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The Dcr2p phosphatase destabilizes Sic1p in *Saccharomyces cerevisiae*

Ritu Pathak, Heidi M. Blank, Jinbai Guo, Sarah Ellis, and Michael Polymenis*

Department of Biochemistry and Biophysics, Texas A&M University, 2128 TAMU, College Station, TX 77843, USA

Abstract

Initiation of cell division is controlled by an irreversible switch. In *Saccharomyces cerevisiae* degradation of the Sic1p protein, an inhibitor of mitotic cyclin/cyclin-dependent kinase complexes, takes place before initiation of DNA replication, at a point called START. Sic1p is phosphorylated by multiple kinases, which can differentially affect the stability of Sic1p. How phosphorylations that stabilize Sic1p are reversed is unknown. Here we show that the Dcr2p phosphatase functionally and physically interacts with Sic1p. Over-expression of Dcr2p destabilizes Sic1p and leads to phenotypes associated with destabilized Sic1p, such as genome instability. Our results identify a novel factor that affects the stability of Sic1p, possibly contributing to mechanisms that trigger initiation of cell division.

Keywords

DCR2; SIC1; START; cell cycle

INTRODUCTION

It is important to understand what determines when cells initiate division because this process determines overall rates of cell proliferation: once cells initiate their division, they are usually committed to complete it. In *Saccharomyces cerevisiae* the decision to initiate division is made in late G1 phase at a point called START [1]. The Sic1p protein is an inhibitor of B-type cyclin (Clb)/cyclin-dependent kinase (Cdk) complexes that catalyze mitotic cell cycle transitions [2]. Phosphorylation of Sic1p by G1 cyclin (Cln)/Cdk complexes destabilizes Sic1p, and destruction of Sic1p allows Clb5,6/Cdk complexes to initiate DNA replication on schedule [3-5]. Phosphorylation of Sic1p was thought to be the only essential function of Cln/Cdk complexes, because the lethality of cells lacking all Clns is rescued by deletion of *SIC1* [6,7]. However, it is now clear that phosphorylation of Sic1p by Cdk is not essential, because of the full viability of cells carrying a mutant Sic1p protein that lacks all Cdk phosphorylation sites [8].

In addition to being a Cdk target, Sic1p is also targeted at multiple sites by several other kinases, including Ime2p [9], casein kinase [10], and the Stress-Activated Protein Kinase (SAPK) Hog1p [11]. How all these phosphorylations affect the stability of Sic1p is not fully understood. Upon SAPK activation [11], or nutrient limitation mimicked by inhibition of the Target of

*To whom correspondence should be addressed. Tel: 1-979-458-3259; FAX: 1-979-845-4946, e-mail: polymenis@tamu.edu

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Rapamycin (TOR) pathway [12], Sic1p is stabilized. Interestingly, the stabilization of Sic1p under these circumstances is not overridden by the Cdk phosphorylations of Sic1p in cells that ectopically express Cln2p [11,12]. Similar pathways may delay cell cycle progression in fission yeast [13,14] and animals [15]. How the stabilizing phosphorylations of Sic1p are reversed is not clear.

We originally identified *DCR2* in a screen for genes that promote cell cycle progression when over-expressed [16,17]. Here we present evidence that Dcr2p destabilizes Sic1p.

MATERIALS AND METHODS

Strains and DNAs

Cell cultivation, media and yeast molecular biology techniques were performed as described by Kaiser et al [18]. The strains used in this study are listed in Table 1. The phenotypes reported for each strain were obtained after examining several independent transformants for the strain in question. The *clnΔ*, *P_{GAL}-CLN3* strain used in Fig. 2C, and the strain used in Fig. 1 to evaluate chromosome loss, were gifts from Mary Miller [19], and Phil Hieter [20], respectively (see Table 1).

All the plasmids used to over-express *DCR2* were high-copy YE_p-based plasmids. The YE_p-*DCR2* (used in Fig. 2C) and the *P_{GAL}-DCR2^{DN}* (used in Fig. 3) plasmids have been described previously [17,21]. To generate the *P_{ADHI}-DCR2* plasmid used in Fig. 2, the *DCR2* ORF was PCR amplified using forward, and reverse, primers carrying sequences at their 5' ends corresponding to the *ADHI* promoter, and terminator, sequences of plasmid p2.5 [22], respectively. The PCR product was then purified after agarose gel electrophoresis and co-transformed into yeast cells together with plasmid p2.5, which was previously linearized with EcoRI and SpeI, which cut between the *ADHI* promoter and terminator sequences of the p2.5 plasmid [22]. The gap repaired plasmid derivative was then recovered from yeast transformants by standard methods [18].

The *P_{GAL}-DCR2* plasmid used in Fig. 1 was from a recently available ORF collection [23], except that we changed the marker from *URA3⁺* to *LEU2⁺* by standard gap-repair based methods [18]. For recombinant expression, the pBAD-*DCR2* plasmid has been described previously [21,24].

Sic1p half-life measurements

The *P_{ADHI}*-based plasmids were transformed into cells carrying a *P_{GAL}-SIC1-TAG* allele (strain YSC3869-9515050), purchased from Open Biosystems (Huntsville, AL). The cells were arrested in G1 phase, and then *SIC1* expression was induced by addition of 2% galactose for 2 h. After a shift to repressive glucose-containing medium, samples were taken every 15 min, and processed for immunoblotting analysis. The intensity of the bands corresponding to each time point, normalized for loading, was then calculated using ImageJ software, from the National Institutes of Health.

Other Techniques

For protein extract preparation from yeast we followed procedures published previously [24]. For immunoprecipitation of GST fusions we used glutathione beads from Pierce (Rockford, IL), according to the manufacturer's instructions. The PAP reagent for detection of the protein-A epitope (present in "TAP" and "TAG" fusions) was from Sigma (used at 1:1000 dilution). The anti GST antibody was from Pierce (Rockford, IL) and used at 1:1000 dilution. For the experiment shown in Fig. 4B, Sic1p from yeast cells and recombinant Dcr2p from bacteria were purified through their 6xHis epitope tag, using TALON Co²⁺ beads from BD Biosciences

(San Jose, CA), according to their instructions. The phosphatase inhibitor cocktails were from Sigma (St. Louis, MO). For phosphoprotein detection we used non-radioactive reagents from Pierce (Rockford, IL) according to their instructions.

RESULTS AND DISCUSSION

Over-expression of *DCR2* leads to chromosome loss

In our attempts to better characterize the cellular roles of Dcr2p, we measured chromosome loss in cells over-expressing *DCR2*, using a standard colony-sectoring assay [20]. Loss of the marker chromosome produces Ade⁻ cells, which form red sectors [20]. Over-expression of *DCR2* elevated substantially the rate of chromosome loss, to $\geq 50\%$ (Fig. 1).

Over-expression of *DCR2* destabilizes Sic1p

Among various regulators of START, loss of the Cdk inhibitor Sic1p leads to genome instability [2]. Because of this correlation we decided to examine if Dcr2p over-expression affects the stability of Sic1p in vivo. We synchronized cells in early G1 phase, and expression of a TAG-tagged *SIC1* allele was induced from a galactose inducible promoter [23]. Synthesis of Sic1p-TAG was then repressed (in glucose containing media), and the cells were released from G1 arrest. Levels of Sic1p-TAG were then evaluated by immunoblotting at different time points after the release from the arrest. We found that the half-life of Sic1p-TAG was significantly reduced in cells with elevated levels of Dcr2p (Fig. 2).

Cells cannot complete START if they lack all three Cln proteins [25], unless these cells also lack Sic1p [6,7]. To genetically test whether Dcr2p destabilizes Sic1p, we asked if over-expression of *DCR2* allows cells lacking all three G1 Cln cyclins to initiate cell division. For this experiment *cln1,2,3Δ* cells were kept alive in galactose-containing media by a *P_{GAL}-CLN3* copy [19]. We then introduced in these cells high copy number plasmids carrying either *DCR2* (YE_p-*DCR2*), or no insert (YE_p-vector), and monitored their viability after expression of *P_{GAL}-CLN3* was turned off, in glucose containing media. For this experiment, exponentially growing cultures (in galactose-containing media) once they reached a cell density of $\sim 5 \times 10^6$ cells/ml were transferred to glucose-containing media, and the cells were counted at regular intervals over several hours. The number of divisions completed after *P_{GAL}-CLN3* was turned off is shown in Fig. 2C. Within 1 or 2 divisions the cells were arrested. Possibly the failure of YE_p-vector cells to arrest within the first cell cycle in some experiments was due to leaky *CLN3* expression. The experiment was done nine times, each time comparing the [YE_p-vector] vs. [YE_p-*DCR2*] transformants. From these pairwise comparisons, it is clear that over-expression of *DCR2* allowed a significant fraction ($\sim 35\%$, $P = 0.0036$ based on a Student's t-test) of *cln1,2,3Δ* cells to undergo one extra round of cell division (Fig. 2C).

Dcr2p and the response to high salt

We examined the role of Dcr2p in the response to high salt, because upon exposure to high salt Sic1p is phosphorylated and stabilized [11]. We had previously shown that loss of *DCR2* by itself does not lead to salt sensitivity [17], unless the system is further sensitized and other genes with roles in G1/S are also deleted (*GID8*, *CLN3*, or *SWI4*). It is possible that in the absence of *DCR2*, other phosphatases with redundant roles target substrates of Dcr2p. Consequently, we decided to reexamine Dcr2p's role in the response to salt using a dominant-negative *DCR2* allele. We had previously introduced an H338A substitution in the phosphatase domain of Dcr2p [17], which in other phosphatases affects catalysis but not substrate binding [26,27]. The Dcr2p-H338A mutant (designated here as Dcr2p^{DN}) should sequester substrates targeted by Dcr2p and possibly other phosphatases. We found that over-expression of this *DCR2^{DN}* allele leads to salt sensitivity (Fig. 3).

Dcr2p might target Sic1p

Interactions between phosphatases and their substrates are usually transient and difficult to detect, but we reasoned that Dcr2p^{DN} might stably bind its substrate(s). We used cells carrying a chromosomal copy of *SIC1* fused to the TAP epitope modules (calmodulin binding protein and Protein A [28]). We then expressed *DCR2^{DN}* in these *SIC1*-TAP cells as an N-terminal GST fusion under the control of a galactose-inducible promoter (from plasmid P_{GAL}-GST-*DCR2^{DN}*). Cells were grown in raffinose (Raf), and upon galactose (Gal) addition a protein of the expected size of a GST-Dcr2p^{DN} fusion was detected after precipitation with glutathione beads and immunoblotting with an anti-GST antibody (Fig. 4A). Sic1p-TAP was co-precipitated from cells transformed with P_{GAL}-GST-*DCR2^{DN}*, but not from cells transformed with a similar plasmid (P_{GAL}-GST) that generates a truncated Dcr2p^{DN} (Fig. 4A).

We then examined if recombinant Dcr2p from bacteria can de-phosphorylate Sic1p. For this experiment, we isolated Sic1p from pheromone-arrested G1 cells after they were exposed to high salt, since under these conditions Sic1p is phosphorylated and stabilized [11]. In the presence of recombinant Dcr2p, Sic1p was de-phosphorylated, but not when phosphatase activity was blocked, or when we used the catalytically inactive recombinant Dcr2p^{DN} mutant (Fig. 4B). Together, our results are consistent with the possibility that Dcr2p targets Sic1p.

Although several kinases are known to target Sic1p, the only phosphatase previously associated with Sic1p is Cdc14p [29]. Cdc14p certainly counteracts the Cdk-dependent phosphorylation of Sic1p [29]. It appears that Cdc14p and Dcr2p might have opposing roles, since Cdc14p over-expression strongly stabilizes Sic1p [29], in contrast to Dcr2p over-expression that destabilizes Sic1p (Fig. 2), possibly reflecting the notion that distinct phosphorylation events differentially affect the stability of Sic1p. Dcr2p has additional roles, independent of Sic1p, such as in the unfolded protein response [21]. In vitro, Dcr2p can de-phosphorylate Sic1p (Fig. 4), but this is not surprising since phosphatases are typically active against numerous substrates in vitro. Although we obtained evidence that Dcr2p could interact with Sic1p in vivo, we relied on over-expression of the dominant-negative Dcr2p^{DN} (Fig. 4). Thus, the role of Dcr2p in destabilizing Sic1p could be direct or indirect.

The Cdk-dependent phosphorylations and subsequent destruction of Sic1p have now been shown not to be essential, and they only contribute to the robustness of START [8]. Sic1p is also targeted by other kinases, and in high salt T173 phosphorylation by the Hog1p kinase has been shown to stabilize Sic1p [11]. However, whether this observation extends to other conditions is not clear, because the corresponding non-phosphorylatable Sic1p-T173A mutant is not destabilized under conditions that do not involve high salt ([4,12]; and H. Blank and M. Polymenis, unpublished observations). Thus, it is possible that additional phosphorylations that stabilize Sic1p exist. It is only after these phosphorylations are identified that one can fully examine how de-phosphorylation of Sic1p by Dcr2p (directly or indirectly) contributes to START. It is also likely that Dcr2p affects START by additional mechanisms, independently of Sic1p. That's because while loss of Sic1p accelerates DNA replication, it does not accelerate budding to the same extent [30]. In contrast, *DCR2* over-expression accelerates both budding and DNA replication [17]. Nonetheless, based on the results we presented in this report, it is reasonable to propose that destabilization of Sic1p by Dcr2p might contribute to the acceleration of START by Dcr2p.

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Figure 1. *DCR2* over-expression leads to chromosome loss

Sectoring assay for chromosome loss, with cells carrying the indicated plasmids. Formation of red sectors indicates chromosome loss.

Figure 2. DCR2 over-expression destabilizes Sic1p

A, The levels of a *GAL*-induced Sic1p-Protein A fusion (Sic1p-TAG) were monitored by immunoblotting, from cells carrying the indicated plasmids after *SIC1* synthesis was turned-off. The Ponceau-stained blots indicate loading. **B**, The relative half-life was determined from (A) from the band intensities. **C**, Over-expression of *DCR2* allows a fraction of *cln* cells to complete one extra cell division. *cln1,2Δ, GAL-CLN3* cells carrying the indicated plasmids were grown in galactose-containing medium, but they were then shifted to glucose-containing medium to turn *CLN3* expression off. From nine independent experiments, the number of divisions the YEp-vector transformants were able to complete is shown on the x-axis, and the corresponding divisions from the paired YEp-DCR2 transformants is shown on the y-axis. The

diagonal is shown to indicate where the data points should have fallen if there was no difference between the YEp-vector and YEp-DCR2 samples.

Figure 3. Over-expression of *DCR2*^{DN} leads to salt sensitivity

Growth of the indicated strains (all in the BY4741 background) was evaluated by spotting 10-fold serial dilutions of the cultures on solid media, as indicated. The plates were incubated at 30°C and photographed after 4 days.

Figure 4. Dcr2p targets Sic1p

A, *SIC1-TAP* cells carrying the indicated plasmids were grown as described in the text, and samples were subjected to SDS-PAGE either directly (bottom panel) or after the *GAL*-induced gene products fused to GST were precipitated with glutathione beads (top panels). For immunoblotting (IB) we used the indicated antibodies. **B**, Sic1p was precipitated with Co²⁺ beads from P_{GAL}-*SIC1-TAG* cells, which were arrested in G1 with α -factor and treated with high salt to promote Sic1p phosphorylation. Sic1p was then incubated with recombinant Dcr2p, Dcr2p^{DN}, or λ -PPase, in the presence (+) or absence (-) of phosphatase inhibitor cocktails, as indicated. The reactions were analyzed by SDS-PAGE and blotting, followed by phosphoprotein-specific detection (top), or immunoblotting with the PAP reagent (bottom).

TABLE 1

Strains used in this study

Strain	Relevant genotype	Source
BY4741	MATa <i>his3Δ leu2Δ met15Δ ura3Δ</i>	Res. Genetics
YSC3869-9515050	[P _{GAL} - <i>SIC1</i> -TAG': :URA3] (BY4741 otherwise)	Open Biosystems
YSC1178-7501699	<i>SIC1</i> -TAP': :his3MX (BY4741 otherwise)	Open Biosystems
1607-2D	MATa <i>cln1-3Δ LEU2</i> : :P _{GAL} -CLN3 <i>ade2 trp1 his3 ura3 can1</i>	Fred Cross Laboratory
YPH363	MATa <i>lys2-801 ade2-101 his3Δ200 leu2-Δ1 CFIII</i> (CEN3.L)	Philip Hieter Laboratory
RPY1	<i>dcr2Δ</i> : :his3MX (BY4741 otherwise)	[17]