Biochemical and Biophysical Research Communications 424 (2012) 385–390

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Cadmium inhibits the protein degradation of Sml1 by inhibiting the phosphorylation of Sml1 in *Saccharomyces cerevisiae*

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ARTICLE INFO

Article history:
Received 6 June 2012
Available online 5 July 2012

Keywords:
Cadmium
Sml1
Dun1
Ubiquitin
Saccharomyces cerevisiae

ABSTRACT

Cadmium is a toxic metal, and the mechanism of cadmium toxicity in living organisms has been well studied. Here, we used *Saccharomyces cerevisiae* as a model system to examine the detailed molecular mechanism of cell growth defects caused by cadmium. Using a plate assay of a yeast deletion mutant collection, we found that deletion of *SML1*, which encodes an inhibitor of Rnr1, resulted in cadmium resistance. Sml1 protein levels increased when cells were treated with cadmium, even though the mRNA levels of *SML1* remained unchanged. Using northern and western blot analyses, we found that cadmium inhibited Sml1 degradation by inhibiting Sml1 phosphorylation. Sml1 protein levels increased when cells were treated with cadmium due to disruption of the dependent protein degradation pathway. Furthermore, cadmium promoted cell cycle progression into the G2 phase. The same result was obtained using cells in which *SML1* was overexpressed. Deletion of *SML1* delayed cell cycle progression. These results are consistent with Sml1 accumulation and with growth defects caused by cadmium stress. Interestingly, although cadmium treatment led to increase Sml1 levels, intracellular dNTP levels also increased because of Rnr3 upregulation due to cadmium stress. Taken together, these results suggest that cadmium specifically affects the phosphorylation of Sml1 and that Sml1 accumulates in cells.

1. Introduction

Heavy metals are toxic metals that inhibit the function of biological processes in cells [1–3]. Cadmium is one of the heavy metals that can induce deleterious effects in cells [4–6]. In humans, cellular cadmium exposure induces many fatal effects, such as cancer [7–9], renal dysfunction [10], and Itai–Itai disease [11].

In the budding yeast, *Saccharomyces cerevisiae*, the mechanism of cadmium toxicity has been well studied at the molecular level [12,13]. One of the targets of cadmium toxicity is the cell cycle pathway, which is mediated by various cell cycle regulatory proteins [9]. Cadmium can activate the expression of genes involved in the DNA repair pathway [14] to overcome cell cycle arrest. The Mec1- and Rad53-mediated cell cycle pathway has previously been studied as a target of cadmium toxicity [3]. Mec1 and Rad53 are known to be involved in DNA damage repair and cell cycle checkpoint control for various cell stresses [15–18]. Mec1 and Rad53 are conserved from yeast to humans, and they are similar to human ATR and CHK2, respectively [19]. Human ATR and CHK2 work upstream of p53 and act at the DNA damage checkpoint.

When yeast cells are exposed to cell-damaging agents, Mec1/Rad53 are activated and phosphorylate Dun1 kinase, which is localized downstream of Mec1/Rad53 [20,21]. Phosphorylated Dun1 then phosphorylates Sml1, which is a ribonucleotide reductase (Rnr) inhibitor [22,23]. Under normal conditions, Sml1 is not phosphorylated and inhibits the activity of Rnr1 while the cellular dNTP concentration is maintained. However, under stress conditions, Sml1 is phosphorylated by the Mec1/Rad53 pathway [39]. Phosphorylated Sml1 is a target of the ubiquitin-dependent protein degradation pathway. Sml1 is directly phosphorylated by Dun1 kinase, and phosphorylated Sml1 is a substrate of the E2 ubiquitin-conjugating enzymes Rad6, Bub1, and Ubr2. Phosphorylated Sml1 fails to inhibit Rnr1, and the cellular dNTP concentration is increased. Increased dNTP concentrations serve as substrates for the DNA repair process [24].

The Rnr family is composed of four subunits. Rnr1 is the major catalytic subunit and synthesizes most of the dNTPs in cells [25,26]. Yeast ribonucleotide reductase is composed of two large and two small subunits [24]. Rnr1 is the large subunit and has an allosteric regulatory site that is regulated by an effector molecule. Rnr2 and Rnr4 are the small subunits and have a tyrosyl residue and a binuclear iron center [27–29]. In addition, *S. cerevisiae* has another large subunit, Rnr3, which is a nonessential gene. Rnr3 has high homology with Rnr1 at the amino acid level and is induced in response to cellular stress, such as DNA damage [30].

Ribonucleotide reductase activity is regulated by two different stress response pathways. One pathway occurs at the Sml1 protein

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http://dx.doi.org/10.1016/j.bbrc.2012.06.103

level, as stated above. The other pathway occurs at the transcriptional level. Dun1 kinase also phosphorylates Crt1, which is a transcriptional repressor of *RNR* genes. In response to stress, phosphorylated Crt1 fails to bind to the upstream region of *RNR2*, *RNR3*, and *RNR4*, and the expression levels of *RNR2*, *RNR3*, and *RNR4* are increased [31], leading to increased synthesis of dNTPs.

In this study, we found that cadmium regulates the yeast cell cycle by regulating the phosphorylation of Sml1. Here, we report the mechanism of cadmium toxicity and how Rnr activity is involved in this process.

2. Materials and methods

2.1. Strains, media and growth conditions

The *S. cerevisiae* BY4741 strain (MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0) and its derivatives were used in this study. The *SML1* overexpression plasmid, pBG1805, was purchased from Open Biosystems. Yeast cells were grown overnight at 30 °C, and cultures were then refreshed with medium to OD 0.2 and grown at 30 °C for 3 h. For the cadmium and hydroxyurea treatments, each strain was grown to mid-log phase (OD600 ~ 0.5) and incubated with both $CdSO_4$ and $CH_4N_2O_2$ for 2 h at 30 °C. Chemical sensitivity or resistance was assessed using YPD containing 0 or 50 μM cadmium and 0 or 100 mM hydroxyurea, respectively. For proteasome inhibition, a *Δpdr5* strain, which blocks proteasome inhibitor efflux [32], was cultured for 5 h in the presence of 40 μM MG132 (Sigma) in 0.1% dimethyl sulfoxide (Sigma).

2.2. Measurement of dNTP levels

Yeast cells were grown overnight at 30 °C in YPD medium. The culture was refreshed to OD 0.5 and grown at 30 °C with a DNA-damaging agent for 2 h. The DNA damage-treated and untreated cells were harvested by centrifugation and resuspended in sterile water. The cell density was adjusted to an OD600 of 1×10^7 cells/ml. The collected cells were suspended in 700 μl of ice-cold extraction solution (12% [w/v] trichloroacetic acid and 15 mM $MgCl_2$). The samples were vortexed for 20 s and centrifuged for 1 min at 13,000 rpm. The aqueous phase was added to 700 μl of the Freon–trioctylamine mixture, and the mixture was vortexed and centrifuged as above. A 475 μl aliquot of the aqueous phase was used to quantitate dNTP levels by HPLC after the addition of 25 μl of 1 M NH_4HCO_3 (pH 8.9). The separation and quantitation of the dNTPs by HPLC was performed as described previously [33,34].

2.3. Immunoprecipitation for detection of ubiquitination

To detect ubiquitinated Sml1–HA, Sml1–HA was immunoprecipitated under denaturing conditions as described previously [35]. The cells were lysed in TNE buffer (100 mM Tris–HCl [pH 7.5], 150 mM NaCl, and 5 mM EDTA) containing protease inhibitors (Complete; Roche), 5 mM *N*-ethylmaleimide, 1 mM PMSF, and 1% Triton X-100 using a bead beater. The supernatant was transferred to a new tube and incubated with an anti-HA antibody for 1 h at 4 °C. After incubation, the mixture was incubated with prewashed Protein A/G PLUS agarose beads (Santa Cruz Biotechnology) overnight. The beads were collected by centrifugation, and the immune complexes were then eluted by incubation at 37 °C for 15 min after adding SDS sample buffer. The samples were analyzed using 12% SDS PAGE, blotted, and detected with an anti-ubiquitin antibody (Santa Cruz Biotechnology).

2.4. SML1 phosphorylation site mutation

To mutate serines 60 and 61, the Muta-Direct™ Site-Directed Mutagenesis Kit was used (iNtRON, KOREA). The pBG1805-*SML1* plasmid (Open Biosystems) and the following primers were used in this experiment: forward primer 5′-CTTCTGCCTCCGCTGCTGCAT TAGAAATGTGGGA-3′ and reverse primer 5′-TCCCACATTTCTAATG-CAGCAGCGGAGGCAGAAG-3′. PCR reactions were performed under the following reaction conditions: 15 cycles at 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 30 s. After PCR, the PCR tube was placed on ice for 5 min, and 1 μl of Mutazyme™ enzyme (10 μ/μl) was added to the PCR mixture. The mixture was then incubated at 37 °C for 1 h before being transformed into *E. coli* DH5α cells.

2.5. Synchronization

Cells were grown to early log phase in YPD medium and arrested at the G1 phase by treating with α-mating factor (3.4 μg/ml; Sigma) in YPD for 3 h. Cells were washed three times with distilled water. The cells were resuspended with fresh YPD at 30 °C and treated with 50 μM cadmium or 100 mM hydroxyurea for 2 h. Cells were collected at the indicated times.

2.6. Cell cycle analysis

A total of 10^7 cells was collected and resuspended with 1 ml of 70% EtOH and incubated at 4 °C overnight. The supernatant was discarded and resuspended with 1 ml of 50 mM sodium citrate buffer, pH 7.0. The cells were sonicated for 15 s, and 0.25 mg/ml RNase A was added. Cells were then incubated for 2 h at 50 °C. Next, 16 μg/ml of propidium iodide was added, and samples were incubated at room temperature for 30 min. Cell cycle analysis was performed with a FACSCalibur flow cytometer (BD).

3. Results

3.1. The SML1 deletion mutant is resistant to cadmium and upregulated by cadmium stress

To identify the genes involved in cadmium stress, a plate assay was performed using a yeast deletion mutant collection. As shown in Fig. 1A , 50 μM cadmium was added to the plate, and replica plating was performed. Hundreds of deletion mutants exhibited resistance to cadmium; in particular, we found that the *SML1* deletion mutant showed strong resistance to cadmium. Sml1 is a known inhibitor of Rnr1, which is a major catalytic component of the Rnr complex [23,36]. Sml1 is phosphorylated by the Dun1 checkpoint kinase and then degraded [24]. The activated Rnr complex synthesizes dNTPs to repair DNA damaged by cell-damaging agents [37]. Next, we attempted to identify the effects of cadmium on Sml1 expression. Cells were treated with 50 μM cadmium for 2 h, and total RNA or protein was extracted from the indicated cells. As shown in Fig. 1B, *SML1* mRNA levels were unchanged after cadmium treatment. However, Sml1 protein levels were upregulated by cadmium treatment. This result indicates that cadmium may regulate Sml1 at the post-translational level. To confirm the effects of cadmium on Sml1 expression, we treated the cells with α-factor and released cells from synchronization. As shown in Fig. 1C, expression of Sml1 was increased in a time-dependent manner from α-factor release. Hydroxyurea is a well-known inhibitor of Sml1, and as shown in Fig. 1C, Sml1 was decreased by hydroxyurea treatment.

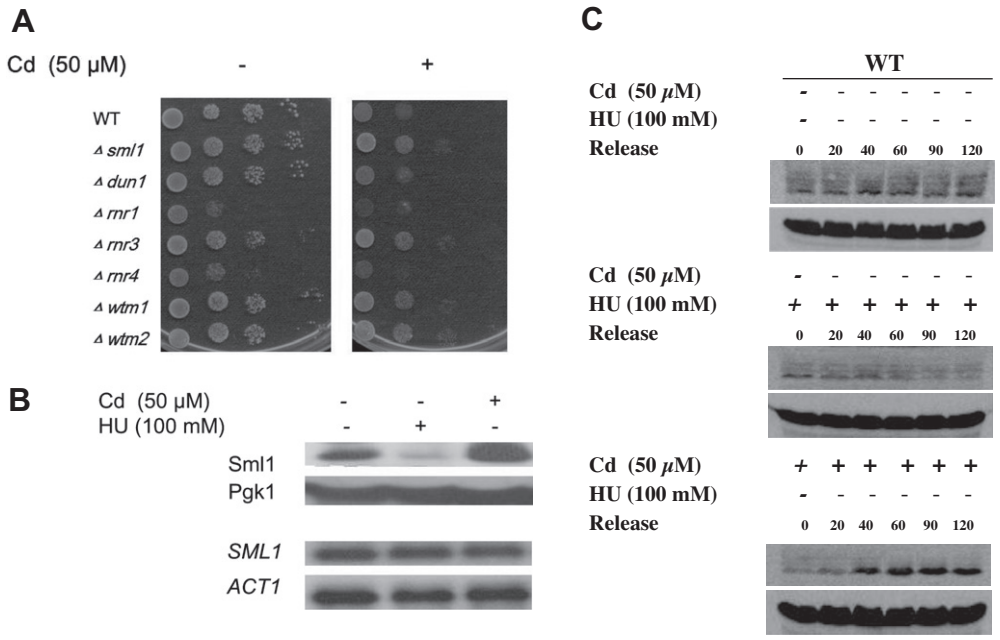

Fig. 1. Cadmium upregulates the expression of Sml1 at the post-transcriptional level and regulates cell cycle progression. (A) The indicated deletion strains were plated on SD plates or plates treated with 50 μM cadmium and diluted by 10-fold serial dilutions. (B) Cells were treated with cadmium or hydroxyurea, and total RNA and protein were extracted from the indicated cells. Northern (lower panel) or western (upper panel) blots were then performed. Hydroxyurea was used as a control. (C) Wild type cells were grown to early log phase and treated with α-factor for 2–3 h. Next, cells were washed with distilled water and were resuspended in fresh YPD medium. Cells were treated with cadmium or HU for the indicated times and then collected, and total protein was extracted.

3.2. Cadmium activates the cell cycle by activating Sml1

To identify the effects of cadmium and Sml1 on cell cycle regulation, we performed FACS analysis with cells in which *SML1* was deleted or overexpressed. As shown in Fig. 2A, the cell cycle progressed faster in wild type cells following cadmium treatment than in untreated cells. Approximately 30 min after cadmium treatment, half of the cells in the sample had progressed to the G2 phase. To identify the effects of Sml1 on cell cycle regulation, cadmium was applied to cells of an SML1 deletion strain. The lower panel of Fig. 2A illustrates the delayed progression from the G1 to the G2 phase of the cell cycle, even in cadmium-treated cells. Furthermore, overexpression of *SML1* accelerated cell cycle progression more quickly than in wild type cells. These results imply that cadmium-dependent cell cycle regulation is mediated by Sml1. To confirm the effects of *SML1* overexpression in response to cadmium stress, we performed a plate assay using the SML1 overexpression strain. A galactose-inducible promoter-derived *SML1* overexpression clone was purchased from Open Biosystems, and a plate assay was performed. As shown in Fig. 2B, cells expressing the *SML1* overexpression clone exhibited slow growth, consistent with the results obtained by FACS analysis.

3.3. dNTP levels are not increased following Sml1 upregulation by cadmium

To confirm the mechanism regulating Sml1, we next investigated intracellular dNTP levels. The levels of the cellular dNTP pools are important for high-fidelity DNA replication and repair [25]. Sml1 is an inhibitor of Rnr1; thus, the cellular dNTP level should decrease upon treatment of cells with cadmium. However, as shown in Fig. 3A, the intracellular dNTP levels were not decreased and were even higher in cadmium-treated cells than in cells not treated with cadmium. Hydroxyurea has been reported to inhibit Sml1 expression *via* the phosphorylation of Sml1 by Dun1 kinase [24,38]. We found that hydroxyurea increased cellular dNTP levels. To investigate why the dNTP level was not changed by cadmium when Sml1 was upregulated, we performed northern blot experiments using wild type and Δ*rnr1* strains to examine the expression levels of the catalytic subunits of the Rnr complex. As shown in Fig. 3B, cadmium upregulated the expression of *RNR3* at the transcriptional level. Furthermore, the deletion of *RNR1* also upregulated the expression of *RNR3* at the transcriptional level, and we noted synergistic effects following cadmium treatment of the *RNR1* deletion mutant. These results indicated that cadmium upregulated the expression of Sml1 at the post-translational level and that upregulation of Sml1 inhibited Rnr1, which upregulated Rnr3. Upregulated Rnr3 led to synthesis of high levels of dNTPs, and a high cellular dNTP level was maintained in the cadmium-treated cells.

3.4. Upregulation of Sml1 by cadmium is not dependent on the DNA damage checkpoint pathway

To investigate how cadmium upregulates Sml1, we tested the function of Dun1 kinase on Sml1 phosphorylation. Dun1 kinase phosphorylates Sml1 when cells are treated with DNA-damaging agents [22]. As shown in Fig. 4A, the northern blots performed with the WT and *Δdun1* strains indicated that hydroxyurea and cadmium did not affect the expression of *SML1* at the transcriptional level in either strain. However, the western blots revealed that hydroxyurea and cadmium affected the expression of Sml1 at the post-translational level in both the WT and *Δdun1* strains. Interestingly, even though Sml1 expression was downregulated by hydroxyurea in the wild-type cells, no difference was found in the *Δdun1* strains. This result was likely due to the failure of Dun1 to phosphorylate Sml1. However, no cadmium-induced changes in Sml1 expression were observed, even in the *Δdun1* strains. These results suggest that regulation of Sml1 is independent of the Dun1-dependent DNA damage checkpoint pathway. To further investigate how Sml1 is regulated, we attempted to examine the degradation pathway for Sml1. First, we tested

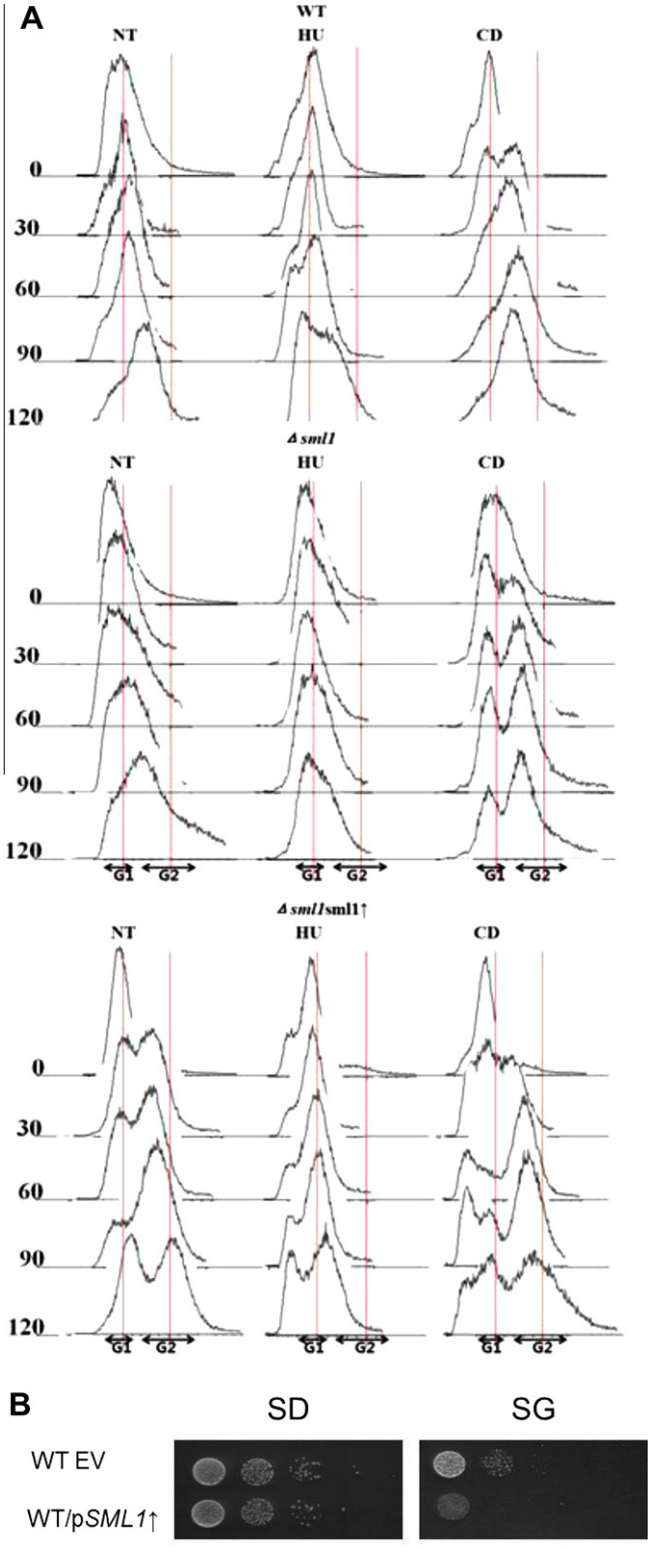

Fig. 2. Cadmium treatment led to cell cycle activation. (A) Cells were treated with α-factor as described in Fig. 1C, and FACS analysis was performed. Wild type, Δ*sml1*, and *SML1* overexpression cells were used to identify the effects of Sml1 on cadmium-induced cell cycle regulation. (B) The growth defect caused by *SML1* overexpression was investigated. An empty vector and the SML1 overexpression vector were transformed into wild type cells and plated on SD or SG media to induce the galactose-inducible promoter.

whether Sml1 degradation utilizes the vacuole-dependent degradation pathway. As shown in Fig. 4B, we examined Sml1

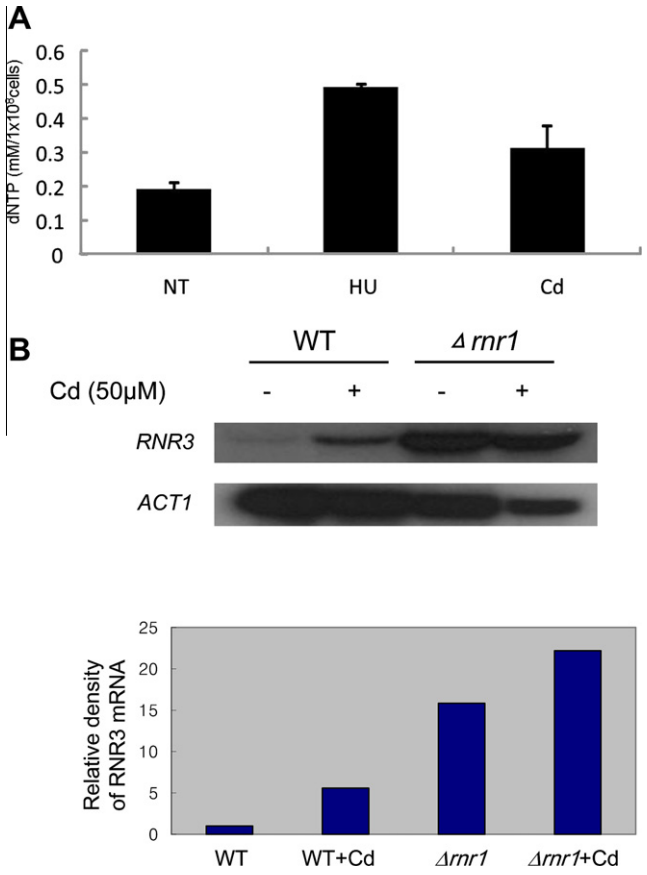

Fig. 3. Cellular dNTP levels increased despite the upregulation of Sml1. (A) Cellular dNTPs were measured in cadmium- and hydroxyurea-treated cells. (B) The expression of RNR3 was upregulated by cadmium and by deletion of *RNR1*. The bottom panel shows the quantitative analysis of the northern blot data.

expression levels in a *Δpep4* strain that is deficient in vacuolar protease activity. Cells were treated with cycloheximide prior to cadmium treatment to follow changes in Sml1 levels. We found that Sml1 underwent degradation at a similar rate in the *Δpep4* background compared with WT cells, and there was no significant effect of vacuolar proteases on Sml1 degradation. Next, we tested whether the degradation of Sml1 was dependent on the proteasome. Sml1 expression levels were measured in the *Δpdr5* strain, which is known to block the efflux of protease inhibitors. As shown in Fig. 4C, when cells were treated with the proteasome inhibitor MG132, Sml1 was not degraded, even following hydroxyurea treatment, and phosphorylated Sml1 was abundant. Furthermore, after cells were treated with cadmium, less phosphorylated Sml1 was found than was observed after hydroxyurea treatment. This result indicates that cadmium inhibits the phosphorylation of Sml1 and inhibits proteasome-dependent protein degradation. We also investigated the effects of cadmium on the ubiquitination of Sml1. As shown in Fig. 4D, hydroxyurea induced Sml1 ubiquitination. However, cadmium inhibited ubiquitination of Sml1 and inhibited degradation by the proteasome. Finally, we tested the effects of phosphorylation of Sml1 on protein degradation. Sml1 has three putative phosphorylation sites. We mutated two sites, serines 60 and 61, to alanine and measured the Sml1 protein level. Others have reported that serines 60 and 61 are phosphorylated before being recognized by the ubiquitin-dependent protein degradation pathway. As shown in Fig. 4E, Sml1 was maintained at the same concentration in cells following cadmium treatment. This result indicates that cadmium inhibits the phosphorylation of Sml1 and inhibits protein degradation.

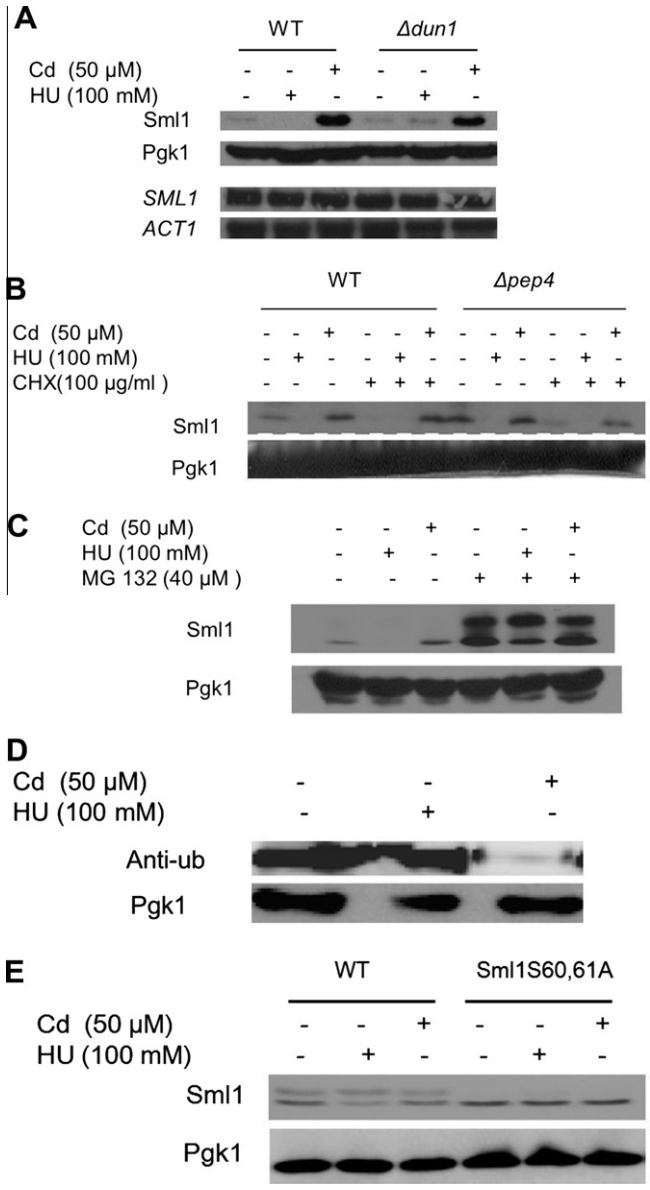

Fig. 4. Cadmium inhibits phosphorylation and degradation of Sml1 by the ubiquitin-dependent protein degradation pathway. (A) The expression of Sml1 by cadmium treatment was not regulated by Dun1 kinase but was regulated by hydroxyurea at the post-transcriptional level. Protein and total RNA were extracted from the indicated cells, and western and northern blots were performed. (B) The expression of Sml1 was also not affected by Pep4. (C) The expression of Sml1 was affected by MG132 treatment. This result indicates that ubiquitination is involved in Sml1 degradation in both cadmium- and hydroxyurea-treated cells. (D) Ubiquitination of Sml1 decreased in the cadmium-treated cells. (E) The putative phosphorylation sites of Sml1 were mutated to alanine, and Sml1 expression levels were investigated. Serine residues 60 and 61 were mutated to alanine by site-directed mutagenesis.

4. Discussion

In this study, we investigated the mechanism of cadmium toxicity on the cell cycle pathway. As shown in Fig. 1, cadmium treatment had differing effects than treatment with hydroxyurea, which is an inhibitor of cell cycle progression at the G2 phase [40]. To better understand the detailed mechanism of cadmium toxicity, we investigated the effects of cadmium on the Sml1 degradation pathway. In particular, we found that cadmium treatment inhibited Sml1 phosphorylation by the Mec1/Rad53 pathway and inhibited Sml1 degradation. Cellular Sml1 levels were increased by cadmium treatment, which was the opposite result than that observed with hydroxyurea treatment. Furthermore, the cellular dNTP concentration was increased by cadmium even though Sml1 was increased. To further examine this phenotype, we first tested the expression levels of the RNR genes following cadmium treatment. The expression of RNR3 was increased by cadmium treatment, and deletion of RNR1 showed a synergistic effect with RNR3 expression. As shown in Fig. 3, the expression of RNR3 depends on cadmium and RNR1 levels. This result explains why cellular dNTP levels were increased even when Sml1 was increased. Sml1 was shown to specifically inhibit Rnr1, and no effect on Rnr3 was observed; most Rnr functions were performed by Rnr3 when cells were treated with cadmium. We speculate that increased levels of Sml1 inhibit the function of Rnr1 and that the decreased activity of Rnr1 activates the expression of RNR3.

Secondly, we examined how cadmium affected the phosphorylation and degradation of Sml1. As shown in Fig. 4, Sml1 accumulated even when *DUN1* was deleted; this result indicates that Sml1 was not affected by Dun1 kinase, a protein kinase in the Mec1/Rad53 pathway that is responsive to cadmium stress. Furthermore, protein degradation was not affected by Pep4, which implies that Sml1 degradation is dependent on another pathway. In addition, MG132, a protease inhibitor, inhibited Sml1 degradation and resulted in high levels of the phosphorylated form of Sml1. As shown in Fig. 4, we found that the phosphorylation sites of Sml1 were involved in protein degradation. Taken together, these results suggest that cadmium specifically affects the phosphorylation of Sml1 and that Sml1 accumulates in cells. However, Rnr3 is upregulated by cadmium due to the inhibition of Rnr1 activity.

Acknowledgments

This work was supported by a National Research Foundation of Korea (NRF) Grant funded by the Korean government (MEST) (No. 2011-0017050).

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