Radicicol Binding to Swo1/Hsp90 and Inhibition of Growth of Specific Temperature-sensitive Cell Cycle Mutants of Fission Yeast

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A panel screening using cdc mutants of Schizosaccharomycetes pombe identified radicicol as a potent growth inhibitor of certain mutants at the permissive temperature. The strains sensitive to radicicol were cdc7, cdc11, and cdc14, all of which are defective in early septum formation. Cytokinesis but not nuclear division of these mutants was inhibited by radicicol, but that of cells with the wild-type background was not. A biologically active derivative of radicicol with a bioin moiety at the C-11 position bound Swo1, an Hsp90 homologue in S. pombe. Increased Swo1 expression partially suppressed radicicol sensitivity of cdc14 and almost completely rescued morphological abnormalities in cdc14 and cdc7 cells induced by radicicol at the permissive temperature. On the other hand, the increased Swo1 expression did not restore septum formation at the nonpermissive temperature. These results suggest that Swo1, as a molecular chaperone, plays a role in stabilizing these temperature-sensitive proteins at the permissive temperature or in activating the cytokinesis signaling cascade.

Key words: cdc mutant; cell cycle; heat-shock protein; radicicol; septum formation

Hsp90 is an essential component of the molecular chaperone, which is highly conserved from bacteria to mammals. Hsp90 suppresses the aggregation of non-native proteins3 and can also promote the refolding of substrates in cooperation with the Hsp70 chaperone system.2,2) The Hsp90 family members contain a conserved pocket that binds ATP/ADP and is important in the regulation of chaperone function. Occupancy of this pocket by several natural products such as geldanamycin has been shown to alter the Hsp90 function and result in the degradation of a subset of proteins such as Src, Raf, and mutated p53.2,3)

Radicicol (also known as monorden)3 is a 14-membered macrolide originally isolated from Monosporium bonorden as an antifungal antibiotic. Recently, radicicol as well as benzoquinone ansamycin antibiotics, such as geldanamycin and herbimycin A, was shown to interact with mammalian Hsp90.6-9) Crystal structure analysis has demonstrated that radicicol acts as a nucleotide mimic, inserting itself into the ATP/ADP-binding pocket of Hsp90.10) Treatment of mammalian cells with radicicol caused multiple phenotypic changes such as reversal of the transformed morphologies to normal ones in v-src, ras, mos,raf, fos and SV40-transformed cell lines,11-14) disruption the Ras-activated signaling pathway,15,16) inhibition of angiogenesis,17) and antitumor effects.18) Inhibition of Hsp90 by radicicol is responsible at least in part for these biological responses. Since the ATP/ADP binding pocket in Hsp90 is highly conserved across the species and 82% of the amino acid residues lining the interior of the pocket are invariant between E. coli and human types, it was expected that radicicol would also to bind to yeast Hsp90. However, molecular details to confirm the involvement of Hsp90 in yeast cell cycle control and effects of radicicol on yeast cell growth were not clear.

During our screening for agents that block the fission yeast cell cycle, using Schizosaccharomycetes pombe cdc (cell division cycle) mutants containing temperature-sensitive cell cycle regulators,19) we found that radicicol specifically inhibited the growth of cells having a cdc14, cdc11, or cdc7 mutation. In this study, we show that radicicol directly binds Swo1, an Hsp90 homologue in fission yeast,20) and an

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increase in Swol expression suppresses the radicicol-induced morphological changes of these mutants. Our data suggest that Swol/Hsp90 serves as a molecular chaperone for maintaining the active protein conformation of certain heat-sensitive cell cycle regulators at the permissive temperature in fission yeast.

**Experimental Procedures**

**Materials.** A radicicol derivative (BR-6) biotinylated at the C-11 position was obtained as described.9

**Yeast strains and media.** The S. pombe strains used were 972 (h-), JY266 (h+ leu1-32), JY746 (h+ leu1-32 ura4-D18 ade6-M210), JY741 (h- leu1-32 ura4-D18 ade6-M216), RA1278 (h- leu1-32 ura4-D18 swol::swol1HA(ura4+)), cdc7 (h- leu1-32 cdc7-24), cdc11 (h- cdc11-119), and cdc14 (h- leu1-32 cdc14-118). Strain RA1278 containing a single integrated copy of swol-HA was kindly provided by Dr. Paul Russell, the Scripps Research Institute. All the cdc mutant strains used in this study were kindly provided by Dr. C. Shimoda, Osaka City University. YES and MM media were used for growing S. pombe cells.20

**Screening.** Microorganisms were isolated on agar plates from samples of soil, mud, and plants. PDA medium (Nissui, Japan) was used for isolation of fungi. The HV agar used for isolation of actinomyces contained 0.1% humic acid, 0.05% Na2HPO4, 0.17% KCl, 0.005% MgSO4·7H2O, 0.001% FeSO4·7H2O, 0.002% CaCO3, B vitamins (0.5 μg/ml each of thiamine-HCl, riboflavin, niacin, pyridoxine-HCl, inositol, Ca-pantothenate, p-aminobenzoic acid, and 0.5 μg/ml biotin), and 50 μg/ml cycloheximide, pH 7.2. Microbial strains isolated were inoculated into a 21-mm test tube containing 10 ml of Q medium (2% glycerol, 1% molasses, 0.5% casein, 0.1% Bactopeptone, and 0.4% CaCO3) and cultivated at 26.5°C for 5 days. An equal volume of acetone was added to the culture broth and the mixture was used for the following assay. The S. pombe strains were maintained on YES medium and were transferred serially at 3-day intervals using the standard microbiological techniques. For the assay, portions of overnight culture of the cdc mutants in liquid YES were seeded into YES agar plates at a final concentration of 0.1% v/v. A sample was put onto an 8-mm filter paper disc (20 μl/disc) and air-dried at room temperature. The discs were put onto the assay plates, and the plates were then incubated at 26.5°C for 2 days. The diameter of the inhibitory zone was measured and recorded.

**Plasmid construction.** The swol open reading frame and its promoter region were amplified by PCR from an S. pombe genomic DNA, using the primers 5’-CGCGGATCCCAACCCAAACCTC-TATGC-3’ (the underline indicates a BamHI site) and 5’-AAACTGCAGTTAGCTTTTGATT-TAATCGAC-3’ (the underline indicates a PstI site). The amplified 2.5-kb DNA fragment was cleaved with BamHI and PstI and cloned into a pAL19 vector (pALswol).

**Binding of Radicicol to Swol.** RA1278 cells were grown in selective medium to the early log phase (OD550 = 0.5–0.6), collected by centrifugation and washed with ice-cold H2O. Cell pellets were resuspended in binding buffer consisting of 10 mM Tris-HCl (pH 7.6), 50 mM KCl, 5 mM MgCl2, 1 mM EDTA, and 0.1 mM Na2VO4. Cells were physically disrupted with glass beads for 5 min at 4°C, and lysates were centrifuged at 14,000 × g for 15 min. For detection of radicicol-bound Swol, the cleared supernatants were incubated with biotinylated radicicol derivatives in the presence or absence of radicicol as a competitor. After incubation for 60 min at 4°C, proteins associated with the biotinylated radicicol derivatives were precipitated with streptavidin agarose, and detected by immunoblotting using an anti-HA antibody (Santa Cruz, Y-11).

**Microscopy.** Cells were observed under a light microscope (Olympus BHS-323N Nomarski differential interference microscopy) or a fluorescent microscope (Zeiss Axiosvert 135). Cells were washed with several times with PEM buffer (100 mM PIPES pH 6.9, 1 mM EGTA, and 1 mM MgSO4) and resuspended in PEM containing 0.5 mg/ml calcofluor (fluorescent brightener 28, Sigma) for 30 min at room temperature. After the cells had been washed three times with PEM, they were mounted with Vectashield mounting medium (Vector, Burlingame, CA) containing 0.05 mg/ml DAPI and then observed under a Zeiss Axiosvert 135 microscope.

**Results**

**Screening and isolation of radicicol**

During our screening program for isolation of microbial metabolites that interfere with the growth of S. pombe cdc mutants, we found an activity in the broth of a fungal strain 2348, which yielded inhibitory zones larger than 20 mm in diameter on cdc7, cdc11, and cdc14 strains but not on other mutants or the wild-type strain (Fig. 1). These three mutants are defective in early septum formation and their terminal phenotypes at the nonpermissive temperature are highly elongated cells with multiple nuclei (see Fig. 4 (B)). The cdc7 and cdc14 cells isolated from the inhibitory area on the agar plate were elongated and multinuclear. For purification of the active compound, mycelium of strain 2348 was harvested by
centrifugation of the 500 ml of culture broth. The active substance was extracted with 500 ml ethylacetate and concentrated by evaporation. The residual materials were fractionated with a silica gel column with a solvent system of chloroform-methanol (98:2). The active fractions eluted were combined and evaporated in vacuo, and the active compound was purified by HPLC with an Aquasil column (Senshu) using a solvent system of chloroform-formic acid (100:0.5). The UV absorption spectrum of the active compound was very similar to that of radicicol (Fig. 2(A)). We therefore compared the HPLC retention times of the active compound and authentic radicicol using an ODS column (Capcell Pack C18) with a solvent system of CH$_3$CN–H$_2$O–trifluoroacetic acid (40:60:0.05). The compounds eluted at the same retention time (data not shown). Furthermore, FAB mass spectrometry had the ion peak (M + H)$^+$ at m/z 365, showing the identity of the compound to radicicol.

Selective growth inhibition of *S. pombe* cdc mutants by radicicol

Minimal inhibitory concentrations (MICs) of radicicol against several *S. pombe* cdc mutants were measured by the conventional agar dilution method. As shown in Table 1, the MICs of radicicol for cdc7, cdc11, and cdc14 were 1.25 μg/ml, 5 μg/ml, and 2.5 μg/ml, respectively. Since wild-type *S. pombe* grew even in the presence of 50 μg/ml radicicol, these mutants were ten-times more sensitive to radicicol than wild-type cells. To find whether the super-sensitivity to radicicol of these mutants is due to the respective cdc gene mutations, we analyzed the phenotypes of their tetrads crossed with the wild-type strain. All the ts segregants were super-sensitive to radicicol (data not shown). These results showed that

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**Table 1.** Minimal Concentrations of Radicicol to Inhibit Growth of cdc Mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (μg/ml)</th>
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<tbody>
<tr>
<td>972 (wt)</td>
<td>&gt;50</td>
</tr>
<tr>
<td>cdc7</td>
<td>1.25</td>
</tr>
<tr>
<td>cdc11</td>
<td>5</td>
</tr>
<tr>
<td>cdc14</td>
<td>2.5</td>
</tr>
</tbody>
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**Fig. 2.** Detection of Swol as a Radicicol-binding Protein.

(A) Chemical structures of radicicol and its biotinylated derivative BR-6. (B) Specific binding of BR-6 to Swol-HA. The cell lysate of strain RA1278 expressing Swol-HA was incubated with BR-6 in the presence or absence of radicicol as a competitor, and the bound proteins were analyzed by immunoblotting with an anti-HA antibody. Lane 1, control without BR6; lane 2, BR-6; lane 3, BR-6 plus radicicol; and lane 4, 1/10 input.
Fig. 3. Effects of Swo1 Expression on Radicicol-induced Growth Inhibition.

The cdc7 or cdc14 cells harboring the empty vector pAL19 or the expression vector pALswol were streaked on agar minimal medium plates containing various concentrations of radicicol, and cultured for 2 days at 26.5°C. The growth inhibition of cdc14 cells by 5 and 10 µg/ml radicicol was apparently suppressed by the Swo1 expression.

the cdc mutations themselves were responsible for the radicicol sensitivity.

Radicicol binding to Swo1

In S. pombe, Swo1 is the only Hsp90 homologue so far identified that is essential for cell growth. Swo1 is highly homologous to the Hsp90 family, showing 60% identity to human Hsp90. To test the possibility of radicicol binding to Swo1, we used BR-6, a biologically active radicicol derivative conjugated with a biotin moiety (Fig. 2(A)). A total cell lysate (100 µg of total soluble protein) from strain RA1278 containing a single integrated copy of swo1-HA was incubated with 1 µg/ml of BR-6 for 1 h and proteins bound to BR-6 were precipitated with streptavidin agarose beads. As a competition experiment, 10 µg/ml radicicol was incubated with the lysate for 1 h at 4°C. Immunoblot analysis using an anti-HA antibody showed that the cell lysate contained HA-tagged Swo1 and small amounts of proteins with faster migration, probably due to the degradation of Swo1-HA. When BR-6 was added to the lysate, Swo1-HA was precipitated with streptavidin agarose beads and this binding was blocked by an excess of radicicol (Fig. 2(B)). These results indicate that radicicol interacts effectively with Swo1, as it does with mammalian Hsp90.

Suppression of radicicol sensitivity of cdc mutants by Swo1 expression

To test whether the interaction of radicicol with Swo1 is concerned with the super-sensitivity to radicicol of the cdc7 and cdc14 strains, we examined the effects of Swo1 overexpression on the radicicol-induced abnormalities in the mutants. Because Swo1 overexpression itself by the strong nmt1 promoter resulted in severe growth inhibition of the strains, we increased the copy number of the swo1 gene using a multicopy vector pAL19,22 in which the Swo1 transcription was directed by its own promoter. As shown in Fig. 3, Swo1 expression suppressed the cell growth inhibition in the cdc14 mutant by 5 µg/ml radicicol. The growth inhibition of the cdc7 strain by radicicol was not suppressed. Surprisingly, however, cell elongation and multinucleation of both the mutant strains caused by radicicol at the permissive temperature were almost completely suppressed (Fig. 4). They formed a single division septum in the presence of radicicol. On the other hand, Swo1 expression could not suppress the growth inhibition or
elongated phenotype induced at the nonpermissive temperature. These results imply that Swo1 is required for the temperature-sensitive Cdc7 and Cdc14 to be active at the permissive temperature.

Discussion

Although Hsp90 is one of the most abundant and ubiquitous molecular chaperones, it appears to have substrate-specific folding activity, unlike the more general Hsp70 and Hsp60 chaperones. The known client proteins for mammalian Hsp90 include protein kinases such as Src, Raf, Akt, and Plk, and transcription factors such as steroid hormone receptors and hypoxia-inducible factor 1α. In fission yeast, Swo1/Hsp90 is essential for viability, but little is known of its substrates. We showed here that radicicol specifically bound Swo1 and inhibited cell growth of several temperature-sensitive cdc mutants of *S. pombe*. Cdc7, Cdc11, and Cdc14 are reported to be required for initiating the synthesis of a division septum. At the nonpermissive temperature, these mutants do not form the septum, but growth, DNA replication, and mitosis continue in the absence of cytokinesis, resulting in the formation of elongated multinucleate cells. The terminal phenotypes of these mutant cells that had been treated with radicicol were almost completely identical with those at the nonpermissive temperature. These results suggest that the mutated Cdc7, Cdc11, and Cdc14 proteins are the substrates for Swo1/Hsp90, and that Swo1 is required for the activity and stability of these proteins at the permissive temperature. Increased Swo1 expression suppressed the radicicol-induced changes in the mutant cell morphology but it failed to rescue those at the nonpermissive temperature, suggesting that Swo1 cannot refold the heat-denatured structures of the mutant proteins. Alternatively, an unknown Swo1 substrate may act as an upstream regulator for these proteins, which mediates Swo1-induced suppression of the radicicol effect by activating the cytokinesis signaling cascade at the permissive temperature. Ppb1, a homologue of calcineurin catalytic subunit involved in cytokinesis and cell polarity in fission yeast, may be one of such the candidates, since calcineurin in budding yeast has recently been shown to be stabilized by Hsp90.

The *swo1* gene was originally identified as a suppressor of *weel* overproduction. *Weel* is a protein tyrosine kinase that negatively regulates Cdc2 serine/threonine kinase, a major cell cycle regulator essential for the onset of mitosis, by phosphorylating tyrosine-15 in Cdc2. *Weel* and Mik1, another kinase regulating Cdc2 activity, were the known substrates for Swo1. Swo1 has been shown to be required for the activity and stability of *weel* and Mik1. Consistent with this, it is reported that one of the Swo1 temperature-sensitive mutants grew well but had a small cell size (weel) phenotype, indicating that partial loss of Swo1 activity advances the onset of mitosis by *weel* inactivation. Inactivation of Swo1 by radicicol might cause rapid degradation and inactivation of *weel*. Indeed, a high concentration of radicicol (>50 μg/ml) induced a weel phenotype in wild type *S. pombe* (data not shown). The observation suggests that a high concentration of radicicol is needed for the complete inhibition of Swo1 function. It is therefore possible that the partial loss of Swo1 activity by a lower dosage of radicicol is sufficient for inactivation of mutated Cdc7, Cdc11, and Cdc14.

It is currently unclear why the increased expression of Swo1 by the multicopy vector could not completely rescue the radicicol-induced growth inhibition of *cdc14* and *cdc7* mutants. During these experiments, a high level expression of Swo1 by the *nmt1* promoter caused growth inhibition with slightly elongated morphology in not only the *cdc* mutants but also wild-type *S. pombe*. This growth inhibition was also observed even in the *weel* disruptant (unpublished results), indicating that *weel* is not involved in the Swo1-induced growth arrest. It seems likely that the toxic effect of Swo1 inevitably associated with its increased expression interferes with the complete recovery from the radicicol-induced arrest in the *cdc* mutants. It is also possible that radicicol targets not only Swo1 but also inhibits the function of the *cdc* gene products directly.

In this study, we showed that temperature-sensitive mutants other than *cdc7*, *cdc11*, and *cdc14* were not sensitive to radicicol, suggesting that Swo1 regulates only a limited number of mutated proteins. Interestingly, all these three gene products are involved in the signaling cascade for early septum formation. It is therefore possible that Swo1 has a role in septum formation also in the wild-type background, which should be clarified in the future.

Acknowledgments

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Selective Growth Inhibition of cdc Mutants by Radicicol 2533


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