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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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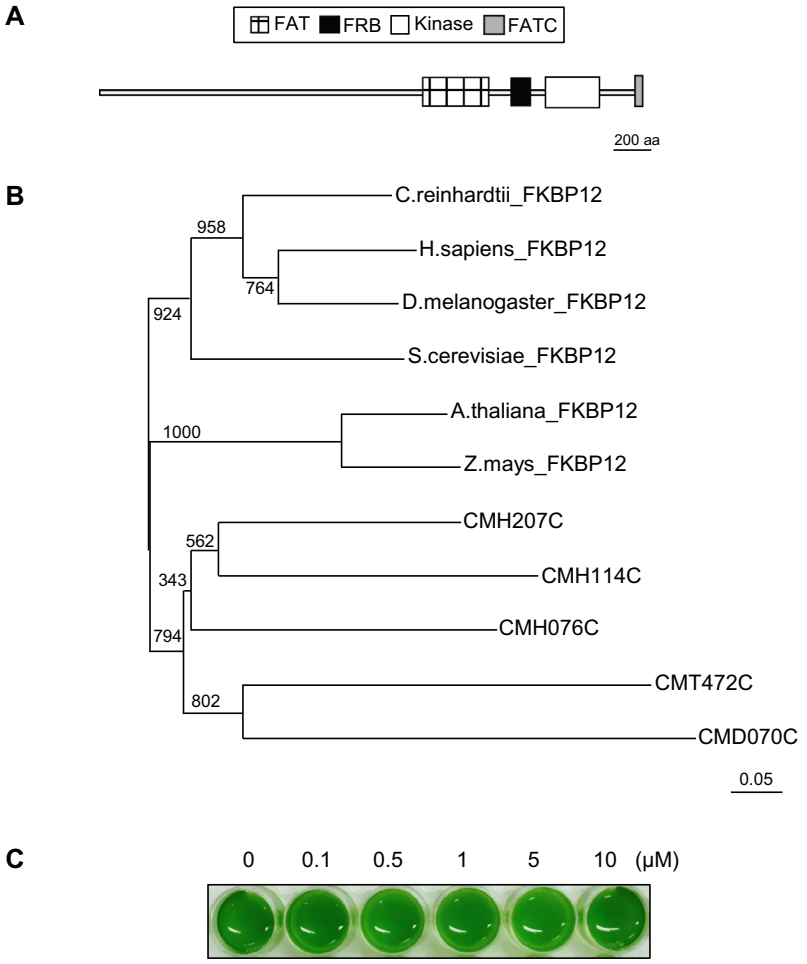

Fig. 1. Resistance of *C. merolae* to rapamycin. (A) A schematic representation of the secondary structure of CmTOR. (B) The evolutionary relationships of FKBP12 and its homologues in *C. merolae*. Bootstrap values from 1000 replicates are shown at each node. Branch lengths are proportional to the number 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.