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Expression of budding yeast FKBP12 confers rapamycin susceptibility to the unicellular red alga *Cyanidioschyzon merolae*

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ABSTRACT

The target of rapamycin (TOR) is serine/threonine protein kinase that is highly conserved among eukaryotes and can be inactivated by the antibiotic rapamycin through the formation of a ternary complex composed of rapamycin and two proteins, TOR and FKBP12. Differing from fungi and animals, plant FKBP12 proteins are unable to form the ternary complex, and thus plant TORs are insensitive to rapamycin. This has led to a poor understanding of TOR functions in plants. As a first step toward the understanding of TOR function in a rapamycin-insensitive unicellular red alga, *Cyanidioschyzon merolae*, we constructed a rapamycin-susceptible strain in which the *Saccharomyces cerevisiae* FKBP12 protein (ScFKBP12) was expressed. Treatment with rapamycin resulted in growth inhibition and decreased polysome formation in this strain. Binding of ScFKBP12 with *C. merolae* TOR in the presence of rapamycin was demonstrated *in vivo* and *in vitro* by pull-down experiments. Moreover, *in vitro* kinase assay showed that inhibition of *C. merolae* TOR kinase activity was dependent on ScFKBP12 and rapamycin.

1. Introduction

Target of rapamycin (TOR) is a serine/threonine protein kinase that plays a central role in the regulation of cell growth and metabolism [1]. This protein is structurally and functionally conserved among eukaryotes [2–4]. In the budding yeast *Saccharomyces cerevisiae*, TOR is encoded by two genes (TOR1 and TOR2) and each TOR interacts with different regulatory proteins to form two distinct complexes, TOR complex 1 (TORC1) and TOR complex 2 (TORC2) [2–4]. TORC1 regulates cell growth and metabolism in response to nutrient and energy requirements [2–4]. TORC1 kinase activity and its functions are specifically inhibited by rapamycin, which is a product of *Streptomyces hygroscopicus* [5]. TORC2 contributes to the regulation of cytoskeleton structure and spatial features of cell growth, and is not inhibited by rapamycin [2–4]. Distinct from budding yeast, mammalian cells have a unique species of TOR called mTOR. However, the mTOR protein forms two independent complexes called mTORC1 and mTORC2 in the cells [3,4]. As in *S. cerevisiae*, the mTORC1 complex modulates a variety of cellular responses, such as translation initiation, ribosome biogenesis, and cell growth, and is rapamycin sensitive [3,4]

Studies in *S. cerevisiae* have uncovered the unique mechanism of action of rapamycin [5]. Rapamycin first binds to the 12 kDa FK506-binding protein (FKBP12) and this complex inhibits the TOR serine/threonine kinase by binding to the FRB domain of TOR. A recent study based on crystal structure at 3.2 Å resolution revealed that the FRB domain of human TOR is one of the substrate binding sites and operates as a gatekeeper of the active site [6]. Thus, the mechanism by which the FKBP12–rapamycin complex inhibits mTOR probably involves sequestering the FRB docking site and steric hindrance of substrate access to the catalytic cleft of mTOR. FKBP12 has peptidyl prolyl cis/trans isomerase activity that is involved in protein-folding processes, but the physiological function of FKBP12 is still poorly understood.

Rapamycin susceptibility is widespread among eukaryotes. However, it has been reported that rapamycin does not significantly inhibit growth in land plants [7,8]. In *Arabidopsis thaliana*, *TOR* knockout strains show an embryonic lethal phenotype [7,9]. This situation has resulted in limited available information about TOR's function in plants [10,11].

Cyanidioschyzon merolae is a unicellular red alga living in acid hot springs (pH 1–3, 40–50 °C), with each cell containing only one mitochondrion, one chloroplast, and one nucleus. The complete genome sequences of these three organelles were determined [12–15], and their extremely simple and minimally redundant

Abbreviations: DMSO, dimethyl sulfoxide; His-tag, histidine-tag; kDa, kilodalton.

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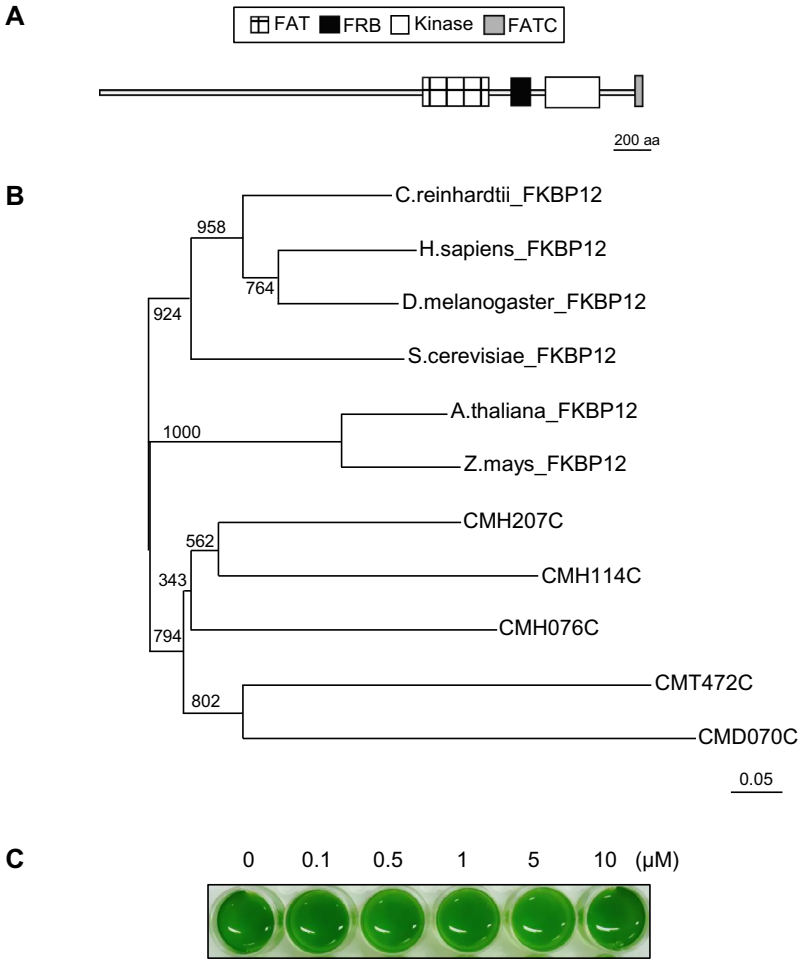

Fig. 1. Resistance of *C. merolae* to rapamycin. (A) A schematic representation of the secondary structure of CmTOR. (B) The evolutionary relationships of FKBP12 and its homologues in *C. merolae*. Bootstrap values from 1000 replicates are shown at each node. Branch lengths are proportional to the number of amino acid substitutions, indicated by the scale bar below the tree. Designations and GenBank accession numbers for sequences are as follows: CMH207C, CMH114C, CMH076C, CMT472C, and CMD070C are gene numbers in the *C. merolae* database (http://merolae.biol.s.u-tokyo.ac.jp/); C.reinhardtii_FKBP12 for *Chlamydomonas reinhardtii* FKBP12 (XP_001693615), H.sapiens_FKBP12 for *Homo sapiens* FKBP12 (NP_000792), D.melanogaster_FKBP12 for *Drosophila melanogaster* FKBP12 (NP_523792), S. cerevisiae_FKBP12 for *Saccharomyces cerevisiae* FKBP12 (NP_014264), A.thaliana_FKBP12 for *Arabidopsis thaliana* FKBP12 (NP_201240), Z.mays_FKBP12 for *Zea mays* FKBP12 (NP_001105537). (C) Wild-type *C. merolae* growth under several concentrations of rapamycin.

gene content was uncovered. Taking advantage of these biological characteristics, we have developed various tools for analysis of *C. merolae* [16–20]. For these reasons, *C. merolae* is thought to be a good model organism to understand TOR function in plants. This study represents the first step toward understanding of TOR function in *C. merolae*, in which we constructed a rapamycin-sensitive strain by expression of the *S. cerevisiae* FKBP12 protein in the cell using transformation techniques.

2. Materials and methods

2.1. Strain and growth conditions

C. merolae 10D wild-type and transformants were grown at 40 °C under continuous white light (50 μmol m^{-2} s^{-1}) in liquid MA2 medium [21] at pH 2.5 with bubbling of air supplemented with 2% CO_2. For the M4 strain, 0.5 mg/mL uracil was added to the medium. For rapamycin treatment experiments, *C. merolae* cells ($OD_{750} \geqslant 10$) were diluted to $OD_{750} = 0.2$ and with rapamycin (dissolved in DMSO; LC Laboratories, Woburn, MA, USA) or DMSO added at the concentrations indicated in the figures

2.2. Phylogenetic analysis

A phylogenetic tree based on 104 unambiguously aligned amino acid positions of six FKBP12 proteins and their five homologues in *C. merolae* was constructed as described previously [22].

2.3. Construction of S. cerevisiae protein-expressing strain

The detailed protocol for construction of the *S. cerevisiae* protein-expressing *C. merolae* strain is provided in the Supplementary methods.

2.4. Immunoblot analysis

Immunoblot analysis was performed as described previously [16].

2.5. Polysome isolation

Isolation of polysomes was carried out as described previously [23] with slight modifications. See Supplementary methods for details.

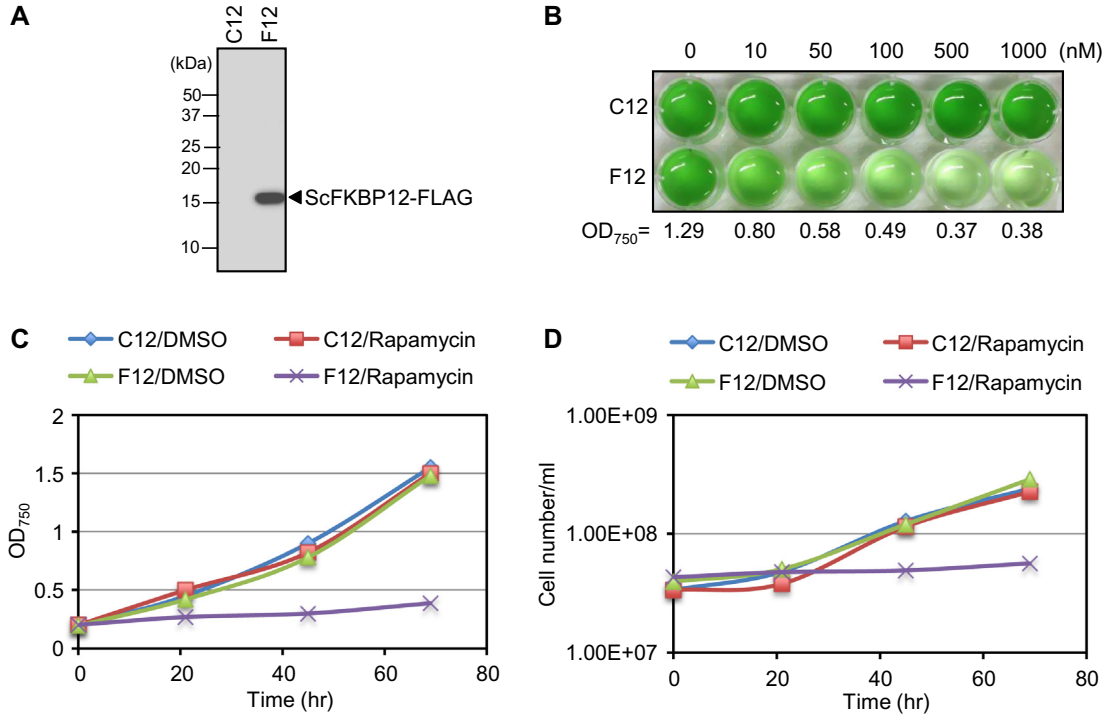

Fig. 2. Sensitivity of the F12 strain to rapamycin. (A) Immunoblot analysis for ScFKBP12. Aliquots of total protein (each 1 μg) prepared from F12 or C12 cells were separated by 15% SDS–PAGE and analyzed by immunoblotting with a polyclonal anti-FLAG antibody. The positions of molecular size markers are indicated in kDa at the left. The arrowhead indicates the expected position of FLAG-tagged ScFKBP12 protein. (B) Growth of the F12 and C12 strains under several concentrations of rapamycin. Optical density at 750 nm (OD_{750}) for each culture of the F12 strain is indicated at the bottom. The others are the same as in Fig. 1C. (C and D) Sequential growth of the F12 and C12 strains in the presence of rapamycin. The growth of both strains in the presence of rapamycin at a final concentration of 0.5 μM was monitored by OD_{750} (C) and cell number (D).

2.6. Preparation of His-tagged S. cerevisiae FKBP12 protein

For cloning of the *S. cerevisiae* FKBP12 gene, the open reading frame was amplified by PCR with *S. cerevisiae* genomic DNA as a template and the primers: ScFKBP12_En_F, CACCATGTCTGA AGTAATTGAAGGTAACGT; ScFKBP12_NT_R, GTTGACCTTCAACAATTCGACGTCG. The PCR-amplified fragment was inserted into pENTR™/D-TOPO (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cloned gene was recombined into the destination vector pDEST-ColdI; a Reading Frame Cassette B of the Gateway Vector Conversion Reagent System (Invitrogen) was cloned into blunt-ended *Xho*I/*Sal*I digested pColdI (TaKaRa Bio, Otsu, Japan), according to the manufacturer's instructions. The overproduction in *E. coli* and purification of His-tagged recombinant protein was performed as described previously for *A. thaliana* pBrp [16].

2.7. Preparation of antibody against C. merolae TOR

Preparation and purification of polyclonal antibodies for *C. merolae* TOR (CmTOR) were carried out as described previously [16]. See Supplementary methods for details.

2.8. Pull-down experiment

A pull-down experiment was performed as described previously [8,22] with modifications. See Supplementary methods for details.

2.9. Immunoprecipitation and in vitro CmTOR kinase assay

Immunoprecipitation and *in vitro* kinase assay was carried out as described previously [24] with modifications. See Supplementary methods for details.

3. Results

3.1. TOR and FKBP12 genes and rapamycin resistance of C. merolae

The 100%-complete *C. merolae* genome sequence [14,15] showed that a unique gene (CMR018C in http://merolae.biol.s.u-tokyo.ac.jp/) encodes the TOR protein (hereafter CmTOR in this study). The well-conserved focal adhesion target (FAT), FKBP12-rapamycin binding (FRB), kinase, and FAT C-terminus (FATC) domains were found in the C-terminal region (Fig. 1A). When *S. cerevisiae* or human FKBP12 was used as a query for blast analysis, five genes were identified that encoded FKBP-type peptidyl-prolyl cis–trans isomerase homologues in *C. merolae* (Supplementary Table S1). To examine the phylogenetic relationships among these, an unrooted neighbor-joining (NJ) tree was constructed with FKBP12 proteins from yeast, animals, green alga, and land plants (Fig. 1B). The resultant phylogenetic tree consisted of three independent groups, and showed that no *C. merolae* protein belonged to a cluster containing rapamycin-sensitive yeast or animals. In the case of the rapamycin-susceptible unicellular green alga, *Chlamydomonas reinhardtii* [25], the FKBP12 protein was positioned in the same clade as FKBP12s from yest and animals (Fig. 1B). We next investigated the effect of rapamycin on *C. merolae* growth. As shown in Fig. 1C, *C. merolae* growth was not affected even in the presence of high concentrations of rapamycin, indicating that wild-type *C. merolae* is insensitive to rapamycin, as are land plants. These results raised the possibility that the rapamycin insensitivity of *C. merolae* is due to divergence of the FKBP12 protein.

3.2. Rapamycin susceptibility of an S. cerevisiae FKBP12-expressing C. merolae strain

If the FKBP12 type is critical for rapamycin sensitivity, heterologous expression of FKBP12 of yeast or animals may confer rapa-

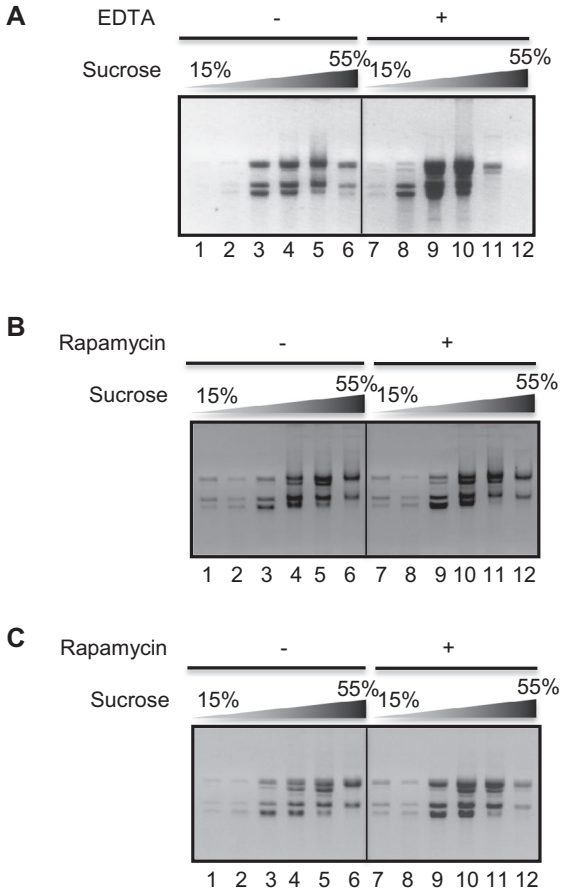

Fig. 3. Inhibition of polysome formation by rapamycin. (A) Polysome isolation in the presence of EDTA. RNA prepared from the F12 strain in the presence of EDTA and isolated from 15% to 55% sucrose gradient fractions was separated by gel electrophoresis and visualized. (B and C) The effect of rapamycin on polysome formation. RNA was prepared from the C12 (B) and F12 (C) strains in the presence or absence of rapamycin. Other factors are the same as in panel A, but $MgCl_2$ was substituted for EDTA.

mycin susceptibility to *C. merolae*. A previous study reported that *S. cerevisiae* FKBP12 (ScFKBP12) was able to bind to *A. thaliana* TOR and that its expression in *A. thaliana* resulted in rapamycin susceptibility [8]. To examine this possibility, a C-terminal FLAG-tagged ScFKBP12-coding region was placed downstream of the strong *APCC* promoter [20], and introduced into *C. merolae* cells to make an ScFKBP12-expressing strain named F12. Expression of the FLAG-tagged ScFKBP12 was confirmed by immunoblot analysis as shown in Fig. 2A. The C12 strain was obtained with the parental plasmid used for construction of the F12 strain, and used as a control strain. Integration of the exogenous DNA into the relevant nuclear genome was confirmed by Southern blot and PCR analyses (Supplementary Fig. S1).

One of the phenotypes of TOR inactivation by rapamycin is growth inhibition [2–4]. Thus, the growth of the F12 strain in liquid culture medium was checked in the presence of rapamycin at the final concentrations shown in Fig. 2B. Growth of the C12 strain was not influenced by addition of rapamycin as in the parental wild-type strain (Fig. 1C). In contrast, growth of the F12 strain was inhibited by relatively low concentrations of rapamycin, such as 10 nM, and the effect appeared to be saturated at concentrations of 500 nM or above (Fig. 2B). We therefore added rapamycin to the culture medium at 500 nM in subsequent experiments. We next examined the influence of rapamycin on growth by measuring two variables, optical density at 750 nm (OD_{750}) and cell number. While the solvent DMSO alone did not affect the growth of the F12 strain, rapamycin addition arrested growth for at least 72 h (Fig. 2C). In contrast, the C12 strain was not affected by rapamycin addition (Fig. 2C). Rapamycin had a similar effect on cell numbers as shown in Fig. 2D. These results indicated that the growth of the F12 strain was inhibited by rapamycin.

3.3. Decrease of polysome formation in the rapamycin-sensitive strain after treatment with rapamycin

Another typical phenotype of TOR inactivation by rapamycin is inhibition of translation [2–4]. Thus, we investigated the effect of rapamycin treatment on polysome formation in the F12 strain. As shown in Fig. 3A, ribosomal RNA (rRNA) signals in lane 12 disappeared in the presence of EDTA, which disrupts polysome formation, indicating that the bottom fraction was enriched for polysomes. The intensities of rRNA signals in the bottom fraction were clearly reduced when F12 cells were treated with rapamycin (Fig. 3C, lanes 6 vs. 12). No reduction was observed when the C12 strain was subjected to the same analysis (Fig. 3B, lanes 6 vs. 12). These results indicated that rapamycin treatment of the F12 strain resulted in decreased polysome formation, i.e., inhibition of translation.

3.4. Binding of CmTOR and ScFKBP12 in the presence of rapamycin inhibits CmTOR kinase activity

To examine whether ScFKBP12 interacts with CmTOR in F12 cells dependent on the presence of rapamycin, we prepared a polyclonal antibody against CmTOR and performed an immunoprecipitation analysis. As shown in Fig. 4A, the CmTOR antibody specifically recognized endogenous CmTOR, producing a single band of expected size (approximately 250 kDa). Results shown in Fig. 4B showed that CmTOR co-immunoprecipitated with ScFKBP12 only in the presence of rapamycin (lane 3), indicating the rapamycin-dependent interaction *in vivo*. To further examine the interaction between ScFKBP12 and CmTOR, ScFKBP12 was overproduced as a His-tagged protein in *E. coli*, purified on a nickel affinity column (Fig. 4C), and subjected to the *in vitro* pull-down experiment. Nickel-agarose bound His-tagged ScFKBP12 was mixed with soluble *C. merolae* proteins with or without rapamycin and the His-tagged ScFKBP12 was eluted after washing the resin. The eluted fraction was subjected to immunoblot analysis. CmTOR was detected together with His-tagged ScFKBP12 in the eluent (Fig. 4D, lane 2), while only His-tagged ScFKBP12 was detected in the absence of rapamycin (lane 1). These results indicated binding of ScFKBP12 to endogeneous CmTOR in the presence of rapamycin. To examine whether rapamycin inhibits CmTOR kinase activity, we first investigated the kinase activity using immunoprecipitants that were obtained under the same conditions as in Fig. 4B. In the assay, we used human 4EBP1 protein as the substrate, which works as a substrate of *A. thaliana* TOR kinase [26]. However, the kinase activities of rapamycin treated cells were almost the same as with those of DMSO treated cells (data not shown). This is probably because the rapamycin concentration was remarkably reduced during the washing steps, and most of the CmTOR-ScFKBP12-rapamycin ternary complex was easily dissociated, which resulted in the little difference of the kinase activities. We next conducted an *in vitro* kinase assay using immunoprecipitants that were obtained from wild-type cells with antibody against CmTOR. CmTOR was able to phosphorylate 4EBP1 *in vitro* in the presence of rapamycin or ScFKBP12 (Fig. 4E, lane 1 vs. lanes 2 and 3), but the kinase activity was severely inhibited when rapamycin and ScFKBP12 were simultaneously added to the reaction mixture (lane 4). No kinase activity was detected when immunoprecipitant with IgG purified from the preimmune serum was used

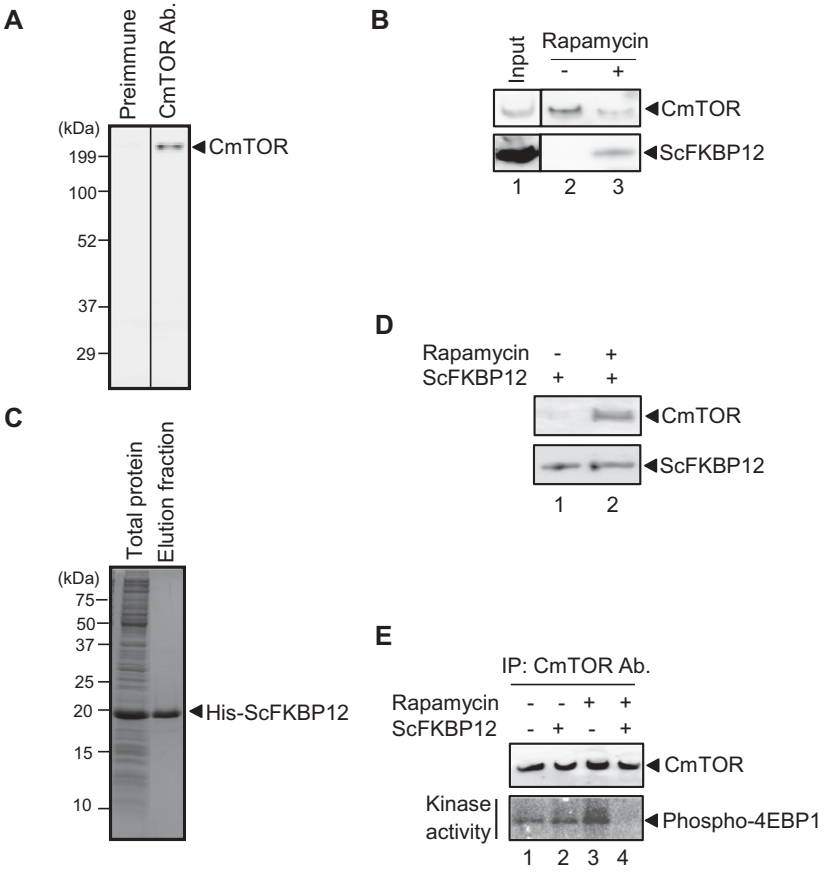

Fig. 4. Binding of CmTOR and ScFKBP12 in the presence of rapamycin leads to inhibition of CmTOR activity. (A) Preparation and specificity of antibody against CmTOR. Aliquots containing 20 μg of *C. merolae* total protein were separated by 15% SDS–PAGE and analyzed by immunoblot analysis with the indicated antibodies. (B) Co-immunoprecipitation of CmTOR and ScFKBP12 *in vivo*. Immunoprecipitation was performed with F12 cell extract and antibodies against CmTOR, and co-immunoprecipitation was analyzed by immunoblot analysis with the indicated antibodies. As a control, 7.5% of cell lysate (Input) was directly subjected to immunoblot analysis. The presence (+) or absence (−) of rapamycin is indicated at the top. (C) Purification of His-tagged ScFKBP12. The overproduced and purified His-tagged ScFKBP12 proteins were resolved on a 15% gel by SDS–PAGE. The gel was stained with Coomassie Brilliant Blue. The positions of molecular size markers are indicated in kilodaltons (kDa) at the left. (D) Pull-down of native CmTOR with His-tagged ScFKBP12. His-tagged ScFKBP12 was incubated with soluble *C. merolae* cell extract in the absence (lane 1) or presence (lane 2) of rapamycin. Upper and lower panel bands were obtained with anti-CmTOR and anti-His-tag antibodies, respectively. (E) *In vitro* kinase assay. Cell extracts of *C. merolae* wild-type cells were subjected to immunoprecipitation with anti-CmTOR antibody. The presence (+) or absence (−) of ScFKBP12 and rapamycin is indicated at the top. The phosphorylation status of 4EBP1 was monitored by the incorporation of [γ-^{32}P] ATP. The amount of CmTOR in each reaction was detected by immunoblot analysis with CmTOR antibody.

(data not shown). These *in vitro* and *in vivo* experiments demonstrated that the CmTOR–ScFKBP12–rapamycin ternary complex was actually formed, and that CmTOR kinase activity was fully inhibited in the presence of ScFKBP12 and rapamycin.

4. Discussion

The TOR of land plants is essential for embryo development and functions in metabolism, growth, and the life span [7,9,26–28]. However, the details of its regulatory mechanisms have been elusive to date. The reasons for this are (i) the high proportion of gene redundancy in land plant genomes, namely complex regulatory systems, (ii) the necessity for investigation of stage- and organ-specific TOR functions, and (iii) TOR activity is not inhibited by the TOR-specific inhibitor rapamycin. As mentioned in the Introduction, the characteristics of *C. merolae* have advantages that overcome the first two obstacles. The rapamycin-sensitive *C. merolae* strain F12 constructed by genetic transformation in this study obviously overcomes the final difficulty. It is conceivable that the underlying roles of this important kinase are highly conserved among photosynthetic eukaryotes. Thus, the F12 strain should be suitable to study the functions of the TOR signaling pathway in plants.

All land plants tested so far appear to be insensitive to rapamycin. The phylogenetic analyses shown in this study and other reports indicate that FKBP12 homologues in *C. merolae* and land plants are not classified into the same clade as FKBP12s from fungi and animals that are rapamycin sensitive (Fig. 1B) [25]. Conversely, *C. reinhardtii* is susceptible to rapamycin and, consistent with this, its FKBP12 is classified into the clade of fungi and animals. Although unique FKBP12-encoding genes have been identified in the organisms shown in Fig. 1B, it is currently not clear which *C. merolae* gene is orthologous to ScFKBP12. Based on comparisons of amino acid sequences, the amino acid residues of human FKBP12, which interacts with rapamycin [29], are well conserved in CMH207C, CMH076C and CMH114C (Supplementary Fig. S2). However, *C. merolae* showed rapamycin sensitivity only when ScFKBP12 was expressed in the cells. The native *C. merolae* TOR protein extracted from the cells was able to bind to ScFKBP12 in the presence of rapamycin *in vivo* and *in vitro*. These results are likely to be due to the unique properties of plant FKBP12s rather than the TOR proteins themselves. Another possibility for the insensitivity of *C. merolae* to rapamycin is the low expression levels of FKBP12-orthologous protein in the cells. In fact, Xiong and Sheen recently reported that rapamycin effectively inhibits *A. thaliana* TOR kinase activity when *A. thaliana* FKBP12 protein is

overexpressed in the cell [28]. In algae, rapamycin sensitivity seems to be dependent on the algal strain, as a unicellular green alga, *Pseudochoricystis ellipsoidea* [30], was rapamycin insensitive like *C. merolae* (Imamura et al., unpublished data). Phylogenetic analysis of the FKBP12s of *P. ellipsoidea* and other algal strains will be helpful to understand the relationship between rapamycin sensitivity and FKBP-type in algae.

Cell growth inhibition of the F12 strain was observed with 10 nM rapamycin, and became more pronounced at higher concentrations, such as 500 nM (Fig. 2B). Although 500 nM rapamycin is about five times the concentration required to inhibit *S. cerevisiae* growth, it is the same as the effective growth inhibitory concentration for *C. reinhardtii* [25], which natively shows rapamycin sensitivity, suggesting that relatively high concentrations of rapamycin are needed for effective inhibition of TOR activity in algae. Recently, several new chemicals, such as Torlin 1 and AZD8055, were identified as ATP-competitive TOR-specific inhibitors [31]. Unlike rapamycin, these inhibitors can inhibit TOR kinase activity in *A. thaliana* [26]. We also checked the effect of these inhibitors on *C. merolae* growth, but no effect was observed, implying a unique structure for the active site of CmTOR or instability of the drugs in the acidic medium.

Taking all of the data in this study into consideration, the cell growth and translation of the F12 strain are controlled by the CmTOR signaling pathway, and its kinase activity is inhibited by rapamycin as in TORC1 and mTORC1. Thus, important subjects, such as CmTOR substrates and the transcriptional and metabolic control by CmTOR, are now able to be analyzed. Further studies will provide new insight into the underlying functions of the TOR signaling pathway in *C. merolae*, which will be helpful to understand how TOR functions in photosynthetic eukaryotes in general.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.045.

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