes essential for S phase onset. How cellular anchorages control these kinases and Cdc6 stability is poorly understood. Here, we report that in rat embryonic fibroblasts, activation of mammalian target of rapamycin complex 1 by a *Tsc2* mutation or overexpression of a constitutively active mutant Rheb overrides the absence of the anchorage and stabilizes Cdc6 at least partly via activating Cdk4/6 that induces Emi1, an APC/C^{Cdh1} ubiquitin ligase inhibitor.

Structured summary:
MINT-7890626: *cdc27* (uniprotkb:Q4V8A2) *physically interacts* (MI:0915) with *Cyclin-A* (uniprotkb:Q6AY13) by *anti bait coimmunoprecipitation* (MI:0006)

1. Introduction

Unless malignantly transformed, fibroblasts require an anchorage to the extracellular matrix (ECM) for S phase entry [1]. However, despite extensive studies, how cellular anchorage to the ECM controls the G_1–S transition has been highly elusive. When deprived of anchorages, cells arrest in G_1 with inactivation of Cdk4/6 and Cdk2, due at least partly to repression of the *Cyclin D1* and *Cyclin A* genes and induction of $p27^{Kip1}$ [2]. Inactivation of Cdk4/6 shuts down the genes essential or important for S phase onset, such as *Cdc6*, *Cyclin A*, *E2F1* and *Emi1* [3,4]. Additionally, expression of Cdc6, which assembles prereplicative complexes for S phase onset [5], is terminated by facilitated proteolysis [6]. Two ubiquitin ligases antagonistically control the G_1–S transition. APC/C^{Cdh1} destroys Cdc6 and Cyclin A to cause G_1 arrest, whereas SCF^{Skp2} destroys the Cdk inhibitor $p27^{kip1}$ to facilitate S phase entry [7]. The activity of APC/C^{Cdh1} is negatively regulated by Cdk4/6-induced Emi1 [4]. Upon anchorage deprivation, in addition to G_1 arrest, apoptosis known as anoikis takes place with activation of caspase 3 that cleaves focal adhesion components and Cdc6 [8,9].

The Tsc1/Tsc2-Rheb-mTOR pathway mediates growth factor and metabolic signals to control cell proliferation [10]. Growth factor-activated AKT/PKB stimulates Rheb by inactivating the Tsc1/Tsc2 complex. Stimulated Rheb activates mammalian target of rapamycin complex 1 (mTORC1) to phosphorylate S6 kinase 1 (S6K1) and eIF4E binding protein (4EBP) to enhance translation. Germinal mutation of *TSC2* or *TSC1* causes multiple benign tumors called familial tuberous sclerosis, a rodent version of which occurs in Eker rats with a *Tsc2* mutation [11]. We have long been interested in understanding how anchorage signals regulate the G_1–S transition and have recently found that the anchorage signal-linked Cdk4/6 activity and Cdc6 stability are critically controlled by the Tsc-Rheb-mTORC1 pathway.

2. Materials and methods

2.1. Antibodies and chemicals

Anti-Cdk4 and anti-β-actin antibodies were purchased from Sigma (St. Louis, MO, USA). Anti-Cdh1, anti-phospho-retinoblastoma

Abbreviations: mTORC1, mammalian target of rapamycin complex 1; ECM, extracellular matrix; S6K1, S6 kinase 1; Rb, retinoblastoma protein; REF, rat embryonic fibroblast; Eker REF, Eker (*Tsc2*$^{-/-}$) rat embryonic fibroblast; 4EBP, eIF4E binding protein; ALLN, N-acetyl-leucinyl-leucinyl-norleucinal; zVAD, benzyloxycarbonyl-valinyl-alaninyl-aspartyl fluoromethyl ketone; MC, methylcellulose medium

* Corresponding author. Fax: +81 3 3815 1419.
E-mail address: okayama@m.u-tokyo.ac.jp (H. Okayama).

0014-5793/$36.00
doi:10.1016/j.febslet.2010.05.005

protein (Rb) (Ser^{780}) and anti-Cyclin D3 were purchased from MBL International (Woburn, MA, USA). Anti-Cdc6 was purchased from NeoMarkers (Fremont, CA, USA). Anti-Tsc2, anti-S6K1, anti-phospho-S6K1 (Thr^{389}), anti-phospho-Rb ($Ser^{807/811}$) and anti-activated caspase 3 (cleaved at Asp^{175}) antibodies were purchased from Cell Signaling Technology (Boston, MA, USA). A polyclonal antibody detecting rat and mouse Emi1 was generated by immunizing rabbits with its C-terminal common 12 amino acids. The remaining antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rapamycin, N-acetyl-leucinyl-leucinyl-norleucinal (ALLN) and ubiquitin were obtained from Biomol International (Plymouth Meeting, PA, USA), and benzyloxycarbonyl-valinyl-alaninyl-aspartyl fluoromethyl ketone (zVAD) was obtained from the Peptide Institute (Osaka, JP).

2.2. Cell culture and plasmids

Rat embryonic fibroblast (REF) and Eker ($Tsc2^{-/-}$) rat embryonic fibroblast (Eker REF) (gifts from O. Hino) were maintained in DMEM with 10% FCS, and when specified to culturing under anchorage deprivation, REF and Eker REF were incubated in DMEM containing 1.17% methylcellulose and 10% FCS. A soft agar assay was carried out with 0.5% Noble agar on the bottom and 0.33% agar on the top. REF and/or Eker REF cell clones constitutively expressing rat Cdc6, human active Rheb mutant [12], mouse Cdh1, Emil and/or the rat counterpart of human TSC2 isoform 4 (NCBI Reference Sequence: NP_001070651.1) [13] from the cytomegalovirus promoter were constructed by using the Retroviral Gene Transfer and Expression System (Clontech) containing the corresponding cDNA and an appropriate drug selectable gene.

2.3. Immunoblot detection and densitometry

Cells were lysed with SDS sample buffer followed by sonication to fragment the DNA. The lysates were electrophoresed in SDS–polyacrylamide gels, transferred to PVDF membranes and immunoblotted for targeted proteins with specific antibodies [6]. For some samples, the intensities of immunodetected bands were quantified with NIH Image software according to the provided protocol.

2.4. In vitro kinase assays

Cdk2 and Cdk4/6 were immunoprecipitated with the specific antibodies, and the activity of each kinase was assayed with a truncated Rb (QED Bioscience) as a substrate followed by immunoblot detection of $Ser^{807/811}$-phosphorylated Rb for Cdk2 activity and Ser^{780}-phosphorylated Rb for Cdk4/6 activity [6].

2.5. Quantitative RT-PCR

Total RNA was extracted from harvested cells by using ISOGEN (Nippon Gene). Isolated RNAs had an A_{260}/A_{280} ratio of 1.86–2.1. The PrimeScript II first-strand cDNA Synthesis kit (Takara-Bio) was used to synthesize first-strand cDNA from the total RNA. For each cDNA synthesis reaction, 2 μg of RNA was denatured at 65 °C for 5 min in 10 μl of 1 mM dNTP mixture with 5 μM oligo-dT primer and quickly chilled on ice. After the addition of 10 μl of PrimeScript buffer containing 200 units of PrimeScript II reverse transcriptase and 20 units of RNase inhibitor, cDNA synthesis was carried out at 45 °C for 30 min and was terminated by heating at 95 °C for 5 min and subsequently cooling on ice.

Quantitative real time PCR was performed with the Smart Cycler System (Cepheid, USA) and SYBR Premix Ex Taq (Perfect Real Time) (Takara-Bio, JP). In parallel, RT-PCR analysis without prior reverse transcription was performed on all RNA samples to assure exclusion of DNA contamination. The primer pairs used were as follows: 5′-gcctatgaggagccaaccta-3′(F) and 5′-cgctaagagcagctggagtag-3′(R) for *Cyclin D3*, 5′-gcacaacgcactttctttcc-3′(F) and 5′-tccagaagggcttcaatctg-3′(R) for *Cyclin D1*, 5′-caaagccatgcagagagtca-3′(F) and 5′-gtcgatgacttttgaacacagg-3′(R) for *Emi1*, 5′-ctctgccggtgctatgatct-3′(F) and 5′-catagcatggggtgattcaa-3′(R) for *Cyclin A*, 5′-aggaaagtgcagaggggatt-3′(F) and 5′-cttcttgctccaggcctaac-3′(R) for *E2F1* and 5′-ttgtaaccaactgggacgatatgg-3′(F) and 5′-gatcttgatcttcatggtgctagg-3′(R) for β-*actin*. β-*actin* transcripts were used to normalize variations generated during preparation of RNA and subsequent cDNA conversion from each sample.

An amplification reaction was performed in 25 μl of SYBR Premix EX Taq containing 0.2 μM of each of the forward and reverse primers and 1 μl of the cDNA. To reduce variability between replicates, PCR premixes, which contained all the reagents except for cDNA, were prepared in advance and aliquoted into 25 μl Smart Cycler Tubes (Cepheid). The thermal cycling conditions used were 15 s at 95 °C for denaturation followed by 30 cycles of 15 s at 95 °C, 20 s at 55 °C and 46 s at 72 °C for β-actin, and 40 cycles of 6 s at 95 °C, 15 s at 57 °C and 10 s at 72 °C for the others. The specificity of single-target amplification was confirmed by examining post-PCR melting curves, and the relative amounts of each transcript to β-actin RNA were determined in triplicate by using the comparative C_T ($\Delta\Delta C_T$) method.

2.6. In vitro ubiquitylation assay

Exponentially proliferating REF-Cdc6 and Eker-Cdc6-aRheb cells were incubated in methylcellulose medium for 24 h and collected with extensive washing. The cells were then lysed with 20 mM Tris–HCl (pH 7.5) containing 100 mM NaCl, 5 mM $MgCl_2$, 0.2% NP-40, 10% glycerol, 1 mM NaF, 1 mM Na_3VO_4, 0.5 mM DTT and protease inhibitor cocktail (Sigma) and then immunoprecipitated for APC/C^{Cdh1} with the anti-Cdc27 antibody and protein G Sepharose beads as reported [14]. APC/C^{Cdh1}-bound protein G beads were washed 5 times with the lysis buffer and added to 12 μl of ubiquitylation buffer containing 50 mM Tris–HCl (pH 7.5), 2.5 mM $MgCl_2$, 0.5 mM DTT, 20 mM ATP, ATP regenerating system (Boston Biochem), 100 nM E1 (ibid.), 500 nM UbcH10 (ibid.), 1.5 μg/μl ubiquitin, the protease inhibitor cocktail and 50 μM roscovitine (Calbiochem) to suppress the kinase activity of the Cdk2-Cyclin A complex used as a substrate. The reaction was initiated by adding 60 ng of Cdk2-Cyclin A complex (Upstate) and took place at 30 °C for 0, 10 and 50 min. After separation of products by SDS gel electrophoresis, ubiquitylated Cyclin A was detected by immunoblotting with the anti-Cyclin A antibody. In parallel, the quantity of Apc8, a component of APC/C, in the reaction samples was determined by immunoblotting.

3. Results

During our studies to understand how cellular anchorage controls the G_1–S transition, we noticed a striking similarity of the effects on Cdc6 stability between anchorage loss and mTORC1 inhibition. When REF overexpressing exogenous Cdc6 (REF-Cdc6) were constructed with retrovirus-mediated gene transfers and deprived of anchorages, Cdc6 protein diminished within 24–36 h despite its constitutive transcription from the strong cytomegalovirus promoter, as previously reported in the rat fibroblast line [6], and this phenomenon was accompanied by a loss of S6K1 Thr^{389} phosphorylation, indicative of inactivation of mTORC1 (Fig. 1A). Similarly, when the same cell, which was grown in an anchorage-furnished culture plate, was treated with rapamycin, an inhibitor of mTORC1, Cdc6 diminished with rapid loss of S6K1 phosphorylation. Density measurements showed that the levels of Cdc6 were reduced to less

than 10% and 15–25% of the original density after anchorage deprivation and rapamycin treatment, respectively. In contrast, in Eker ($Tsc2^{-/-}$) REF engineered similarly (Eker-Cdc6) and deprived of anchorages, Cdc6 was stably expressed (Fig. 1B). However, when the rat counterpart of human TSC2 isoform 4, which lacks exons 25 and 31 but can effectively suppress the onset of tuberous sclerosis in Eker rats [13], was re-expressed in the cell (Eker-Cdc6-$Tsc2^{*}$), it became unstable with diminished S6K1 phosphorylation, as observed for the original REF. While the level of Cdc6 remained within 85–110% in Eker-Cdc6, it was lowered to 10% in Eker-Cdc6-$Tsc2^{*}$, as estimated by densitometry. This suggests that the Tsc1/Tsc2-Rheb-mTORC1 cascade was crucially involved in the control of Cdc6 stability.

3.1. Caspase 3 is not responsible for the destruction of Cdc6 early in methylcellulose culture

We sought to understand the mechanistic basis for this remarkable phenomenon. To achieve this goal, we constructed REF-Cdc6-aRheb and Eker-Cdc6-aRheb (Fig. 1C), in which REF-Cdc6 and Eker-Cdc6 cells were further engineered to express a constitutively active mutant Rheb (aRheb) [12] by retrovirus-based gene transfer.

Because anchorage loss induces anoikis with activation of caspase 3 that can fragment Cdc6, we first investigated whether caspase 3 was responsible for the degradation of Cdc6 within 36 h in methylcellulose medium by monitoring caspase 3 activation and the effects of zVAD, a pan-caspase inhibitor, and/or ALLN, a proteasome inhibitor, on the Cdc6 levels (Fig. 1C). In REF-Cdc6, cleavage of caspase 3 occurred slightly upon anchorage loss (see REF-Cdc6, lane 2) and heavily with fragmentation of Cdc6 after additional ALLN treatment (see REF-Cdc6, lane 5). zVAD alone had no effects on Cdc6 in all of these cells regardless of mTORC1 activation (compare lanes 2 and 3 for all the cells). By contrast, ALLN markedly stabilized Cdc6 in all of these cells (see lane 5 for all the cells), but co-treatment with zVAD had no additional effects besides suppression of its ALLN-induced fragmentation in REF-Cdc6 (see REF-Cdc6, lane 4). The lack of zVAD effects was not the result of an improper use of this compound because in the same samples, this compound completely suppressed the anchorage deprivation as well as the ALLN-induced caspase 3 activation (see REF-Cdc6, lanes 3 and 4) [15]. Interestingly, activation of caspase 3 was largely suppressed in the cells where Tsc2 was inactivated or active Rheb was overexpressed, even after ALLN treatment. These data indicate that proteasomes and/or other ALLN-sensitive proteases, but not caspase 3, were responsible for the destabilization of Cdc6 detected early in methylcellulose culture. Furthermore, despite the complete destruction of Cdc6 protein, approximately 70% of the REF-Cdc6 cells harvested at 36 h in methylcellulose medium were viable and could form colonies after transfer to culture dishes, indicating that cell death was not linked to the destabilization of Cdc6.

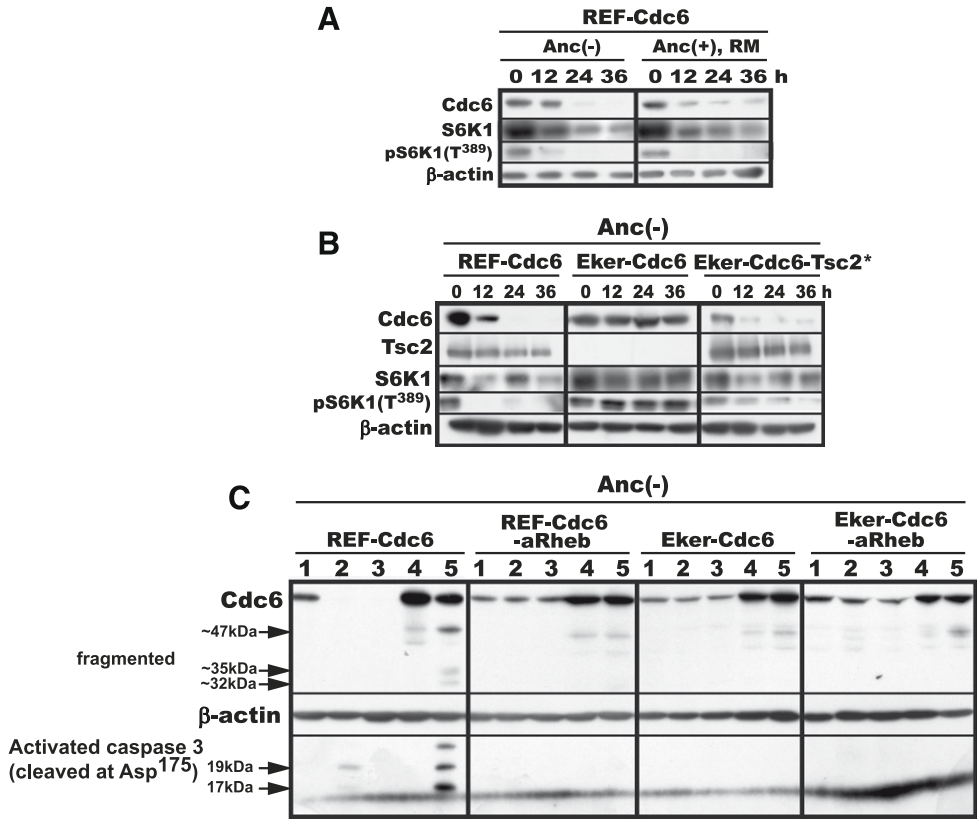

Fig. 1. Inhibition of mTORC1 destabilizes Cdc6 in the presence of anchorages, whereas inactivation of the *Tsc2* gene stabilizes Cdc6 in the absence of anchorages. (A) Treatment with rapamycin destabilizes Cdc6. Rapidly proliferating REF-Cdc6 was cultured in methylcellulose medium (MC) [Anc(−)] or were treated with 50 nM rapamycin (RM) in anchorage-furnished culture plates and analyzed for levels of Cdc6, S6K1 and S6K1 Thr^{389} phosphorylation. β-actin was used as a loading control. (B) In Eker-Cdc6, Cdc6 is stable without anchorage, but in Eker-Cdc6-$Tsc2^{*}$ where the Tsc2 isoform 4 is re-expressed, Cdc6 becomes unstable. Rapidly proliferating REF-Cdc6, Eker-Cdc6 and Eker-Cdc6-$Tsc2^{*}$ were cultured in MC and analyzed as in (A). (C) Effects of caspase and proteasome inhibitors on the stabilization of Cdc6. Logarithmically proliferating REF-Cdc6, REF-Cdc6-aRheb, Eker-Cdc6 and Eker-Cdc6-aRheb cells, for which two independently isolated clones expressing similar levels of Cdc6 were combined, incubated in MC for 24 h and treated for 12 h with or without 75 μM zVAD and/or 90 μM ALLN. Cells were collected at the start of MC culture (lane 1), 36 h after with no addition (lane 2), zVAD (lane 3), zVAD with ALLN (lane 4) or ALLN (lane 5), and the levels of Cdc6 and activation of caspase 3 (cleaved at Asp^{175}) were determined by immunoblotting.

3.2. Activation of mTORC1 suppresses virtually all of the effects of anchorage deprivation on G_1 cell cycle factors

Given the above results, we next examined the levels of major G_1 cell cycle factors and some relevant proteins expressed in these four cells during anchorage deprivation (Fig. 2A). In anchorage-deprived REF-Cdc6, Cdc6, Cyclin A, D-type Cyclins and E2F1 all markedly diminished within 36 h to approximately 10%, 15%, 5-15% and less than 5% of their original levels, respectively, as analyzed by densitometry. Additionally, Rb shifted to a lower position, in agreement with a previous report [16], and confirming the data presented in Fig. 1A and B, S6K1 lost its Thr^{389} phosphorylation and eventually the protein itself, indicating inactivation of Cdk2 and mTORC1, respectively. The levels of the remaining factors decreased to various degrees. By contrast, in cells where mTORC1 was active, all of these factors, including Cdc6, continued to be expressed. Additionally, Emi1 was up-regulated three–fourfold over its levels in REF-Cdc6 as estimated by densitometry. On the other hand, $p27^{Kip1}$ was initially down-regulated but accumulated during anchorage deprivation, unlike the results obtained for REF-Cdc6.

Thus, virtually all the effects of anchorage loss on these G_1 cell cycle factors were effectively suppressed by inactivation of Tsc2 or overexpression of active Rheb. However, Rb remained shifted to a lower position, indicating that Cdk2 was still inactive. By contrast, the continued production of Cyclin A and E2F1 suggested that unlike Cdk2, Cdk4 and/or Cdk6 remained active. These observations were confirmed by in vitro kinase assays (Fig. 2B). In the Eker-Cdc6-aRheb, Cdk2 was inactivated within 12 h of methylcellulose culture. On the other hand, Cdk4 activity was severely reduced but remained present for 36 h (see Fig. 5 for Cdk6). Likely due to the persistent Cdk2 inactivation, these mTORC1-activated REFs were still unable to effectively proliferate in the absence of anchorages (Fig. 2C).

3.3. Emi1 mRNA remains up-regulated in anchorage-deprived Eker-Cdc6-aRheb

We next examined whether or not the elevated Emi1 protein in Eker-Cdc6-aRheb was at least partly ascribable to an up-regulation of its mRNA. Like *Cyclin A* and *E2F1*, the *Emi1* gene is activated by Cdk4/6 via E2F [3,4]. Consistently, *Emi1* mRNA was up-regulated like *Cyclin A* and *E2F1* in Eker-Cdc6-aRheb, where Cdk4/6 remained active despite the absence of anchorages (Fig. 3). In the same cell, *Cyclin D1* mRNA accumulated fourfold, whereas the level of *D3* mRNA was virtually unchanged and similar between the two cells.

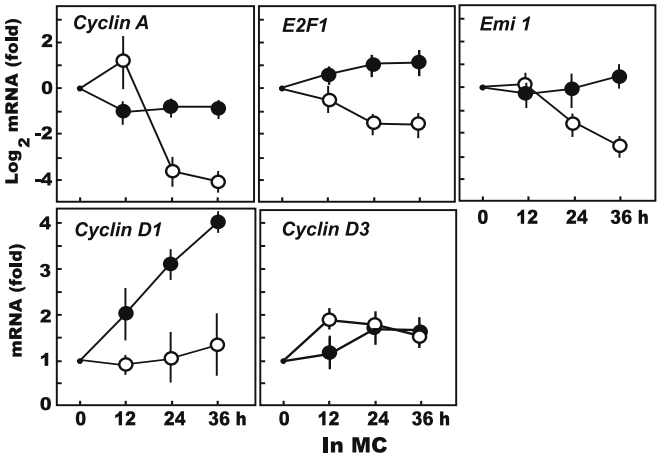

Fig. 3. *Emi1* mRNA remains up-regulated, and *Cyclin D1* mRNA accumulates, in anchorage-deprived Eker-Cdc6-aRheb. Transcripts for *Emi1*, *E2F1*, *Cyclins A*, *D1* and *D3* were extracted from MC-cultured REF-Cdc6 and Eker-Cdc6-aRheb and quantified by real time RT-PCR as described in Section 2. All of the data obtained from at least two separate experiments were normalized to the levels of β-*actin* mRNA and are expressed as average values with standard deviations. Open and closed circles denote mRNA from REF-Cdc6 and Eker-Cdc6-aRheb cells, respectively.

3.4. Up-regulated Emi1 is at least partially responsible for the stabilization of Cdc6

As shown above, the *Emi1* gene was up-regulated, with elevated levels of its protein product in the anchorage-deprived Eker-Cdc6-aRheb (Figs. 2A and 3). Because APC/C^{Cdh1} was a major ubiquitin

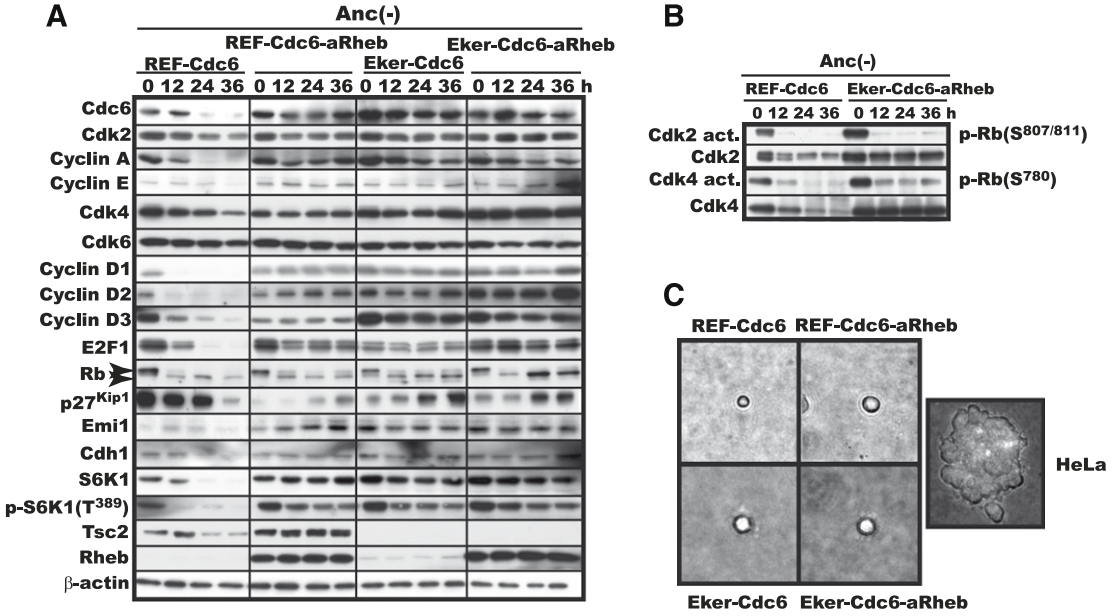

Fig. 2. Activation of mTORC1 overrules anchorage loss and stabilizes Cdc6. (A) REF and Eker REF cells constitutively expressing Cdc6 (REF-Cdc6 and Eker-Cdc6) and those with active Rheb expression (REF-Cdc6-aRheb and Eker-Cdc6-aRheb) were incubated in MC [Anc(−)] and analyzed for the G_1 cell cycle proteins and related factors by immunoblotting. (B) In vitro kinase assays for Cdk2 and Cdk4 in REF-Cdc6 and Eker-Cdc6-aRheb cultured in MC. In vitro assays of these kinase activities were carried out as described in Section 2. (C) Eker REFs overexpressing Cdc6 alone or both Cdc6 and the active Rheb poorly proliferate in soft agar. REF-Cdc6, REF-Cdc6-aRheb, Eker-Cdc6 and Eker-Cdc6-aRheb cells were cultured for 10 days in soft agar with HeLa cells as a positive control.

ligase that degraded both Cdc6 and Cyclin A and could be inactivated by Emi1 [4,7], we hypothesized that the elevated Emi1 was responsible, at least partially, for the stabilization of Cdc6. Therefore, we tested this idea initially by attempting *Emi1* knockdown in Eker-Cdc6-aRheb, but despite vigorous trials with various doses (up to fivefold over the dose sufficient to transfect all of the cells) of two sets of 23/27mer duplex siRNA constructs, there was no reduction in the level of Emi1 protein. Although under the same conditions, virtually all of the cells were successfully transfected with a fluorescent labeled 23/27mer duplex control RNA. Therefore, we modified our strategy by stably expressing Emi1 in REF-Cdc6 to a level comparable to that in Eker-Cdc6-aRheb and examined its effects on Cdc6 levels. REF-Cdc6 expressing exogenous Emi1 (REF-Cdc6-Emi1) (roughly to a twofold higher level of Emi1 as estimated by densitometry) and empty vector-introduced REF-Cdc6 were deprived of anchorage and analyzed likewise (Fig. 4A). As expected, expression of both Cdc6 and Cyclin A was partially restored in REF-Cdc6-Emi1 despite translational repression, as indicated by the diminished S6K1 Thr^{389} phosphorylation. To confirm that Cdh1 was the target of the up-regulated Emi1 in Eker-Cdc6-aRheb, we overexpressed Cdh1 in these cells and examined its effects (Fig. 4B). As expected, both Cdc6 and Cyclin A disappeared upon anchorage deprivation despite persistent mTORC1 activation and three times more protein loading, as indicated by the intensities of β-actin. These results indicate that the up-regulated Emi1 was at least partially responsible for the stabilization of Cdc6 presumably via inactivating APC/C^{Cdh1} in Eker-Cdc6-aRheb, as established [4].

3.5. APC/C^{Cdh1} is inactive in Eker-Cdc6-aRheb

To verify inactivation of APC/C^{Cdh1} in Eker-Cdc6-aRheb, we performed in vitro ubiquitylation assays of the APC/C^{Cdh1} immunoprecipitated from the anchorage-deprived REF-Cdc6 and Eker-Cdc6-aRheb cell lysates. Their APC/C^{Cdh1} activities were assayed with Cyclin A as a substrate and compared with equal amounts of immunoprecipitated Apc8, a key component of this ubiquitin ligase complex (Fig. 4C). Consistent with the in vivo data, unlike the results obtained for REF-Cdc6, APC/C^{Cdh1} from the Eker-Cdc6-aRheb was highly inactive, as confirmed by performing assays with various reaction times.

3.6. Inhibition of mTORC1 inactivates Cdk4/6 and destabilizes Cdc6

Finally, we investigated whether the *Tsc2* inactivation- and/or active Rheb expression-induced stabilization of Cdc6 was mediated by mTORC1. When 24-h methylcellulose-cultured Eker-Cdc6-aRheb was treated with rapamycin, Cdc6 was destabilized with inactivation of Cdk4/6, as shown by a loss of Rb Ser^{780} phosphorylation and in vitro kinase assays (Fig. 5A). Consistent with their inactivation, E2F-dependently expressed Cyclin A, E2F1 and Emi1 also diminished or disappeared. On the other hand, all of the D-type Cyclins decreased but were still present even after 24 h treatment with rapamycin. Quantitative RT-PCR analysis showed that the levels of *Cyclins D1* and *D3* mRNAs were virtually uninfluenced by rapamycin treatment. Thus, the levels of these transcripts were not controlled by mTORC1.

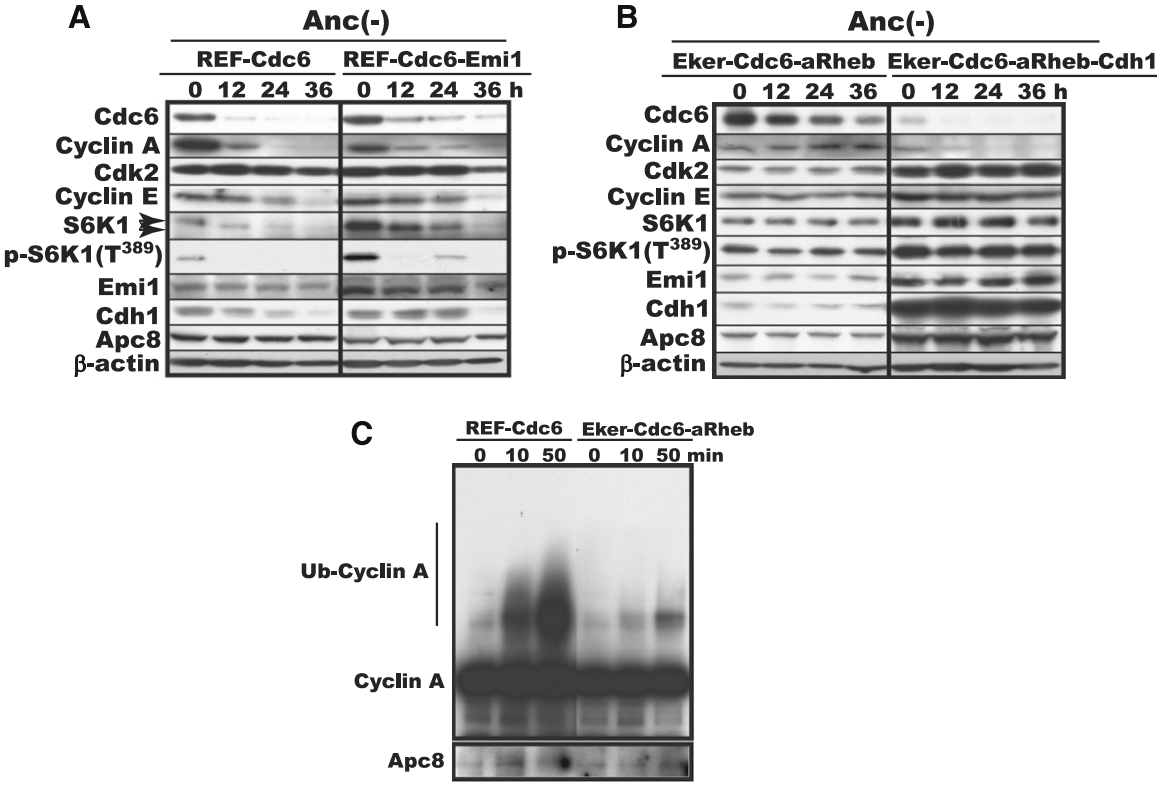

Fig. 4. Enforced Emi1 expression stabilizes, and Cdh1 overexpression destabilizes Cdc6 in anchorage-deprived cells. (A) REF-Cdc6 and REF-Cdc6 constitutively expressing Emi1 (REF-Cdc6-Emi1) were incubated in MC and analyzed for the indicated factors by immunoblotting. (B) Eker-Cdc6-aRheb and Cdh1-overexpressing Eker-Cdc6-aRheb cells (Eker-Cdc6-aRheb-Cdh1) were similarly analyzed as in (A). Roughly three times more protein was loaded for immunoblot analysis of Eker-Cdc6-aRheb-Cdh1 to clearly show diminished levels of Cdc6 and Cyclin A. (C) APC/C^{Cdh1} is inactive in anchorage-deprived Eker-Cdc6-aRheb. APC/C^{Cdh1} was immunoprecipitated from lysates of the REF-Cdc6 and Eker-Cdc6-aRheb cultured for 24 h in MC and assayed for its ubiquitin ligase activities with Cyclin A as a substrate and for the amounts of co-precipitated Apc8 as described in Section 2. After an in vitro ubiquitylation reaction for 0, 10 and 50 min followed by separation by SDS gel electrophoresis, ubiquitylated Cyclin A was detected with the anti-Cyclin A antibody.

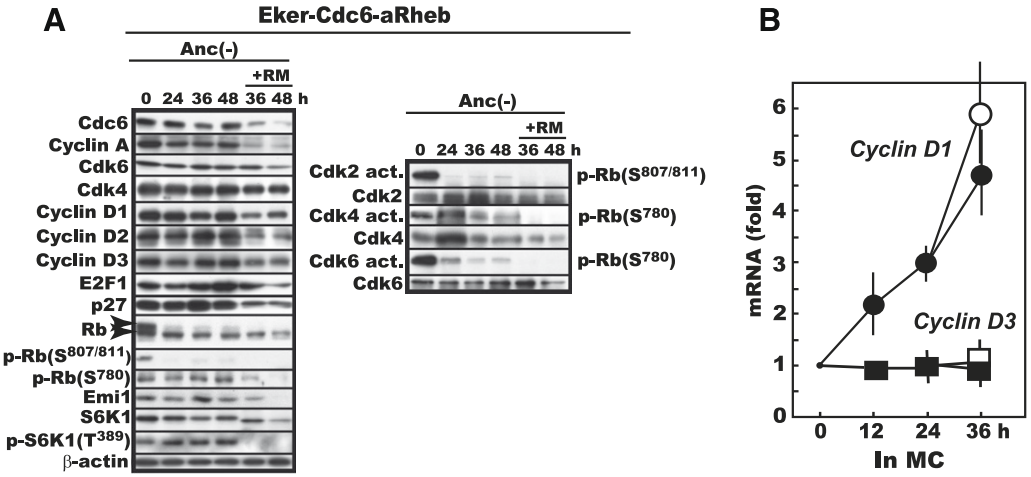

Fig. 5. Inhibition of mTORC1 inactivates Cdk4/6 with diminished expression of the critical G_1 factors in anchorage-deprived Eker-Cdc6-aRheb. (A) Eker-Cdc6-aRheb was cultured for 24 h in MC, treated with or without 50 nM rapamycin for an additional 12 and 24 h and analyzed for the indicated factors by immunoblotting. In vitro kinase assays were performed for Cdk2, Cdk4 and Cdk6 as in Fig. 2B. (B) Levels of *Cyclins D1* and *D3* transcripts are not controlled by mTORC1. *D1* and *D3 Cyclin* mRNAs were extracted from the Eker-Cdc6-aRheb cultured in MC with or without rapamycin as in (A) and quantified by real time RT-PCR and data processing as in Fig. 3. Circles and squares denote *D1* and *D3* mRNAs, whereas closed and open symbols denote the mRNAs from the untreated and rapamycin-treated cells, respectively.

4. Discussion

The Tsc1/Tsc2-Rheb-mTORC1 pathway mediates growth factor and nutrient signals to control cell proliferation by regulating general translation [10]. Our studies with anchorage-deprived rodent embryonic fibroblasts indicate that the Tsc1/Tsc2-Rheb-mTORC1 cascade is also critically involved in the activation of Cdk4/6 to promote S phase onset, consistent with a previous observation with a malignantly transformed cell line [17]. Activated Cdk4/6 activates genes important or essential for S phase onset including *Cdc6*, *Cyclin A* and *Emi1*, via the known cascade involving Rb and E2F [3,4]. Induced Emi1 inhibits APC/C^{Cdh1}, thereby stabilizing nascent Cdc6 and Cyclin A proteins that are efficiently translated under S6K1 activation and 4EBP inactivation.

In addition to regulating whole cell levels of D-type Cyclins, as previously reported [18,19] and shown herein, how does mTORC1 control Cdk4/6 activities? It is unclear at present, but the association of neither D-type Cyclins nor $p27^{Kip1}$ with these kinases might be controlled by mTORC1. The involvement of p27 has been suggested in mTORC1 control of cell cycle progression in some cells [20,21]. In our experiments, however, the whole cell level of p27 was rather reduced by treatment with rapamycin, which led to inactivation of Cdk4/6 (Fig. 5A). Moreover, when we tentatively analyzed the quantity of the D-type Cyclins and the p27 bound to the Cdk4/6 from rapamycin-treated and untreated Eker-Cdc6-aRheb, their quantities were similar regardless of the treatment (unpublished data). This result provisionally suggests that at least the quantities of these Cyclins and the inhibitor associated with Cdk4 might be neither directly reflected by their levels in the whole cell nor regulated by mTORC1.

One realistic scenario raised in this study is that an anchorage signal might actually be mediated by the mTORC1 pathway, and the rationale for this is as follows. Anchorage deprivation markedly reduced S6K1 Thr^{389} phosphorylation, indicative of mTORC1 inactivation, whereas activation of mTORC1 by a *Tsc2* mutation or active Rheb overexpression suppressed virtually all of the effects of anchorage deprivation on the G_1 cell cycle factors. Consistent with this scenario, we have tentatively identified Rho-associated kinase as a candidate for the molecule that directly links the anchorage signal to Tsc2 (Park et al., unpublished).

Acknowledgments

We thank Drs O. Hino for wild type and Eker REF and the *Tsc2* cDNA, P. Jackson for the *emi1* cDNA, H. Saya for the *cdh1* cDNA and R. Lamb for the constitutively active *Rheb* cDNA. This work was supported by Grants-in-Aid for Scientific Research (S) and for the Global Center of Excellence, from the Ministry of Education, Science and Culture of Japan.

References

[1] Otsuka, H. and Moskowitz, M. (1975) Arrest of 3T3 cells in G1 phase in suspension culture. J. Cell. Physiol. 87, 213–219.
[2] Danen, E.H. and Yamada, K.M. (2001) Fibronectin, integrins, and growth control. J. Cell. Physiol. 189, 1–13.
[3] Blais, A. and Dynlacht, B.D. (2004) Hitting their targets: an emerging picture of E2F and cell cycle control. Curr. Opin. Genet. Dev. 14, 527–532.
[4] Hsu, J.Y., Reimann, J.D.R., Sorensen, C.S., Lukas, J. and Jackson, P.K. (2002) E2F-dependent accumulation of hEmi1 regulates S phase entry by inhibiting APC^{Cdh1}. Nat. Cell Biol. 4, 358–366.
[5] Mendez, J. and Stillman, B. (2000) Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis. Mol. Cell. Biol. 20, 8602–8612.
[6] Jinno, S., Yageta, M., Nagata, A. and Okayama, H. (2002) Cdc6 requires anchorage for its expression. Oncogene 21, 1777–1784.
[7] Peters, J.M. (2006) The anaphase promoting complex/cyclosome: a machine designed to destroy. Nat. Rev. Mol. Cell Biol. 7, 644–656.
[8] Chiarugi, P. and Giannoni, E. (2008) Anoikis: a necessary death program for anchorage-dependent cells. Biochem. Pharmacol. 76, 1352–1364.
[9] Pelizon, C., d'Adda di Fagagna, F., Farrace, L. and Laskey, R.A. (2002) Human replication protein Cdc6 is selectively cleaved by caspase 3 during apoptosis. EMBO Rep. 3, 780–784.
[10] Sarbassov, D.D., Ali, S.M. and Sabatini, D.M. (2005) Growing roles for the mTOR pathway. Curr. Opin. Cell Biol. 17, 596–603.
[11] Yeung, R.S., Xiao, G., Jin, F., Lee, W., Testa, J.R. and Knudson, A.G. (1994) Predisposition to renal carcinoma in the Eker rat is determined by germ-line mutation of the tuberous sclerosis 2 (TSC2) gene. Proc. Natl. Acad. Sci. USA 91, 11413–11416.
[12] Yan, L., Findlay, G.M., Jones, R., Procter, J., Cao, Y. and Lamb, R.F. (2006) Hyperactivation of mammalian target of rapamycin (mTOR) signaling by a gain-of-function mutant of the Rheb GTPase. J. Biol. Chem. 281, 19793–19797.
[13] Momose, S., Kobayashi, T., Mitani, H., Hirabayashi, M., Ito, K., Ueda, M., Nabeshima, Y. and Hino, O. (2002) Identification of the coding sequences responsible for Tsc2-mediated tumor suppression using a transgenic rat system. Hum. Mol. Genet. 11, 2997–3006.
[14] Grossberger, R., Gieffers, C., Zachariae, W., Podtelejnikov, A.V., Schleiffer, A., Nasmyth, K., Mann, M. and Peters, J.M. (1998) Characterization of the DOC1/APC10 subunit of the yeast and the human anaphase-promoting complex. J. Biol. Chem. 274, 14500–14507.
[15] Zhu, W., Murtha, P.E. and Young, C.Y.F. (1995) Calpain inhibitor-induced apoptosis in human prostate adenocarcinoma cells. Biochem. Biophys. Res. Commun. 214, 1130–1137.

[16] Gan, B., Yoo, Y. and Guan, J.L. (2006) Association of focal adhesion kinase with tuberous sclerosis complex 2 in the regulation of S6 kinase activation and cell growth. J. Biol. Chem. 281, 37321–37329.
[17] Gaben, A.-M., Saucier, C., Bedin, M., Barbu, V. and Mester, J. (2004) Rapamycin inhibits Cdk4 activation, $p21^{WAF1/CIP1}$ expression and G1-phase progression in transformed mouse fibroblasts. Int. J. Cancer 108, 200–206.
[18] Nelsen, C.J., Rickheim, D.G., Tucker, M.M., Hansen, L.K. and Albrecht, J.H. (2003) Evidence that cyclin D1 mediates both growth and proliferation downstream of TOR in hepatocytes. J. Biol. Chem. 278, 3656–3663.
[19] Gao, N., Flynn, D.C., Zhang, Z., Zhong, X.-S., Walker, V., Liu, K.J., Shi, X. and Jiang, B.-H. (2004) G_1 cell cycle progression and the expression of G1 cyclins are regulated by PI3K/AKT/mTOR/p70S6K1 signaling in human ovarian cancer cells. Am. J. Physiol. Cell Physiol. 287, 281–291.
[20] Luo, Y., Marx, S.O., Kiyokawa, H., Koff, A., Massague, J. and Marks, A.R. (1996) Rapamycin resistance tied to defective regulation of $p27^{Kip1}$. Mol. Cell. Biol. 16, 6744–6751.
[21] Kawamata, S., Sakaida, H., Hori, T., Maeda, M. and Uchiyama, T. (1998) The upregulation of $p27^{Kip1}$ by rapamycin results in G1 arrest in exponentially growing T-cell lines. Blood 91, 561–569.