Hydroxyurea Sensitivity Reveals a Role for ISC1 in the Regulation of G2/M*

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Saccharomyces cerevisiae cells lacking ISC1 (inositol phosphophingolipase C) exhibit sensitivity to genotoxic agents such as methyl methanesulfonate and hydroxyurea (HU). Cell cycle analysis by flow cytometry revealed a G2/M block in isc1Δ cells when treated with methyl methanesulfonate or HU. Further investigation revealed that the levels of Cdc28 phosphorylated on Tyr-19, which plays an essential role in the regulation of the G2/M checkpoint, were higher in synchronized and asynchronous cells lacking ISC1 in response to HU. Use of a Cdc28-Y19F mutant protected isc1Δ from the G2/M block. In wild type cells, HU induced a loss of the Swe1p kinase, the enzyme that phosphorylates Cdc28-Tyr-19, correlating with resumption of the cell cycle. In the isc1Δ cells, however, the levels of Swe1p remained at sustained high levels in response to HU. Significantly, deletion of SWE1 in an isc1Δ background overcame the G2/M block in response to HU. The double isc1Δ/swe1Δ mutant also overcame the growth defect on HU. Taken together, these findings implicate Isc1p as an upstream regulator of Swe1p levels and stability and Cdc28-Tyr-19 phosphorylation, in effect signaling recovery from the effects of genotoxic stress and allowing G2/M progression.

In Saccharomyces cerevisiae, ISC1 (inositol phosphophingolipase C) is the homologue of the mammalian neutral sphingomyelinase (nSMase 2). These enzymes catalyze the degradation of complex sphingo- and dihydroceramides to produce phytoceramide/dihydroceramide in yeast and ceramide/dihydroceramide in mammals (1). Ceramide is a known bioactive lipid and has been shown to play an important role in cell processes such as growth, death, senescence, and response to stress (2, 3). ISC1-deficient budding yeast exhibits a slower growth rate than wild type (WT)2 strains (4) and higher sensitivity to high salt (NaCl) and hydrogen peroxide (5, 6). A genome-wide screen revealed that ISC1 deletion (isc1Δ) mutants were sensitive to the DNA alkylating agent methyl methanesulfonate (MMS) and to the DNA replication blocking drug hydroxyurea (HU) (7). However, the role of ISC1 in protecting cells from MMS or HU and its mechanism have not been investigated.

MMS is a carcinogen that methylates DNA on N7- and N3-deoxyguanine (8–10). HU is a ribonucleotide reductase inhibitor that blocks the synthesis of DNA. Yeast cells treated with HU activate the S phase checkpoint, and cells arrest in early S phase (11, 12). After a few hours in the presence of HU, WT cells resume cell division, overcoming the S phase block.

In this study, we investigate the mechanism and role of ISC1 in the response to HU. Cell cycle analysis using flow cytometry showed unexpectedly that deletion of ISC1 resulted in an additional and subsequent block in the G2/M phase in the presence of MMS or HU. The G2 phase progression into M phase is regulated by the cyclin-dependent kinase Cdc28p (Cdk2 in mammals). Cdc28p is an essential regulator of the cell cycle in the yeast S. cerevisiae (13, 14), and its activity is dependent on the binding of a cyclin subunit. During the different phases of cell cycle progression, Cdc28p is coupled with different cyclins. There are two classes of Cdc28 cyclins, G1 and B type cyclins: three G1 cyclins (cln1 to cln3) and six B-type cyclins (clb1 to clb6). The CDC28 kinase activity is under multiple complex controls, and kinase activity is required for both initiation of DNA replication and G2/M transition (15–20). Activation of the G2/M checkpoint is achieved through the inhibitory phosphorylation of Cdc28 at Tyr-19 when Cdc28p is complexed with Clb2cyclin (21), which is regulated by the Swe1 kinase, the S. cerevisiae homologue of Schizosaccharomyces pombe Wee1 (22). The action of Swe1 on Cdc28 is cyclin-specific; it phosphorylates Clb2-Cdc28 but not Cln2-Cdc28 (22).

This study shows that loss of Isc1p results in activation of the G2/M checkpoint as revealed by the increased phosphorylation of Cdc28 on Tyr-19 following HU treatment. The Swe1 kinase was found to have a crucial role in isc1Δ sensitivity to HU, and the levels of Swe1p were increased and remained high for several hours following treatment with HU in an ISC1-dependent manner. The role of Swe1p was confirmed by deletion studies, which showed that the double mutant isc1Δ/swe1Δ was protected form HU toxicity. These studies reveal an unexpected G2/M checkpoint in response to HU and role for Isc1p in negotiating this checkpoint.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—The S. cerevisiae strains used in this study are: JK9-3d (MATa trp1 leu2-3 his4 ura3 ade2 rme1), JK9-3d isc1Δ (MATa trp1 leu2-3 his4 ura3 ade2 rme1 isc1Δ::KanMX) from our laboratory (23), and JMY1469 (MATa SWE1myc:HIS2 ade2 his2 leu2-3, 112 trp1-1 ura3Δns) from the
Role of ISC1 in G2/M

Daniel Lew laboratory (24). ISC1 was deleted in JMY 1469 strains to obtain JMY 1469 isc1Δ using the following primers (forward, 5′-TCCGAAAATGCGTCTATGCT-3′, and reverse, 5′-TGGTTACGGTGTCCTTCAA-3′) to amplify the ISC1 locus containing KanMX sequence from a genomic DNA of J9K-3d isc1Δ. The PCR product was transformed into JMY 1469, and we then selected for G418-resistant cells (yeast lithium acetate transformation methods). The deletion of ISC1 was verified by PCR and sequencing to confirm the replacement of ISC1 locus by KanMX sequence. Strains were grown on solid or liquid Minimum dextrose without leucine (SD-Leu). HU (U. S. Biologicals) was added to YPD at the concentration of 10 mg/ml. All cells were grown at 30 °C. SWE1 was deleted using primers (forward primer, 5′-GTAT-GCGTGTGTTAATCTATCCTGC 3′, and reverse primer, 5′-AAACCTCATCAGGTTTTATCGG 3′) to amplify the deleted locus from strain DLY 690 Mat a/cdc24-1 swe1Δ::LEU2 bar1 (ade his2 leu-2, 3, 112 trpl-1 uralans) received from Dr. Daniel Lew (24). The PCR product was used to transform J9K-3d isc1Δ, and transformants were selected on SD-Leu. The deletion of SWE1 gene was verified by PCR.

Plasmids—We received a plasmid expressing Cdc28-Y19F as a kind gift from Dr. George F. Sprague (25). Cells were transformed with the plasmid expressing the mutated Cdc28 or with vector control pRS415.

Cell Cycle Analysis—Asynchronous MATa (J9K-3d and J9K-3d isc1Δ) cells were grown to logarithmic phase on YPD or SD-Leu to an A400 of 0.3. Half of the culture was transferred to flasks containing 10 mg/ml or 7.5 mg/ml HU, and the other half served as untreated control. Aliquots were taken for Western blot studies, and others were fixed in 70% ethanol in 0.2M TE, pH 7.5, and stored at 4 °C. For subsequent fluorescence-activated cell sorting (FACS) analysis, the fixed cells were centrifuged, the supernatant (ethanol) was discarded, and the cells were resuspended in 200 μl, 1 mg/ml RNase in 0.2M TE, pH 7.5, and incubated at 37 °C for 2 h. Cells were centrifuged, and the supernatant was discarded. The cells were resuspended in 10 μl of 20 mg/ml proteinase K in 0.2M TE, pH 7.5, and incubated at 50 °C for more than 2 h. Cells were centrifuged and resuspended in 0.2M TE, pH 7.8, containing 4 μg/ml of propidium iodide, left for a few hours at 4 °C, and then taken for FACS analysis.

To obtain cells in the G2/M phase, we treated the cells with α-factor 5 mg/ml (U. S. Biologicals), washed them, and then released them in nocodazole (Sigma) or in nocodazole + HU. Aliquots were taken for Western blot analysis at appropriate time points.

Western Blot Analysis—Cell lysates were prepared using lysis buffer containing TE, EDTA, and proteinase inhibitors followed by the addition of cold glass beads. The tubes were vortexed for 3 min at 4 °C and then placed on ice for 1 min. This cycle was repeated a total of five times to obtain the whole cell lysate. The tubes were then centrifuged at 4 °C for 10 min at 6000 rpm. The supernatant was transferred to new tubes, and the protein concentration was determined using the Bio-Rad protein assay reagent. Sixty micrograms of proteins were loaded onto 4–20% Tris–HCl gel and then transferred to nitrocellulose membrane, both from Bio-Rad. The membranes were blocked, and immunoblotting was performed using a phospho-Cdc2 (Tyr-15) antibody (Cell Signaling) that recognizes yeast Cdc2–28 when phosphorylated at Tyr-19. Anti-Swe1 antibody was obtained from Santa Cruz Biotechnology (Swe1 (y-311): sc-7171). We used anti-myc antibody to detect Swe1–myc in the time course experiment done with synchronized cells. The Pstaire antibody (recognizing a conserved PSTAIRE domain of Cdc2 p34) was used in all the westerns as a loading control.

Statistical Analysis—The specific statistical methods and the number of replicates for each experiment are indicated in all figure legends.

RESULTS

ISC1 Deletion Renders Cells Sensitive to Genotoxic Agents—In a genome-wide screen for MMS sensitivity, ISC1 deletion increased the sensitivity of the cells to MMS as well as to HU (7). To confirm these results, we investigated the sensitivity of the yeast strain J9K-3d and isc1Δ to three types of genotoxic agents: MMS, HU, and camptothecin. On a spot test shown in Fig. 1, isc1Δ showed clear sensitivity to MMS and HU and partial sensitivity to camptothecin. These results raised important questions as to the role and molecular mechanisms that connect an enzyme involved in the degradation of complex sphingolipids to survival in the presence of a genotoxic drug.

HU Induces a G2/M Block in isc1Δ Cells—Because isc1Δ showed sensitivity to HU treatment, it was important at the outset to determine whether the progression of the cell cycle in the mutant cells was any different from the WT. A time course FACS analysis was performed in WT and isc1Δ, untreated and treated with HU, 10 mg/ml. The results of these experiments, shown in Fig. 2, reveal no differences in the progression of the cell cycle in the absence of HU (Fig. 2, A and B). When cells were treated with HU, both WT and isc1Δ exhibited a characteristic G2/M phase arrest in response to HU following the first 2 h of treatment (Fig. 2, C and D). Thus, ISC1 does not appear to be involved in the initial response to HU. On the other hand, the cell cycle profile became distinct between WT and isc1Δ after a few hours of treatment with HU as isc1Δ cells accumulated substantially in the G2/M phase, whereas the WT cells progressed normally out of the S phase block (Fig. 2, E and F). Thus, cell cycle analysis demonstrated an unexpected G2/M block in response to HU revealed through the deletion of ISC1.

To determine whether the G2/M phase block was due to the HU treatment specifically or in response to genotoxicity, the effects of MMS on the cell cycle in WT and isc1Δ cells were evaluated. The results showed that MMS treatment had the same effect as HU on isc1Δ cells; the G2/M block was evident at 8–12 h in isc1Δ (Fig. 2H) when compared with WT cells, which
resume cycling (Fig. 2). These results indicated that the G₂/M block is not specific to HU. Thus, the S phase checkpoint resulting from HU treatment is not specifically involved in the successive G₂/M block in isc1Δ.

**Phosphorylation Status of Cdc28 Kinase in isc1Δ⁻/⁻ HU**—Next, studies were undertaken to determine the mechanism of this G₂/M block. Initially, the phosphorylation of Cdc28 at Tyr-19 (Cdc28-Tyr-19-Pi) was evaluated as a measure of its activation status, employing an anti-phospho-Cdk2-Tyr-15 antibody that recognizes yeast Cdc28-Tyr-19-Pi. In Fig. 3A, a time course Western blot of asynchronous cultures showed that the levels of Cdc28-Tyr-19-Pi were higher in isc1Δ when compared with WT in the presence of HU. To more specifically evaluate the phosphorylation status of Cdc28-Tyr-19 at G₂/M phase, both WT and isc1Δ were synchronized using α-factor and then released in medium containing nocodazole alone or nocodazole and HU. Nocodazole blocks the cells at G₂ phase where normally Cdc28-Tyr-19-Pi is low. As shown in Fig. 3B, Cdc28-Tyr-19-Pi levels increased in both strains but persisted in the isc1Δ strain, and the effects were much more pronounced following treatment with HU (Fig. 3C). These results indicate that the G₂/M checkpoint regulator Cdc28 was significantly inactivated by phosphorylation in response to HU in isc1Δ cells.

To further corroborate these results and extend them, we employed a mutated form of Cdc28-Tyr-19, the active Cdc28-Y19F (the tyrosine in position 19 was replaced by a phenylalanine), which cannot be phosphorylated by Swe1p, the kinase responsible for the phosphorylation of Cdc28 at the Tyr-19 site. When Cdc28-Y19F was expressed in isc1Δ/H9004 cells, the G₂/M phase block was no longer detected when cells were treated with HU (Fig. 4A). However, the isc1Δ cells containing the vector alone treated with HU showed a G₂/M block (Fig. 4B). These results indicate clearly that the phosphorylation of Cdc28-Tyr-19 is necessary for the G₂/M block to occur in cells lacking ISC1 gene in the presence of HU.

**Swe1 Kinase Is Implicated in the Sensitivity of isc1Δ to HU**—The results revealing higher Cdc28-Tyr-19-Pi in isc1Δ in Fig. 3, A–C, suggested that isc1Δ might have a more active Swe1 kinase. Initially, the levels of Swe1 were evaluated, and for these
studies, an anti-myc antibody to Swe1p-myc was employed. The results showed that HU induced a time-dependent decrease in Swe1 in WT cells, which is consistent with the ability of the WT cells to negotiate the G₂/M checkpoint (Fig. 5A). On the other hand, the elevated levels of Swe1 persisted longer in isc1Δ cells in the presence of HU. The levels of Swe1 were also evaluated in α-factor synchronized cultures released in nocodazole to synchronize the cells primarily in the G₂ phase when usually the levels of Swe1 are very low (Fig. 5B). Again, it was found that Swe1 levels failed to down-regulate in isc1Δ cells in response to HU (Fig. 5C). These results demonstrate that HU-treated cells require a drop in Swe1 levels to negotiate the G₂/M checkpoint and that this occurs in an Isc1-dependent manner.

Next, the role of Swe1p in mediating the effects of ISC1 deletion on the G₂/M checkpoint were evaluated using a deletion of SWE1 because the loss of Swe1p should cause less phosphorylation of clb2-Cdc28-Tyr-19, resulting in an unphosphorylated active form of the cyclin-dependent kinase. FACS analysis was performed in the isc1Δ/swe1Δ double mutant, and the results showed that loss of expression of the SWE1 gene was sufficient to overcome the G₂/M block after HU treatment (Fig. 6A). Furthermore, a spot test was performed to determine whether the deletion of the SWE1 gene would rescue the viability of isc1Δ on HU. The results showed that the double deletion isc1Δ/swe1Δ rescued growth on HU (Fig. 6B). These results support a mechanism by which Swe1p plays a key role in the G₂/M block of isc1Δ in response to HU.

**DISCUSSION**

This study reveals that Isc1p, a phosphosphingolipase C involved in sphingolipid metabolism and ceramide generation, is required for cell cycle progression and protects cells from the genotoxic effect of MMS and HU. When cells lack the ISC1 gene, both MMS and HU lead to a G₂/M block due to activation of the Swe1p/Cdc28-dependent G₂/M checkpoint.

Mechanistically, the current investigation implicates Isc1p action upstream of Swe1p. The results revealed that cells lacking Isc1p exhibited higher levels of phosphorylated Cdc28 kinase when compared with WT cells following treatment with HU. Cdc28 kinase is the major regulator of cell cycle progression (13, 14), and its phosphorylation on tyrosine 19 results in inhibition of its activity and consequently an inability to cross the G₂/M checkpoint (22, 26, 27). This increased phosphorylation was implicated mechanistically in mediating the action of HU, as demonstrated by studies showing that expression of a mutated form of Cdc28 kinase, Y19F, which keeps the Cdc28p kinase in its active form, reversed the G₂/M block in isc1Δ cells, which now behaved similar to WT in their response to HU. These results strongly suggest that Isc1p mediates its action on the G₂/M block checkpoint activation through the phosphorylation of Cdc28-Tyr-19.

Additional mechanistic studies implicated Swe1p, the kinase responsible for Cdc28-Tyr-19 phosphorylation in G₂/M, in mediating the effects of HU on G₂/M in the isc1Δ cells. Western blotting results showed that the levels of Swe1p decreased in HU-treated cells as these cells resumed cell cycle progression and negotiated the G₂/M checkpoint. On the other hand, the levels of Swe1p were elevated and more persistent over time in isc1Δ cells in response to HU. Also, in α-factor synchronized cells released in nocodazole, to block them at metaphase when the levels of Swe1 are normally low, the levels of Swe1 were elevated in isc1Δ in response to HU. These results suggest failure of down-regulation of Swe1 in isc1Δ cells in response to HU.

Functionally, deletion of SWE1 restored cell cycle progression and overcame the G₂/M block in response to HU in isc1Δ cells. Deletion of Swe1 also overcame the sensitivity of isc1Δ cells to HU. These results define Swe1p as a major downstream mediator of the action of HU in isc1Δ cells.

Taken together, these results suggest the following scenario (Fig. 6C).
The recovery of yeast cells from the action of HU (and other genotoxic stresses) requires loss of Swe1p in order for these cells to negotiate the G2/M checkpoint. Indeed, a previous study has shown that HU caused the accumulation of Swe1 (28); however, the kinetics of that effect and the potential role of Swe1 were not investigated. The results from this study reveal that the subsequent decrease in Swe1p is essential for cells to traverse the G2/M checkpoint. From these findings, it emerges that ISCI-deleted cells fail to decrease Swe1p following HU treatment. This in turn causes increased phosphorylation of Cdc28 on Tyr-19, which leads to cell cycle block and increased sensitivity to HU. Thus, Isc1p emerges as a key regulator in the cell cycle progression such that recovery of cells from genotoxic agents requires the action of Isc1 upstream of Swe1p/Cdc28 in G2/M regulation.

Isc1p is an enzyme of sphingolipid metabolism, and multiple studies have begun to implicate various sphingolipids in regulation of the cell cycle. Mammalian ceramide has been implicated in regulating both G1 and G2/M cell cycle progression. Several studies have shown that ceramide induces dephosphorylation of the retinoblastoma protein (Rb), leading to G0/G1 cell cycle arrest (29). Other studies have shown that accumulation of endogenous ceramide due to inhibition of glucosylceramide synthase results in a G2/M block. In yeast, sphingolipid bases were found to be involved in heat stress-induced cell cycle arrest in G0/G1 (30).

Although the results from this study suggest a key and previously unidentified role for sphingolipids in regulation of cell cycle, they do not define the key lipid involved and whether it is dihydrophyto-ceramide (product of ISCI), complex sphingolipids (substrates of the enzyme), or additional metabolites. Ongoing studies, using mutants involved in ceramide metabolism, suggest a role for ceramide itself and not other metabolites. Such studies, along with lipidomic approaches and additional mutants in sphingolipid metabolism, should provide a better understanding of the specific lipid(s) involved in the regulation of SWE1 and the cell cycle progression in G2/M.

Taken together, the results suggest that Isc1p appears to hold the power of a stop–go signal when the cells are challenged with genotoxic agents. The results raise the hypothesis that when Swe1p accumulates in the cell in response to genotoxic stress, Isc1p must act to signal the “all clear” as cells recover from genotoxic stress. This is accomplished by causing a decrease of Swe1p levels, leading to a go signal and normal cycle progression.

In conclusion, this study offers novel insight on the regulation of the cell cycle in response to stress generated by genotoxic agents such as MMS and HU. The data implicate Isc1p, the enzyme involved in sphingolipid metabolism, in the regula-

**FIGURE 5. Effect of isc1Δ on the levels of Swe1p.** Asynchronous WT and isc1Δ cells were treated with HU, whole cell proteins were isolated at the indicated times (see “Experimental Procedures”), and the levels of Swe1p were detected using an antibody for myc-tagged Swe1p (A). WT and isc1Δ were synchronized using α-factor and then released in YPD medium containing 20 μg/ml nocodazole (B) or nocodazole and HU 10 mg/ml (C). Pstaire antibody was used as loading control. These blots were repeated independently three times.

**FIGURE 6. Effect of Swe1 deletion on the G2/M progression and survival of isc1Δ cells.** A, the role of Swe1 in mediating the cell cycle effects of HU in isc1Δ cells. Double mutant isc1Δ/ swe1Δ cells were grown as described under “Experimental Procedures,” treated with HU, and harvested at the indicated times. An aliquot of cells at each time point was analyzed by FACS. B, the role of Swe1 in mediating the growth effects of HU in isc1Δ cells. Exponentially growing WT, swe1Δ, isc1Δ, and isc1Δ/swe1Δ cells were spotted onto agar YPD plates without HU or containing 10 mg/ml HU. The plates were incubated for 3–4 days at 30 °C. C, schematic description of the role of Isp1 in G2/M block in response to HU. In the presence of HU, the levels of Swe1 increase in both strains. Swe1p levels decrease in WT cells, Cdc28p is in an active form, and the G2/M phase progresses normally. When Isp1p is missing, Swe1p levels remain elevated, and isc1Δ cells fail to decrease Swe1 kinase levels. Persistent high levels of Swe1p lead to an increase of phosphorylated Cdc28p (Cdc28p-Pi), which is inactive. The G2/M checkpoint is activated, and cells are blocked in G2/M phase. Experimental analysis in A represents the average of two independent experiments. The experiment in B was repeated three times with similar results.
tion of the G₂/M progression and checkpoint activation under genotoxic stress.

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