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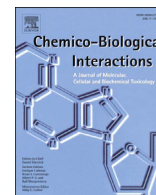

# Cadmium induces the activation of cell wall integrity pathway in budding yeast

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ABSTRACT

MAP kinases are important signaling molecules regulating cell survival, proliferation and differentiation, and can be activated by cadmium stress. In this study, we demonstrate that cadmium induces phosphorylation of the yeast cell wall integrity (CWI) pathway_MAP kinase Slt2, and this cadmium-induced CWI activation is mediated by the cell surface sensor Mid2 through the GEF Rom1, the central regulator Rho1 and Bck1. Nevertheless, cadmium stress does not affect the subcellular localization of Slt2 proteins. In addition, this cadmium-induced CWI activation is independent on the calcium/calcineurin signaling and the high osmolarity glycerol (HOG) signaling pathways in yeast cells. Furthermore, we tested the cadmium sensitivity of 42 paired double-gene deletion mutants between six CWI components and seven components of the HOG pathway. Our results indicate that the CWI pathway is epistatic to the HOG pathway in cadmium sensitivity. However, gene deletion mutations for the Swi4/Swi6 transcription factor complex show synergistic effects with mutations of HOG components in cadmium sensitivity.
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## 1. Introduction

As one of the major components in cigarette smoke, cadmium is listed as one of the major soil and river heavy metal pollutants, and classified as a Category I human carcinogen by the International Agency for Research on Cancer (IARC) [1]. Chronic exposure to cadmium due to dietary sources and cigarette smoking has emerged as a significant threat for human health, since cadmium is associated with various cancers and cardiovascular diseases [3–8]. In addition, recent studies show that cadmium is a possible etiological factor for human neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease [9,10].

The underlying molecular mechanisms for cadmium carcinogenicity seem to be complex, which include interactions with DNA repair processes, tumor suppressor functions and signal transduction pathways, but are not completely understood. In the cell, cadmium ions disrupt reactive oxygen species (ROS)/$Ca^{2+}$ homeostasis, which leads to downstream cell death and adaptive signaling cascades [1,2]. These signaling pathways include the $Ca^{2+}$/calmodulin-dependent pathways and the mitogen activated protein kinases (MAPKs), such as the high osmolarity glycerol (HOG) pathway in yeast cells as well as the p38 (mammalian counterpart of yeast Hog1), Erk1/2 and c-Jun N-terminal kinase (JNK) MAP kinase pathways in mammalian cells [11,12]. A previous study shows that high levels of extracellular cadmium concentrations induce a sharp increase in cytosolic calcium ions in *Saccharomyces cerevisiae* cells [13].

A sequential activation (phosphorylation) of two MAP kinases, Hog1 and Slt2, is required for cellular adaptation to cell wall damage caused by zymolyase (β-1,3 glucanase) [14]. In addition, a systemic genomics study has revealed that deletion of each of sixty-four genes, which are mainly related to cell wall construction and morphogenesis and the cell wall integrity (CWI) signaling pathway, induces the phosphorylation of Slt2 [15]. From a previous genome-scale genetic screen, we have identified gene deletion mutants of six components for the CWI pathway are sensitive to cadmium stress [11]. Here we show that the CWI pathway is activated by cadmium stress, but this cadmium-induced activation is independent on the calcium/calcineurin signaling and the HOG signaling pathways in yeast cells.

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## 2. Materials and methods

### 2.1. Yeast strains and media

Unless indicated, all diploid and haploid gene deletion mutants are from the diploid and haploid yeast deletion libraries, respectively, which were purchased from Invitrogen Inc. and used in our previous studies [11,16–18]. The *smf1*::*nat*MX4 strain and the double-gene deletion strains, *smf1*::*nat*MX4/*smf2*::*kan*MX4, was constructed with the *Eco*RI-linearized plasmid p4339 that contains the *nat*MX4 cassette [19]. Yeast strains were maintained on standard yeast extract/peptone/dextrose (YPD) medium. Synthetic drop (SD) lacking appropriate amino acids were made as described [20]. A serial dilution assay was used to evaluate ion sensitivity of yeast cells as described [21].

### 2.2. Construction of double-gene mutants between genes encoding the CWI and HOG pathway components

To study the interaction between the CWI pathway and the HOG pathway in cadmium sensitivity, we created the double-gene mutants between genes encoding the CWI and HOG pathway components through the synthetic lethality array approach described previously [22,23].

### 2.3. DNA manipulations

To construct SLT2-HA fusion protein, we amplified with primers, ScSLT2-F (5′ GTCCATTCAT AAGGCACAGG 3′; a *Sal*I site 43 base downstream of this primer site) and ScSLT2-R (5′ CGGCATGCAA AATATTTTCT ATCTAATCCA AAC 3′; *Sph*I site underlined), the DNA fragment including the 655-bp promoter region and the 1452-bp coding sequence without its stop codon, and cloned it into the *Sal*I and *Sph*I sites of pHAC111 [19] to yield pHAC111-SLT2. To examine the subcellular localization of Slt2 proteins, we subcloned the same *SLT2* PCR product above into the *Sal*I and *Sph*I sites of pGFP33 [20], which resulted in the generation of pGFP33-SLT2 expressing the ScRCH1-GFP fusion protein. All DNA constructs were confirmed by DNA sequencing.

### 2.4. Western blot analyses

To investigate the phosphorylation state of Slt2 (Mkp1), we introduced the plasmid pHAC111-SLT2, expressing the SLT2-HA fusion protein under the *SLT2* promoter in the pHAC111 vector (centromeric; *LEU2*) [19], into the wild type BY4743 or BY4741 and their isogenic gene deletion mutants. Protein extraction and Western blot analysis was carried out as described [11,24]. Protein levels were quantified with Coomassie blue using BSA as a standard. Phosphorylated proteins of SLT2-HA were detected by the Phospho-p44/42 MAPK Rabbit mAb purchased from Cell signaling (Product number: 4370) [16], membranes of which were washed with 0.1 M glycine (pH2.8) solution at 35 °C for 1 h before they were used for detection of total SLT2-HA proteins by a monoclonal anti-HA antibody purchased from Abcam Inc. (Hong Kong, China). Signals were quantified and analyzed using the Bio-Rad Gel DocTM XR + System and the Image Lab software (Version 4.0.1).

### 2.5. Fluorescent microscopy

Subcellular localization of SLT2-GFP was examined in BY4741 cells containing pGFP33-SLT2, and visualized with 1000 × magnification, exposure for 1 s and analog gain for 2.0x under the Nikon 80i Fluorescence Microscopy as described [25,26].

## 3. Results

### 3.1. Gene deletion mutants of components for the CWI pathway are sensitive to cadmium stress

In our previous genome-scale screen for cadmium-sensitive gene deletion mutants, we have identified gene deletion mutants for six CWI pathway components are sensitive to 100 μM $CdCl_2$, which include Mid2 (the plasma membrane sensor), Bck1 (the MAP kinase kinase), Slt2 (the MAP kinase) as well as their two downstream transcription factors, Rlm1 and the Swi4/Swi6 complex (Fig. 1 and Supplementary Fig. 1, [11]). Through a serial dilution assay we further examined the cadmium sensitivity of deletion mutants for all known components of the CWI pathway and their regulators [27]. At the end, we observed additional deletion mutants for two components of the CWI pathway were also sensitive to 100 μM $CdCl_2$, which were Sac7 (a negative regulator as the GTPase activating protein for Rho1) and Fab1 (the positive regulator as the guanine nucleotide exchange factor for Rho1) (Supplementary Fig. 1). Gene deletion mutants of these CWI pathway components show elevated calcium contents in response to cadmium stress [11]. Taken together, these results indicate that the CWI pathway is involved in the response of yeast cells to cadmium stress.

### 3.2. The CWI pathway is activated in response to cadmium stress

Although previous four genetic screens have also identified key components of both HOG and CWI MAP kinase pathways are involved in cadmium sensitivity [11,28–31], the activation of the CWI pathway in response to cadmium stress has not been further investigated. Therefore, we measured the Slt2 (Mpk1) phosphorylation in response to cadmium stress in this study. We first constructed the plasmid pHAC111-SLT2, expressing the SLT2-HA fusion protein, which complemented the function of Slt2 in cadmium sensitivity (Fig. 2A). We then introduced this plasmid into the wild type BY4743 cells. SLT2-HA phosphorylation was not detected in log-phase growing cells in the absence of cadmium, but detected when these cells were exposed to 20 μM $CdCl_2$ (Fig. 2B). SLT2-HA phosphorylation level elevated as the cadmium concentration was increased to 50 μM and 100 μM (Fig. 2B). The phosphorylation nature of SLT2-HA was demonstrated by the treatment of phosphorylated SLT2-HA proteins with calf intestine alkaline phosphatase (CIP) (Fig. 2C). The phosphorylation level of SLT2-HA increased gradually as the exposure of yeast cells to 50 μM $CdCl_2$ prolonged, and reached a peak level around 150 min after the treatment (Fig. 2D).

### 3.3. Contributions of cell surface sensors to the cadmium-induced CWI activation

To examine the contribution of the CWI pathway components to the cadmium-induced phosphorylation of Slt2, we introduced the plasmid pHAC111-SLT2 to their deletion mutants. We first examined the effects of five cell surface sensors, Wsc1 (also called Hcs77 and Slg1), Wsc2, Wsc3, Mid2 and Mtl1 that are implicated in transmitting CWI signaling [27], on the cadmium-induced activation of the CWI pathway. As compared to the wild type BY4743 cells, the cadmium-induced phosphorylation of Slt2-HA in the gene deletion mutant of *MID2*, but not in gene deletion mutant of *WSC1*, *WSC2*, *WSC3* and *MTL1*, were abolished (Fig. 3A). These results indicate that Mid2 mediates the cadmium-induced CWI signaling. However, it is interesting to note that the phosphorylation level of Slt2-HA, under the condition without cadmium treatment, is highly elevated in deletion mutants of *WSC1*, *WSC2* or *WSC3*, but not in deletion mutants of *MID2* and *MTL1* (Fig. 3A). Together, these

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