Genetic defects in SAPK signalling, chromatin regulation, vesicle transport and CoA-related lipid metabolism are rescued by rapamycin in fission yeast

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1. Introduction

Rapamycin is an antifungal metabolite discovered in the 1970s in Streptomycin hygroscopicus. It inhibits cell proliferation and antibody formation by T cells, which prompted its use as an immunosuppressive drug for organ transplantation (known by the generic name Sirolimus) [1]. Rapamycin forms a complex with peptidyl-prolyl-isomerase, FKBP12, to inhibit serine/threonine protein kinases designated TOR (target of rapamycin) [2–5]. TOR kinases comprise two distinct protein complexes inside cells that regulate cell proliferation [6]. Various studies have shown that many TOR pathway components are associated with cancer [7–10], and as a result, rapamycin has been used as an anti-tumour drug. Clinical trials have confirmed its effectiveness against mammary tumours, colon cancer, melanocarcinoma and ependymoblastoma [11]. In addition, rapamycin also extends the lifespans of yeast, fruit flies and mice [12–15], probably by reducing calorie consumption. Recently, rapamycin became the first drug in clinical trials for the treatment of fibrodysplasia ossificans progressiva, using patient-derived induced...
pluripotent stem cells [16]. Most recently, rapamycin has also proved effective in treating Pompe disease, which causes lysosomal glycogen accumulation in skeletal muscle and heart [17].

The fission yeast Schizosaccharomyces pombe has proved to be an excellent model for studying cellular functions of rapamycin and the TOR pathway, owing to the availability of genetic methods, comprehensive mutant libraries and high conservation of mammalian TOR pathway components [18]. In our previous report, we discovered that temperature sensitivity (ts) of cut1/separase and cut2/securin mutants was rescued by rapamycin, illuminating a new aspect of TOR signalling in cell growth and division [19]. This discovery suggested that there still exist undiscovered ts mutants that could offer a more comprehensive understanding of rapamycin. Accordingly, in the hope of discovering novel applications of this drug, we tested the capability of rapamycin to rescue mutants in our ts mutant library, which contains 1014 applications of this drug, we tested the capability of rapamycin and the TOR pathway, owing to the availability of genetic methods, comprehensive mutant libraries and high conservation of mammalian TOR pathway components [18]. In our previous report, we discovered that temperature sensitivity (ts) of cut1/separase and cut2/securin mutants was rescued by rapamycin, illuminating a new aspect of TOR signalling in cell growth and division [19]. This discovery suggested that there still exist undiscovered ts mutants that could offer a more comprehensive understanding of rapamycin. Accordingly, in the hope of discovering novel applications of this drug, we tested the capability of rapamycin to rescue mutants in our ts mutant library, which contains 1014 strains [20]. Mutants defective in 12 genes were found to be rescued by rapamycin. Among them, sty1 mutants were further studied to show that rapamycin alleviated abnormal growth and division of these mutants. Also, we report a metabolome analysis to reveal the cellular impact of rapamycin. Metabolomic results strongly suggested that purine biosynthesis is implicated in the critical regulation targeted by rapamycin.

2. Results

2.1. Screening of the ts mutant library for rapamycin rescue

For library screening, a pilot spot test using a control strain (cut1-21) [19] showed that a rapamycin concentration of 0.1 µg ml⁻¹ was useful (figure 1a), because higher concentrations (e.g. greater than 1 µg ml⁻¹) inhibited growth of the parental strain of this library (leu1-32 arp8-GFP) at the restrictive temperature (36°C). Therefore, using an automated robot system, screening was conducted by spotting 1014 ts mutants [20] onto YPD plates with or without 0.1 µg ml⁻¹ rapamycin (figure 1b). At 36°C, 62 strains showed better colony formation on YPD plates with rapamycin than without (electronic supplementary material, figure S1). These strains were further tested by serial dilution and manual spotting on plates to eliminate false positives (electronic supplementary material, figure S2). In 45 strains, it was confirmed that rapamycin rescued the ts phenotypes (figure 2a). Then, the mutated genes responsible for ts sensitivity were identified by suppression analysis and by either tetrad analysis or whole-genome sequencing (electronic supplementary material, table S1, Material and methods).

2.2. Rapamycin-rescued ts phenotypes of mutations in 12 genes

Among these 45 strains, 12 genes proved responsible for the ts phenotype (electronic supplementary material, table S1). They must be sole responsible genes for each strain, because plasmids containing WT genes for other background mutations could not rescue the ts phenotype. Based on reported functions, they belonged to four categories (figure 2b): stress-activated protein kinases (SAPK) (two genes), chromatin regulation (three genes), transport (four genes) and lipid metabolism (three genes).

The largest number of strains involved either of two SAPK mutants, sty1 (MAPK; five strains) and wis1 (MAPKK; five strains) (figure 2a). These genes are central to the stress-responsive signalling pathway, and Wis1 phosphorylates Sty1 [21,22]. Mutations in all sty1 and wis1 strains with amino acid substitutions were located in or near their protein kinase domains, and all with nonsense mutations (sty1-791, wis1-887, sty1-989) lacked the C-terminal half of the kinase domains (electronic supplementary material, table S1). Given their abundance, we examined these mutants further.
In the chromatin regulation gene group, one cut1 mutant (cut1-109) was identified in addition to the previously reported cut1-21 [19]. Cut1 cleaves Rad21, which is a subunit of cohesin complex. It should be noted that 18 other cut1 mutants exist in this library, so further spot tests were performed for them. In fact, most of them revealed a more temperature-sensitive phenotype, which was also rescued by rapamycin (electronic supplementary material, figure S3A). Mutation sites were concentrated in Cut1’s peptidase and central domains (electronic supplementary material, figure S3B). Other chromatin regulation genes included mis4, a cohesion loader for establishing sister chromatid cohesion [23,24], and htb1, a histone H2B (figure 2c). Four other htb1 mutants have been identified in this library. Of those, two were mutated at the N-terminus: htb1-377 (G30D) and htb1-442 (E35 K) and showed the rescue phenotype, while htb1-72 (G52D) and htb1-223 (P102 L) did not [25]. The N-terminal end of H2B is phosphorylated under stress conditions [26,27], suggesting the basis for this allelic difference.

In the vesicle transport gene group, one clathrin-binding gene (ucp7), one exocytic Rab-type GTPase gene (ypt2) and its GTP exchange factor (sec2), and one Rab geranylgeranyl transferase-related gene (mrs6) were identified (figure 2b).

In the metabolism gene group, there were three genes related to CoA metabolism and the mevalonate pathway: erg10 (acyl-CoA C-acetyltransferase), cem1 (3-oxoacyl-[acyl-carrier-protein]-synthase condensing enzyme) and fps1 (farnesyl diphosphate synthase) (figure 2b).

2.3. Rapamycin-induced cell division at 36°C, in contrast to SAPK mutations

All SAPK ts mutants showed identical phenotypes, and we also found a deletion strain of sty1 (Δsty1) that was similar. As with ts mutants, the Δsty1 strain showed reduced viability at 36°C, which was rescued by rapamycin (figure 3d). Therefore, Δsty1 was used to study functional implications of SAPK and rapamycin, in order to eliminate potential dominant negative effects of ts mutants.

At the permissive temperature, 26°C, Δsty1 cells multiply slightly more slowly than WT, displaying longer cell lengths (figure 3a,b,c). At 26°C, addition of 200 nM rapamycin did not have a significant effect upon the rate of cell division, a negative result reported previously [28]. At 36°C, Δsty1 showed a ts phenotype with retarded mitotic increase, while WT cells divided much more rapidly (figure 3a,b). At this temperature, rapamycin rescued the ts phenotype of Δsty1, accelerating cell division approximately 1.63-fold (figure 3b). Similar effects were also observed in the ts strain, sty1-989 (electronic supplementary material, figure S4).

2.4. Rapamycin-rescued nuclear abnormality and viability loss of the SAPK mutant, Δsty1, at 36°C, even under nitrogen deprivation

Cell morphology of Δsty1 was further examined at 36°C. Six hours after the temperature shift, cell size was abnormally elongated and septated cells were frequently observed (figure 3c). Also, nuclei appeared enlarged and deformed in Δsty1. Such deformation was moderated by rapamycin. In fact, subsequent FACS analysis of Δsty1 showed that its 2C DNA peak expanded to the right at 36°C, but this expansion was alleviated when rapamycin was added (figure 3f). Thus, Sty1 and rapamycin may maintain proper nuclear and cell shapes at 36°C.

Previously, we reported that SAPK mutants showed abnormally expanded nuclei and severely decreased viability after nitrogen deprivation (−N) [29], so we tested whether rapamycin could also rescue this phenotype. After −N, WT cells undergo two divisions without cell growth, resulting in a roughly fourfold increase in cell number. G0 phase cells are small and round, and most have 1C DNA content (figure 3g,i). They complete G0 phase entry within 24 h after −N, and maintain high viability (figure 3h). However, Δsty1
2.5. Rapamycin induces stress-responsive metabolites and basic amino acid derivatives at 36°C

In order to examine effects on metabolism induced by rapamycin, we conducted quantitative metabolomic analysis of WT and Δsty1 cell cultures incubated for 6 h at 36°C with or without 200 nM rapamycin, the smallest concentration able to inhibit TOR signalling [28] (figure 4a). Since cell size varied among stains and conditions, data were normalized by protein concentration (electronic supplementary material, table S2).

The experiment was run in triplicate and reproducibility was confirmed using principal components analysis (figure 4b).

In WT, compared with DMSO (the solvent used for rapamycin, as a control), rapamycin increased nine metabolites greater than twofold (figure 4c). Those metabolites were trehalose, basic amino acids and their acetylated forms (arginine, histidine, lysine, N-acetyl-arginine, N-acetyl-lysine, 4-guanidinobutanoate, ornithine), and glyceraldehyde-3-phosphate. Trehalose, which showed the greatest increase, is well known as a stress-responsive metabolite [30]. Interestingly, it was also significantly induced in WT by rapamycin addition, but in Δsty1, the trehalose level was already high before rapamycin addition (figure 4d), implying that Sty1 might control stress response intermediates, nucleotide derivatives and CoA related metabolites at 36°C

In WT, rapamycin caused 13 metabolites to decrease to less than half (figure 4e). The majority of these were purine intermediates, nucleotide derivatives and CoA related metabolites at 36°C
biosynthesis intermediates (FGAM, FGAR, PRPP) and nucleotide-related metabolites (cytidine, mimethyl-guanosine, N-methylguanosine, CDP-choline). They accounted for more than half of the decreased metabolites, implying that nucleotide metabolism is a major target of rapamycin. Beside nucleotide derivatives, HMG-CoA and acetyl-CoA decreased by 78% and 51%, respectively (figure 4f).

In the SAPK mutant, Δsty1, rapamycin abolished overproduction of purine biosynthesis intermediates and nucleotide derivatives

Metabolomic results indicated that many metabolites were overproduced in Δsty1 cells. In those cells, 37 metabolites were found at concentrations greater than twofold the level seen in WT cells (figure 5c, DMSO; electronic supplementary material, table S2). However, with rapamycin addition, most of these overproduced metabolites returned to WT levels, and only 11 remained greater than twofold (figure 5d, Rap; electronic supplementary material, table S2). Among those adjusted to WT level by rapamycin, those that decreased more than 50% included biosynthesis intermediates (FGAM, SAICAR; figure 5c) and nucleotide derivatives (CDP, CDP-choline, CDP-ethanolamine, CTP, GDP, GDP-glucose, GTP, UTP; figure 5d). This implies that purine biosynthesis and nucleotide metabolism might be key regulatory targets of SAPK and rapamycin.

On the other hand, only three metabolites in Δsty1 decreased to less than 50% of the WT (figure 5b, DMSO; electronic supplementary material, table S2). Those were ergothioneine and methylated histidines, which could be related to stress response. When rapamycin was added, three more metabolites in Δsty1 decreased to less than 50% of the WT (figure 5b, Rap; electronic supplementary material, table S2). Again, two were purine biosynthesis intermediates (AICAR, FGAR), further suggesting that the purine pathway could be rapamycin’s physiological target.

3. Discussion

In this study, we identified mutants in which the ts phenotype was rescued by rapamycin. Although roles of TOR have been reported in diverse cellular processes from protein synthesis to autophagy [6], the 12 genes identified here were rather narrowly focused.
One possibility was suggested by our metabolome analysis, rapamycin target for restoring cell viability. DNA content. It is thus critically important to determine the D

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rapamycin screening. The chromatin regulation group comprised three genes rescued by rapamycin. Among them, Cut1 and Mis4 are for cohesin release and loading of chromatin, respectively [23,24,32,33], while Htb1 is for nucleosomal histone H2B function [25]. Previously, a possible explanation for the rescue of cut1 mutants by rapamycin was thought to be that the crucial balance between TORC1 and Cut1/separase might prevent premature sister chromatid separation, in showing that overproduction of purine biosynthesis intermediates and nucleotide derivatives in \( \Delta \text{sty1} \) was abolished by rapamycin. In excess, these metabolites in \( \Delta \text{sty1} \) cells might cause cell death. Rapamycin especially reduced the level of purine intermediates, like FGAM. In support of such a conclusion, mTORC1 was recently reported to be linked to de novo purine synthesis via the mitochondrial tetrahydrofolate cycle [31]. These are just our initial findings for complex regulation between SAPK and TOR signalling for cell growth and division. If growth and division are well balanced, continuous cell proliferation is maintained. However, if division becomes excessive, as in –N conditions, after several mitotic divisions, continuous proliferation is abandoned. Twelve genes identified in this study may be important for maintaining the appropriate balance.

The most abundant were SAPK mutants, \( \text{wis1} \) and \( \text{sty1} \), each comprising five ts alleles. Four \( \text{wis1} \) and three \( \text{sty1} \) mutants involved single amino acid substitutions. Further, all but one (\( \text{wis1}-982 \)) of these seven missense mutations occurred at conserved amino acids in the catalytic domains of Wis1 and Sty1, strongly suggesting that the loss of function for these protein kinases might be compensated by rapamycin-dependent inactivation of TOR signalling. Thus, the TOR and SAPK signalling pathways seem most likely to have opposing principal cellular functions.

The most striking aspect of suppression of the SAPK \( \Delta \text{sty1} \) mutant by rapamycin is that cell viability is restored in both +N and –N media when rapamycin is added (figure 3d,h), whereas the abnormal rod-like shape of \( \Delta \text{sty1} \) cells remains in –N media (figure 3i). Furthermore, the DNA content of \( \Delta \text{sty1} \) in –N media remains 2C (figure 3i), indicating that rapamycin cannot restore G0 phase quiescent cell shape and DNA content. It is thus critically important to determine the rapamycin target for restoring cell viability.

The mechanism of rescue required further investigation. One possibility was suggested by our metabolome analysis, showing that overproduction of purine biosynthesis intermediates and nucleotide derivatives in \( \Delta \text{sty1} \) was abolished by rapamycin. In excess, these metabolites in \( \Delta \text{sty1} \) cells might cause cell death. Rapamycin especially reduced the level of purine intermediates, like FGAM. In support of such a conclusion, mTORC1 was recently reported to be linked to de novo purine synthesis via the mitochondrial tetrahydrofolate cycle [31]. These are just our initial findings for complex regulation between SAPK and TOR, and we would like to continue more detailed metabolome analyses in future to reveal the mechanisms.

In addition to SAPK, we also identified chromatin regulation, vesicle transport and CoA metabolism genes from rapamycin screening. The chromatin regulation group comprised three genes rescued by rapamycin. Among them, Cut1 and Mis4 are for cohesin release and loading of chromatin, respectively [23,24,32,33], while Htb1 is for nucleosomal histone H2B function [25]. Previously, a possible explanation for the rescue of cut1 mutants by rapamycin was thought to be that the crucial balance between TORC1 and Cut1/separase might prevent premature sister chromatid separation, in
which low TORC1 activity alleviates the Cut1 requirement [19]. However, it may be that in these mutants, the delicate balance between cell elongation and cell division is modulated by rapamycin through its influence over TORC signalling responding to nutritional cues.

The vesicle transport group comprised four genes. Regulation of endosome maturation and endocytosis/exocytosis by TOR signalling and/or rapamycin has been reported [34–38], so some strains rescued by inhibiting the TOR pathway may involve this mechanism.

The metabolism group was related to lipid metabolism and CoA regulation, comprising three genes, Erg10, Fps1 and Cem1. Erg10 catalyses reactions between acetyl-CoA and acetoacetyl-CoA [39]. Both are substrates for the mevalonate pathway to produce geranyl diphosphates for prenylation of Rheb and Rab GTPases [40–42] (figure 2c). Lipids are required for homeostatic synthesis of new membranes during G0 quiescence. TOR signalling is reported to activate the mevalonate pathway via the sterol-responsive element-binding protein (SREBP) [43,44]. Notably, because mrs6 (Rab geranylgeranyl transferase) was also identified in addition to fps1, these metabolism genes could contribute to prenylation of vesicle transport proteins. Cem1 is a beta-keto-acyl synthase that directs the use of acetyl-CoA in fatty acid biosynthesis [45]. These metabolism genes may also work to balance synthesis and degradation of lipids.

Previously, large-scale screening of yeast deletion libraries has been conducted to test rapamycin sensitivity [46,47]. Genes affecting rapamycin sensitivity are mainly involved in upregulation of TOR signalling, as gene deletions cause hypersensitivity to rapamycin. By contrast, our screening employed a ts mutant library in which cell proliferation is disrupted at the restrictive temperature, but is rescued by rapamycin. Rapamycin actions apparently compensate for these deletions and ts mutants. Genes identified in the present study seem to be required to balance TOR signalling for continuous cell proliferation (figure 5e) and raise the possibility of therapeutic applications for rapamycin. Notably, human orthologues of Erg10, Mis4 and MrS6 are reportedly associated with beta-ketothiolase deficiency, Cornelia de Lange syndrome and intellectual disability, respectively [48–50]. In addition, Cut2/PTTG, a regulator of Cut1/ESPL1, is reportedly associated with cancer [51], while the SAPK cascade is a key therapeutic target of inflammatory disease [52,53]. Our results suggest the potential use of rapamycin to cure or ameliorate these diseases resulting from these genes.

4. Material and methods

4.1. Strains

A collection of 1014 ts mutant strains made by random mutagenesis was used [20]. To identify the mutation responsible for the phenotype, first, a plasmid set containing fragments of an S. pombe genomic library was introduced to each strain. Plasmids that rescued the ts phenotype were sequenced to identify suppressor genes. Second, if suppressor genes were definitive, tetrad analysis was conducted to identify the responsible gene. Or if several suppressor genes were identified, whole-genome sequencing was employed as follows. Mutant strains were backcrossed with WT to collect segregants showing the ts phenotype (ts−). DNA samples from several ts− segregants were extracted, mixed equally and sequenced using a Genome Analyzer IIx sequencer (Illumina). Background mutations were eliminated and mutations common to all ts− segregants, which were also identified previously among suppressor plasmids, were confirmed as the responsible genes. The other strain used in this study was h− Δsty1::ura4+ ura4-D18 (KS1366) [21] for the Δsty1 study.

4.2. Screening method and spot test assays

Rough screening of all 1014 strains was conducted using an automated robot system (Biomek FX, Beckman Coulter). Cells were cultivated in YPD liquid media and adjusted to a concentration of 1 × 10^6 cells ml⁻¹, after which 3 μl were spotted on YPD agar plates with or without 0.1 μg ml⁻¹ rapamycin (figure 1a). Plates were incubated at 36°C for 3 days, and colony formation was compared. Subsequent manual spot tests were conducted by cultivating cells to 1 × 10^7 cells ml⁻¹ and serially diluting them in five steps (10-fold dilution in each step). Then 5 μl of each dilution were spotted on new plates. Spotted plates were incubated at 36°C.

4.3. Nitrogen deprivation, cell number, viability assays and flow cytometry

Nitrogen deprivation was accomplished by switching the culture media of exponentially growing cells from EMM2 to EMM2-N (EMM2-N lacks NH₄Cl) by vacuum filtration, as described previously [54]. Cell number was measured using a Multisizer3 Coulter counter (Beckman Coulter), and cell viability was measured by plating 300 cells on YPD plates and determining the percentage of the number of colonies formed per plated cell. To measure the DNA content, FACS analysis was conducted using a FACS Calibur (Becton Dickinson).

4.4. Microscopy

For DAPI staining, cells were fixed with 2% glutaraldehyde for 10 min on ice, washed 3× with phosphate-buffered saline (PBS), and observed under a fluorescence microscope (Axioplan2, ZEISS) after mixing with DAPI (25 μg ml⁻¹).

4.5. Metabolome analysis

WT and Δsty1 samples in liquid EMM2 with DMSO or 200 nM rapamycin were incubated for 6 h at 36°C and 40 ml of 5 × 10^6 cells ml⁻¹ were harvested. Metabolome samples were prepared as described previously [55,56] and protein amount for each sample was measured using Direct Detect (Merck). Samples were spiked with two internal standards, PIPES and HEFES, corrected for protein quantity, extracted and separated by liquid chromatography on a ZIC-pHILIC column (Merck), and then measured using an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific). MZmine2 software was used for raw mass spectrum analysis [57]. From areas of detected peaks, principal components analyses were calculated and the two principal components were plotted using SIMCA P+ software (Sartorius Stedim) to show clear differentiation of the triplicates under each condition (figure 4b).

Among detected peaks, 95 metabolites were identified by comparing m/z values and retention times with authentic standards. Additionally, FGAM, FGAR and SAICAR were identified as previously explained [56].
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