Proteomic Study for the Cellular Responses to Cd\textsuperscript{2+} in Schizosaccharomyces pombe Through Amino Acid-coded Mass Tagging and Liquid Chromatography Tandem Mass Spectrometry*\textsuperscript{S}

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Cadmium (Cd\textsuperscript{2+}) is one of well-known toxic heavy metal ions. To gain a global understanding how Cd\textsuperscript{2+} affects cells at the molecular level, we systematically studied the cellular response of the fission yeast Schizosaccharomyces pombe to Cd\textsuperscript{2+} using our integrated proteomic strategy of amino acid-coded mass tagging (AACT) and liquid chromatography-tandem mass spectrometry. Our proteome-wide investigation unequivocally identified 1133 S. pombe proteins. Of which, the AACT-based quantitative analysis revealed 106 up-regulated and 55 down-regulated proteins on the Cd\textsuperscript{2+} exposure. The most prevalent functional class in the up-regulated proteins, –28% of our profile, was the proteins involved in protein biosynthesis, showing a time-dependent biphasic expression pattern characteristic with rapid initial induction and later repression. Most significantly, 27 proteins functionally classified as cell rescue and defense were up-regulated for oxygen and radical detoxification, heat shock response, and other stress response. Furthermore, the large precursor sequence coverage of our AACT approach allowed us to unequivocally identify and quantify different isozymes from their amino acid sequence. Our quantitative dataset for glutathione-\textsuperscript{-}transferase, which have close similarity in their amino acid sequence. Our quantitative dataset also showed that 80% of the up-regulated proteins found in the S. pombe response were different from those in the Saccharomyces cerevisiae response. The function of some of the key identifications was validated through biochemical assays. It is very interesting that the induction of cysteine synthase expression was not observed in our study, although it has been proven as a critical enzyme to supply free cysteines for the enhancing synthesis of Cd\textsuperscript{2+}-sequestering molecules such as glutathione and phytochelatins in plants and some yeasts. Our quantitative proteomic result instead suggested that, as an alternative mechanism for the detoxification of Cd\textsuperscript{2+}, S. pombe produced significantly higher level of inorganic sulfide to immobilize cellular Cd\textsuperscript{2+} as a form of CdS nanocrystallites capped with glutathione and/or phytochelatins. Molecular & Cellular Proteomics 3:596–607, 2004.

Contamination of soil and ground water with heavy metals poses a major environmental and human health problem because they are very toxic and have intrinsically persistent nature. The toxicities of heavy metal ions are originated mainly from their strong binding affinity to metal-sensitive groups such as thiol or histidyl moieties in the cells even at low concentrations, resulting in the blocking of functional groups of biologically important molecules, the displacement and/or substitution of essential metal ions such as Ca\textsuperscript{2+}, Fe\textsuperscript{3+}, Cu\textsuperscript{2+}, Zn\textsuperscript{2+} ions from biomolecules, conformational modification, denaturation and inactivation of enzymes, and disruption of cellular and organelle integrity (1–3). Cd\textsuperscript{2+}, a designated human carcinogen, is one of well-known toxic heavy metal ions, and its toxicity has been extensively studied (4–9).

Living organisms have evolved several defensive mechanisms to overcome Cd\textsuperscript{2+} toxicities (1–3, 10–13). In eukaryotes, cells sequester Cd\textsuperscript{2+} as biologically inactive forms with cysteine-rich peptides such as glutathione (GSH),\textsuperscript{1} phytochelatins (PCs), and/or metallothioneins (MTs) (3, 11–13). The mechanisms by which mammalian cells protect themselves against this toxic metal ion are very complex and not well understood (13, 14). However, the structural and functional similarity of genes in lower eukaryotes and mammals suggests that more in-depth understanding of the molecular basis of the cellular responses to toxic Cd\textsuperscript{2+} in lower organisms will provide useful insights into the mechanisms in human cells (15). A fission yeast Schizosaccharomyces pombe and a budding yeast Saccharomyces cerevisiae have become valuable tools for the study of basic cellular functions of eukaryotic cells because of the ease of genetic manipulation and the availability of the complete genomic sequences of the both yeast species (16–19).

Compared with the molecular/cellular biological studies of individual genes or proteins one at a time as has traditionally

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\textsuperscript{1} The abbreviations used are: GSH, glutathione; AACT, amino acid-coded mass tagging; MS, mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; ORFs, open reading frames; PCs, phytochelatins, MTs, metallothioneins; ROS, reactive oxygen species; SD medium, synthetic minimal medium; DTPP, 2,2'-dithiodipyridine; TFA, trifluoroacetic acid; ICAT, isotope-coded affinity tag; DTT, dithiothreitol; 2D, two-dimensional; 1D, one-dimensional; GST, glutathione S-transferase.

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been done, the global analysis either at a genomic or at a proteomic level allows for a systematic overview of thousands of genes or their products in a species at the same time (20–23). Proteomics can produce more accurate and comprehensive information than what genomic studies can provide because protein expressions are regulated not only at transcriptional but also at translational levels, resulting in more details about mature proteins and their interactions than genome-based prediction (23). Therefore, significant discrepancies between mRNA and protein levels have also been found in several studies (24–26). It is inevitable that study of the cellular responses to different stresses at the proteomic level will inform us what gene products are actually expressed and their changes. In this regard, proteomics complements other functional genomics approaches such as microarray-based expression profiles, systematic phenotypic profiles at the cell and organism level, and small-molecule-based arrays (20).

A number of affinity- or mass-tagging methods such as isotope-coded affinity tag (ICAT) and other isotopic labeling strategies have been introduced for mass spectrometry (MS)-based quantitative proteomics and have proved to be useful for both the identification and quantification of proteins on a large scale (27, 28). Our strategy of residue-specific or amino acid-coded mass tagging (AACT) with stable isotopes through in vivo/in vitro cell culturing has provided internal quantitative markers for cellular proteins expressing in different conditions on a proteome scale (29–31). Without the need for two-dimensional (2D) gel electrophoresis-based high-resolution protein separation, we apply this AACT strategy to large-scale protein identification and concurrent high-throughput quantification to determine all possible protein factors sensitive to Cd\(^{2+}\) stress. We have further investigated the nature of those proteins in the cell rescue and defense to counteract Cd\(^{2+}\) toxicities. Alternative mechanism for scavenging free Cd\(^{2+}\) ions through the production of inorganic sulfide to form a nanocrystalline complex, CdS-GSH/PCs, has been postulated based on our proteomic dataset.

**EXPERIMENTAL PROCEDURES**

**Reagents and Yeast Strain**—The deuterium-enriched amino acid precursor, L-leucine-5,5,5-d\(_3\)-98% (Leu-d\(_3\)), was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Unlabeled amino acids, protease inhibitors, and other chemicals for yeast cell culture, gel electrophoresis, peptide extraction, and sample preparation for mass spectrometric analysis were obtained from Sigma (St. Louis, MO), Diilithiothreitol (DTT), sequencing-grade trypsin, and yeast nitrogen base were purchased from Fisher Scientific (Pittsburgh, PA). All the chemicals were sequence or high-pressure liquid chromatography grade if not specifically mentioned. S. pombe strain 972h was obtained from the American Type Culture Collection (Manassas, VA).

**Yeast Cell Culture and Protein Extraction**—Yeast cells were inoculated into 10 ml of either normal leucine-containing synthetic minimal medium (SD medium; light) or isotope-labeled leucine (Leu-d\(_3\))-containing SD medium (heavy) and incubated overnight at 30 °C. The SD medium consists of 0.17% yeast nitrogen base containing all the essential vitamins, salts, and trace elements for cell growth, 0.5% ammonium sulfate, 2% dextrose, and 0.01% of leucine. The medium components were sterilized by autoclaving for 20 min at 120 °C. Cd\(^{2+}\) ions in the form of CdSO\(_4\) (in 0.01 N HCl) were added to the medium exponentially growing culture (A\(_{600}\) ~ 0.35) at a final concentration of 1 mM. To precisely determine the regulated proteins caused only by Cd\(^{2+}\) exposure, control cells were separately prepared under the same conditions except for the absence of Cd\(^{2+}\) treatment. Cells were harvested at designated time points after Cd\(^{2+}\) addition and washed twice with milli-Q H\(_2\)O (Millipore Corp., Bedford, MA) to remove excess medium. The cells were then resuspended in 50 mM Tris HCl (pH 8.0), 5% β-mercaptoethanol, 0.3% SDS, and protease inhibitor mixture (1 tablet/50 ml). Protein samples for the quantification were prepared by mixing the control cells grown in the light medium with the cells treated with Cd\(^{2+}\) in the heavy medium at a 1:1 ratio and by vortexing with glass beads for 10 min at 4 °C followed by centrifugation at 14,000 rpm for 10 min at 4 °C to clarify the soluble proteins.

**Separation and Tryptic Digestion of Proteins**—Extracted proteins (50 μg) were mixed with SDS-PAGE sample buffer and heated at 95 °C for 5 min. The denatured proteins were then separated on 12.5% polyacrylamide SDS gels and stained with Coomassie dye (G-250). All protein bands were sliced from the gel, destained with 50% (v/v) acetonitrile in 50 mM NH\(_4\)HCO\(_3\), and completely dried in a speed-vacuum centrifuge. Then 15 μl of sequencing-grade modified trypsin (12.5 μg/ml in 50 mM NH\(_4\)HCO\(_3\)) was added to the dried gel slices that were incubated for 30 min on ice. The unsorbed solution was removed before 45 μl of 50 mM NH\(_4\)HCO\(_3\) was added to the rehydrated slices. These samples were incubated at 37 °C overnight. Tryptic digestion was stopped by adding 5 μl of 2% trifluoroacetic acid (TFA). The digested peptides were extracted from each gel slice by sonication in 0.1% TFA and 50% acetonitrile/0.1% TFA for 45 min. Both supernatants were combined and lyophilized.

**Nanospray Microcapillary Liquid Chromatography Tandem Mass Spectrometry (μLC-MS/MS)**—The digested peptides were analyzed by LC-MS/MS using a QSTAR Pulsar I mass spectrometer (Applied Biosystems, Foster City, CA) coupled with LC Packings Ultimate Microcapillary LC system (Dionex, Sunnyvale, CA). Five microliters of sample using an autosampler was loaded into a 10-μl sample loop and then pumped onto the C18 preconcentration column at a flow rate of 30 μl/min by a sample-loading pump. Three minutes after the sample loading, the 10-port valve was switched to a preconcentration cartridge in line with the nano-flow solvent delivery system, thus enabling the trapped peptides to be eluted onto the analytical column. The peptides were eluted from the analytical column with a linear gradient of solvent B (5% for 5 min, 5–50% for 50 min, then 75% for 10 min) at flow rate of 200 nl/min. Solvent A is 0.1% formic acid and 5% acetonitrile. Solvent B is 0.1% formic acid and 95% acetonitrile. The end of the analytical column was connected with a 10-μm inner diameter PicoTip nanospray emitter (New Objective, Woburn, MA) by a stainless steel union (Valco Instrument, Houston, TX) mounted on the nanospray source (Protacon Engineering, Odense, Denmark). The spray voltage (usually set between 1800 and 2100 V) was applied to the emitter through the stainless steel union and tuned to obtain the best signal intensity using standard peptides. The two most intense ions with the charge states in between 2 and 4 in each survey scan were selected for MS/MS experiments. The rolling collision energy feature was employed to fragment the peptide ions according to their charge states and m/z value.

**Protein Identification and Quantitative Analysis**—The identity of both peptides of each isotope pair was confirmed by MS/MS sequencing. The QSTAR instrumental default for mass isolation window is usually set on 3 Da, and this mass window is adjustable. Depending on where the lower mass end of the ion cursor will be set, which is usually 0.5 Da less than the light isotope signal, each individual
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isotope peak (light or heavy) could programatically be selected for a MS/MS experiment. Also, because there is only a 3-Da split for each Leu-\(d_3\)-containing peptide, we could set the low-mass ion cursor end close enough to the light isotope peaks so that both isotope peaks were fragmented simultaneously in a pair that could serve as pair signal validation. Tracking the paired signals in each MS/MS spectrum, we were able to determine the real isotope pairs for quantitation. Once confirmed, LC-MS/MS data were searched against the Sanger Institute \( \text{S. pombe} \) protein database using the ProID program available on the QSTAR instrument. Peptides hits with scores better than 30 in ProID were acceptable as good matches. Because we used Leu-\(d_3\) in cell culture for amino acid-specific mass tagging, those peptides containing leucine were further analyzed for the quantification of differentially expressed proteins. If the database search identified certain peptides as the leucine-containing peptides, we first manually inspected the raw MS/MS data for characteristic 3n-Da mass-split patterns (\( n \) is the number of leucine) as previously described (30). This process can also validate the database search results. For those Leu-\(d_3\)-containing peptides satisfying both criteria, the quantitative results for their corresponding proteins were obtained by examining the peptide mass map of certain precursor ions on chromatographic profiles, and the induction ratios of proteins were obtained both by measuring the mono-isotopic peak intensity of unlabeled (light) and Leu-\(d_3\)-labeled (heavy) peptides and by comparing the integral peak areas of the pairs. Both measurements showed similar ratios unless there was severe signal overlapping in mass spectra. In cases where the light and heavy isotope distributions overlapped, the ratios were determined by applying an isotopic correction factor as following. The peptide sequence was submitted to the web-based tool MS-isotope, which is part of the ProteinProspector package (prospector.ucsf.edu), for determining theoretical isotope intensity distribution. The theoretical isotope pattern of the light peaks in the peptide pair was then subtracted from the apparent isotope pattern to obtain the correct intensities of the heavy peaks.

**Assays for Thiol Groups, GSH, Inorganic Sulfide, and Cysteine Synthase Activity**—Total nonprotein thiols and GSH were estimated following the methods described by Grassetti and Murray (32) and Anderson (33), respectively. Briefly, 1 ml of cells was harvested, washed twice with distilled water, resuspended in 0.2 ml of 5% sulfosalicylic acid, and disrupted by vortexing with 0.1 ml of glass beads. For the analysis of thiols, 50 \( \mu \)l of the supernatant was mixed with 930 \( \mu \)l of 0.2 M sodium acetate (pH 4.0) and 20 \( \mu \)l of 2.2'-dithiodiopyridine (DTDP) stock solution (7 mg of DTDP in 2 ml of 1 N HCl and 8 ml of 0.2 M sodium acetate, pH 4.0). The reaction mixture was vortexed and incubated for 1 h at room temperature. The amount of the thiols was estimated by reading absorbance at 343 nm. GSH and cysteine were used as standards for calibration. Then 20 \( \mu \)l of the supernatant was used for the analysis of GSH using cyclic assay.

To analyze the acid-labile inorganic sulfide using a method by King and Morris (34), 1 ml of the culture was harvested by centrifugation at 18,000 \( \times g \) for 1 min. The cell pellet was resuspended in 0.4 ml of 1.5 M NaOH and incubated at 95 °C for 15 min. The suspension was vigorously vortexed, mixed with 0.25 ml of zinc acetate (2.6% in water) and 0.125 ml of \( N,N \)-dimethyl-p-phenylenediamine dihydrochloride (0.1% in 5 N HCl), and vortexed for 1 min. Then 0.05 ml of ferric chloride was quickly added (11.5 ml in 0.6 N HCl), vortexed, and incubated at room temperature for 30 min. Then, 0.425 ml of deionized water was added, vortexed, and centrifuged at 18,000 \( \times g \) for 10 min. The absorbance of the supernatant was recorded at 670 nm.

The activity of cysteine synthase was measured following the method by Hirase and Molin (35). One milliliter of cells were broken in phosphate-buffered saline buffer containing glass beads. Then 50 \( \mu \)l of the supernatant was transferred to 50 mm phosphate buffer (1 ml, pH 7.5) containing 5 mm O-acetyl serine, 1 mm sodium sulfide, 1 mm DTT, and 0.025 mm pyridoxal-5'-phosphate. The reaction mixture was incubated for 60 min at 30 °C. The reaction was stopped by adding 0.5 ml of 20% trichloroacetic acid and centrifuged for 10 min at 2000 \( \times g \). A total of 250 \( \mu \)l of the supernatant was used for assaying the synthesized cysteine by a ninhydrin method (36).

**RESULTS**

Large-scale Identification and Quantification for the Proteins Sensitive to \( \text{Cd}^{2+} \) Exposure—The integrated strategy of AACT and LC-MS/MS was used to elucidate the cellular responses to \( \text{Cd}^{2+} \) in the fission yeast \( \text{S. pombe} \) at the proteomic level (Scheme 1). Leucine was selected as a coding amino acid tag because it is the most prevalent amino acid in the cellular proteins of this organism (distribution abundance of 9.89%). The \( \text{S. pombe} \) cells grown in the heavy medium containing Leu-\(d_2\) were exposed to 1 \( \mu \)M \( \text{Cd}^{2+} \) at the mid-exponentially growing stage (\( \text{OD}_{600} \sim 0.35 \)), and culture aliquots were collected at 1, 4, and 12 h after \( \text{Cd}^{2+} \) exposure, respectively, whereas the control cells in the light medium containing normal leucine were harvested at the same time points without adding \( \text{Cd}^{2+} \). We selected 1 \( \mu \)M \( \text{Cd}^{2+} \) to treat the exponentially growing cells for inducing observable cell responses at the protein level after carefully examining the cell survival curve (Fig. 1), and minimum cell death was observed at this \( \text{Cd}^{2+} \) concentration, which was used in other recent studies (37–39). Proteins were analyzed from the cell cultures collected at three different time points to see whether the
change is transient or constitutive. Cadmium treatment produced varying kinetics of the global change at the proteomic level (Supplementary Tables I and II). During the experimental time span, proteins generally showed a conserved change even with different ratios, either up- or down-regulation. However, some proteins, especially many ribosomal proteins, showed to be up-regulated at early time points, but later down-regulated (Table I). It is not clear yet whether this inverting regulation of some ribosomal proteins is related to the increasing demand for the synthesis of heavy metal scavenging molecules at an early stage. Reduction in the synthesis of ribosomal proteins at a later stage would permit energy and other resources to be diverted toward other mechanisms involved in surviving under cadmium stress. With the assistance of leucine-specific mass tagging, a total of 1133 proteins that represent ~25% of the total annotated genes were identified for the fission yeast cells from more than 23,000 MS/MS spectra.

In the quantification analyses of differentially expressed proteins in response to Cd\(^{2+}\) exposure, cell populations rather than protein concentrations were used as a parameter to mix the cells from both light and heavy pools. In order to prevent bias due to cell growth differences between the light and heavy medium, protein lysates were also quantitated from the swapped cell mixture, i.e. the control cells were grown in the heavy medium while the sample cells treated with Cd\(^{2+}\) in the light medium. Protein expressions were quantitated for both cell mixtures to calibrate the induction ratio. Overall, AACT-assisted quantification analysis discovered 106 up-regulated (Supplementary Table I), 55 down-regulated (Supplementary Table II), and 158 unchanged proteins in S. pombe on the exposure to Cd\(^{2+}\). The remaining identified proteins were not precisely quantitated due to either insufficient peak

![Image of graph showing cell density vs. incubation time](FIG.1)

**Fig. 1. Effect of cadmium ions (Cd\(^{2+}\)) on the growth of S. pombe cells in the SD minimal medium.** S. pombe cells were grown at 30 °C and exposed to Cd\(^{2+}\) at mid-exponential growth phase (A\(_{600}\) 0.35). Cell density was monitored up to 48 h after Cd\(^{2+}\) treatment with the absorbance at 600 nm. The Cd\(^{2+}\) concentrations used in the cellular treatment were given as indicated.

**Table I**  
Induction ratios of up-regulated proteins involved in protein biosynthesis at different time points after exposure to Cd\(^{2+}\)

Proteins are regarded as up-regulated if it has an induction ratio higher than 1.3 in at least one of the time courses. Induction ratio (IR) is presented as an average value from three independent experimental sets and one swapped experiment. Two numbers in the No. column show the number of peptides used to identify each protein (first) and the number of the peptide pair used to calculate the induction ratio of each protein (second), respectively. ND, not determined.

| Protein name | 1 h IR | 1 h No. | 4 h IR | 4 h No. | 12 h IR | 12 h No. |
|--------------|--------|---------|--------|---------|---------|---------|
| Expression trend induced and subsequently repressed | | | | | | |
| 40S ribosomal protein S10-A | 1.79 | 5.2 | 0.77 | 19.7 | 0.43 | 8.3 |
| 40S ribosomal protein S17-A | 1.35 | 6.2 | 1.05 | 14.5 | 0.71 | 4.2 |
| 40S ribosomal protein S24-B | 1.56 | 7.2 | 0.95 | 7.3 | 0.73 | 2.1 |
| 40S ribosomal protein S9-A | 1.55 | 17.6 | 0.95 | 17.6 | 0.72 | 21.7 |
| 60S acidic ribosomal protein P2-alpha | 1.66 | 12.4 | 1.19 | 26.10 | 0.52 | 3.1 |
| 60S ribosomal protein L10-A | 1.47 | 9.3 | 0.95 | 4.2 | 0.7 | 6.2 |
| 60S ribosomal protein L13 | 1.41 | 4.1 | 0.86 | 9.4 | 0.75 | 8.2 |
| 60S ribosomal protein L15.2 | 1.66 | 7.2 | 0.9 | 16.7 | 0.63 | 7.2 |
| 60S ribosomal protein L27-A | 1.35 | 11.3 | 0.91 | 8.3 | 0.69 | 15.5 |
| 60S ribosomal protein L28-A | 1.44 | 7.2 | 0.77 | 18.7 | 0.6 | 9.4 |
| 60S ribosomal protein L4-A | 1.51 | 22.7 | 0.68 | 31.13 | 0.26 | 40.15 |
| 60S ribosomal protein L8 | 1.41 | 20.5 | 0.73 | 20.9 | 0.54 | 13.5 |
| 60S acidic ribosomal protein p0 | 1.53 | 11.4 | 0.79 | 10.4 | 0.69 | 7.2 |
| 40S ribosomal protein S20 | 1.37 | 2.1 | 0.73 | 4.2 | 0.71 | 4.1 |
| 60S ribosomal protein L3-A | 1.38 | 15.5 | 0.79 | 16.6 | 0.75 | 4.2 |

**Induced and subsequently restored back to normal level**

| Protein name | 1 h IR | 1 h No. | 4 h IR | 4 h No. | 12 h IR | 12 h No. |
|--------------|--------|---------|--------|---------|---------|---------|
| Eukaryotic initiation factor 4A | 1.43 | 6.2 | 0.82 | 27.11 | 1 | 10.4 |
| 40S ribosomal protein S23 | 1.82 | 5.1 | 0.89 | 4.2 | 1.1 | 2.1 |
| 40S ribosomal protein S5-B | 1.64 | 6.2 | 1.26 | 2.1 | 0.89 | 3.1 |
| 40S ribosomal protein S14 | 1.51 | 9.3 | 1.12 | 14.6 | 1.1 | 7.3 |
| 60S ribosomal protein L5-A | 1.36 | 11.3 | 0.77 | 22.9 | 0.87 | 4.2 |
| 40S ribosomal protein S11 | 1.61 | 7.3 | 0.74 | 15.6 | 0.91 | 2.1 |

**Others**

| Protein name | 1 h IR | 1 h No. | 4 h IR | 4 h No. | 12 h IR | 12 h No. |
|--------------|--------|---------|--------|---------|---------|---------|
| 60S ribosomal protein L20 | 1.3 | 9.3 | 0.89 | 23.10 | 2.82 | 4.2 |
| 60S ribosomal protein L35 | ND | 0.56 | 5.2 | 2.02 | 5.2 |
intensities, peak overlapping, or the lack of leucine residue in the peptides. Proteins were determined as up-regulated if their induction ratios would be higher than 1.3 in at least one of the time courses, whereas down-regulated proteins were determined with any induction ratio less than 0.78. These cutoff values were selected on the basis that there was a significant consistency in the range of 0.78–1.3, i.e. less than 5% variations from peptide to peptide in the induction ratios obtained from different peptides of a same protein (Supplementary Table III) or less than 20% ratio variations from experiment to experiment for three independent experiments and one swapped experiment (Supplementary Table IV).

Molecular Mass and pI Distributions of the Identified Proteins—The distribution of molecular masses of the proteins identified is shown in Supplementary Fig. S1A. More than 75% of total gene products are located in the range of 10–70 kDa. Our dataset showed 70% of the identified proteins are in this range. The majority of the up-regulated proteins are in the range of 10–40 kDa (62.3%), whereas relatively few proteins have molecular masses greater than 70 kDa (10.4%). The dominance of the low-molecular-mass proteins might be due to those proteins related to protein biosynthesis, protein metabolisms, and detoxification (see “Functional Classification of the Proteomic Dataset”). The down-regulated proteins also showed a similar pattern in the molecular mass distribution (data not shown). Our method was very effective to identify various proteins cross the entire range of molecular masses, covering 21 ± 2.5 and 35 ± 1.5% for the proteins in the range of 10–70 and above 70 kDa, respectively.

pI values of the identified proteins showed a bimodal distribution (Supplementary Fig. S1B). It has been found in both prokaryotes and eukaryotes that cytosolic proteins cluster around pI 5.0–6.0, whereas integral membrane proteins around pI 8.5–9.0 (40, 41). Our proteomic results showed the first cluster centered at 5.0–6.0 originated from the cytosolic proteins. The second cluster showed a slightly broader distribution around 8.5–9.5 due to the abundance of the expressed ribosomal proteins. Most of the up-regulated proteins clustered around pI values of 5.5–6.5, whereas a second cluster was further shifted to a more basic region, around 10.0–11.0. The second cluster was found to exclusively consist of ribosomal proteins. It should be noted that the ribosomal proteins with high pI values can be easily missed from 2D-PAGE-based analysis because the isoelectric focusing separation step excludes the basic proteins (pI > 9.5). However, our method was able to identify more than 80% of total ribosomal proteins because a one-dimensional (1D) SDS-PAGE gel separates proteins according to only their molecular masses, regardless of their pI values.
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Functional Classification of the Proteomic Dataset—On the bases of annotations from Swiss-Prot and TrEMBL, MIPS (mips.gsf.de), and the S. pombe database at Sanger Institute (www.sanger.ac.uk/Projects/S_pombe/), 106 up-regulated and 55 down-regulated proteins were functionally classified (Supplementary Fig. S2, A and B, respectively). The complete list of the functionally classified up-regulated proteins is available in Supplementary Table V. The protein factors involved in protein biosynthesis were the most prevalent class including both up-regulated and down-regulated proteins (28 and 41%, respectively). The protein class for protein biosynthesis was also reported in S. cerevisiae as the most prevalent class containing the repressed proteins after Cd^{2+} treatment, but not among induced proteins (37). Currently, it is not clear whether the up-regulated proteins involved in protein biosynthesis are related to the increased synthesis of proteins for either directly scavenging Cd^{2+} ions or the detoxification mechanisms of toxic effects produced by Cd^{2+} ions. The next prevalent class of the up-regulated proteins represented the proteins related to cellular defense mechanism (26%), including heat shock response (11%), oxygen and radical detoxification (8%), and stress response (7%), whereas the proteins involved in nucleotide metabolism belonged to the second most prevalent class of down-regulated proteins (16%). There were no defense-related proteins that were down-regulated except for yeast chaperone hsp78 homolog. As many as five hypothetical proteins were found to be up-regulated, but none of them were down-regulated. These hypothetical proteins may be participated in cellular detoxification against Cd^{2+} toxicities, of which functions have not yet elucidated. In addition, six oxidoreductases were up-regulated, indicating that various active cellular responses were invoked against the toxicity of Cd^{2+} because many oxidoreductases have shown to be involved in the stress-responsive processes (Supplementary Table V) (6, 42–44).

Ribosomal Proteins—The proteins involved in protein biosynthesis represented the major functional category among both up- and down-regulated proteins (Supplementary Fig. S2, A and B). There are 42 proteins in the small subunit (40S) and 75 in the large subunit (60S) in the Swiss-Prot/TrEMBL protein database. All of the small subunit proteins were identified in our study. Among the identified small subunit proteins, 10 were up-regulated on the exposure to Cd^{2+}, whereas 10 were down-regulated. We identified 61 large-subunit proteins, indicating 80% identification coverage. Among them, 16, 9, and 12 of identified proteins were up-regulated, down-regulated, and not changed on the exposure to Cd^{2+}, respectively. Two of a total of five cytoplasmic elongation factors were down-regulated, whereas the rest remained unchanged. No elongation factors in the mitochondria were identified. In addition, we identified nine translation initiation factors with five unchanged. The other four proteins were not quantitated. Interestingly, most of the up-regulated ribosomal proteins showed a time-dependent manner on the exposure to Cd^{2+} (Table I). Fifteen of the 30 up-regulated ribosomal proteins were induced in the cells at 1 h after being exposed to 1 mM Cd^{2+}. However, they were down-regulated later up to 12 h after exposed to Cd^{2+}. The expression level of six proteins, which were induced at 1 h after exposed to Cd^{2+}, were restored back to normal level at either 4 or 12 h later. Two large subunit proteins (L20 and L35) showed the highest expression level at 12 h after Cd^{2+} exposure. Among 23 down-regulated ribosomal proteins, the expression level of two proteins was restored back to the normal level later, whereas 17 showed the minimal expression level at 12 h after exposure to Cd^{2+} (data not shown).

Detoxification Proteins—Our AACT-assisted quantitative analysis identified a total of 27 up-regulated proteins from S. pombe exposed to Cd^{2+} that are involved in the cellular detoxification mechanisms, including oxygen and radical detoxification, heat shock responses, and other responses to a stress (Table II). The production of reactive oxygen species (ROS) is known as one of the major mechanisms of the toxicities exerted by Cd^{2+} ions (7, 8, 45, 46). It has been recently reported that proteins with antioxidant properties were up-regulated in the budding yeast S. cerevisiae exposed to Cd^{2+}, such as alkyl hydroperoxide reductase, superoxide dismutate [Mn], thioredoxin, and thioredoxin peroxidase (37). Our experiments discovered that all proteins functionally classified as oxygen and radical detoxification were significantly up-regulated, including both S. pombe ortholog proteins of the above proteins identified in S. cerevisiae and catalase, glutaredoxin, superoxide dismutate [Cu-Zn], and thioredoxin reductase (Table II). These antioxidant proteins showed the enhancing induction with increasing exposure time to Cd^{2+}. These data clearly indicate that Cd^{2+} causes oxidative stress in this microorganism and that the S. pombe cells respond against the Cd^{2+} stress by enhancing the expression of proteins with antioxidant properties. Fig. 2 shows a quantitative analysis of pmp20, the role of which has been suggested to detoxify ROS (47). Up to 17 leucine-containing peptides were quantitated to determine the expression level of pmp20. The quantification values of the protein from different peptides showed within 3% difference. The expression level of pmp20 remained the same up to 4 h after Cd^{2+} treatment (Fig. 2B). However, it was enhanced 2.6 times in the cells exposed to Cd^{2+} for 12 h in comparison to that of the control cells (Fig. 2C).

Induction of heat shock proteins has been reported as defense mechanisms against a wide range of stress conditions, including heat shock, oxidative stress, heavy metals, and pathologic conditions (3, 4, 48). However, there have been no available data in regards to heat shock proteins from S. pombe exposed to Cd^{2+}. Here, we identified 12 up-regulated, one down-regulated, and two unchanged heat shock proteins from this microorganism on the exposure to Cd^{2+} (Table II and Supplementary Table II). The up-regulated heat shock proteins generally increased their expression level as
the exposure time to Cd$^{2+}$ increased. Two heat shock proteins, HSP16 and HSP90 homologs, were down-regulated initially (1 h after exposure), but later significantly activated (the induction ratio increased from 0.19 to 6.48 and from 0.56 to 1.7, respectively). We also identified seven up-regulated stress-responsive proteins, which do not belong to either oxygen and radical detoxification class or heat shock proteins (Table II). Among these proteins, the levels of six proteins...
Proteomic Responses to Cd\(^{2+}\) in \(S. \text{ pombe}\)

Fig. 3. Primary amino acid sequence alignment of the GST isozymes of \(S. \text{ pombe}\), GST-I, GST-II, and GST-III using ClustalW (ebi.ac.uk/clustalw/). Sequences underlined indicate the identified peptides for quantitative measurements. Amino acids that are identical in all sequences are marked with *, **, and *** indicate the semi-conserved and the conserved substitutions, respectively.

were gradually increased as exposing time increased, whereas plasma membrane ATPase was induced 1 h after Cd\(^{2+}\) treatment, but down-regulated later (data not shown).

Most significantly, our AACT method allowed us to unequivocally identity and quantify the isozymes that have close similarity in their amino acid sequence because of the larger distribution of leucine residues used as the internal markers (Fig. 3). There are three isozymes for glutathione S-transferase (GST) in \(S. \text{ pombe}\), GST-I, -II, and -III, which are important in the detoxification of many xenobiotic compounds and in protecting cells from oxidative stress by detoxifying some of the secondary ROS produced when ROS react with cellular components (49, 50). GST-I and GST-II share 79% identical sequences, which makes their identification difficult using other quantitative proteomic approaches such as ICAT. We found two peptide sequences for GST-II (24–38 and 185–202) distinguishable from those in GST-I, and one sequence (143–154) overlapped each other. To unambiguously quantify the expression of these two isozymes, we first measured the induction ratio of GST-II using two other nonoverlapping sequences, and the value was 3.44 in up-regulation. An overlapped peptide between GST-I and GST-II showed that its heavy isotope peak was 7.36 times higher than the light isotope peak. We subsequently subtracted the value of 3.44, which was contributed from GST-II, from the GST-I and GST-II combined induction value of 7.36. Therefore, GST-I was found to be activated by 3.92 times in \(S. \text{ pombe}\) on the exposure to Cd\(^{2+}\). GST-III was 1.63 times up-regulated with the two distinguishable peptide sequences (52–73 and 212–226).

The Systematic Comparison Between \(S. \text{ pombe}\) and \(S. \text{ cerevisiae}\)—Cd\(^{2+}\) exerts its toxic effect either directly by blocking functional groups of biologically important molecules or indirectly by producing ROS and subsequent damages in the cellular components (7, 8, 46). Consequently, cells need to activate both biological machineries to remove the Cd\(^{2+}\) and the produced ROS and to repair the damaged cellular components. \(S. \text{ cerevisiae}\) has long been used as a model system to study a wide variety of cell biology problems including cellular responses to heavy metals (Ref. 16 and references therein). It has been shown there are both some similarities in common environmental response/environmental stress response genes (38, 51) and also significant differences in the mechanism for scavenging Cd\(^{2+}\) between \(S. \text{ pombe}\) and \(S. \text{ cerevisiae}\) (11, 13). Once again, it should be underscored the relevance of studying the cellular responses to stresses at the proteomic level because there are significant discrepancies between genomic and proteomic data and only proteomics can provide full information of proteins regarding posttranslational modifications, regulation of protein function by proteolysis, and composition of functional protein complexes (24–26).

Among 56 up-regulated proteins identified through our proteomic analysis, only eight were shown that their corresponding genes were up-regulated from total mRNA microarray analysis in \(S. \text{ cerevisiae}\) exposed to Cd\(^{2+}\) ions (37, 39). Furthermore, there is clear inconsistency in induction ratios even among proteins identified from both analyses. Likewise, only 23 among 106 up-regulated proteins in our study were matched to the corresponding genes induced in \(S. \text{ pombe}\) (38). Our proteomic result allows us to systematically compare the similarity and difference between these two yeast species in response to Cd\(^{2+}\) exposure and to draw useful conclusion with respect to those critical factors involved in this biological process. Among 106 up-regulated proteins in the \(S. \text{ pombe}\), only 21 proteins were matched to those in the \(S. \text{ cerevisiae}\) (37). These common proteins are mainly proteins with antioxidant properties and heat shock proteins, suggesting the two yeasts might similarly respond to the oxidative stress produced by Cd\(^{2+}\).

Yeasts and plants respond on the Cd\(^{2+}\) stress by the enhanced synthesis of GSH and PCs to immobilize the toxic metal ions (3, 11–14). Therefore, cells need to synthesize more cysteine for enhancing the synthesis of the Cd\(^{2+}\)-scavenging molecules, which are cysteine-rich peptides. Accordingly, \(S. \text{ cerevisiae}\) overexpressed the enzymes involved in the synthesis of cysteine from methione (37, 39). On the other hand, \(S. \text{ pombe}\) synthesizes cysteine not from methione but directly from O-acetyl serine through cysteine synthase (52). However, we were not able to identify the enzyme activated in our analysis. We therefore assayed the enzyme activity as shown in Fig. 4A. The enzyme activity did not show any detectable change in the control cells up to 72 h and was not different from those in the literatures (33, 53). Interestingly, this enzyme activity in the Cd\(^{2+}\)-treated cells was only 50% of that in the control cells. Although the enzyme activity showed a significant difference, there was no detectable difference in the amount of free cysteine in both cells (data not shown). On exposure to Cd\(^{2+}\), \(S. \text{ pombe}\) produced slightly increased
GSH and other thiol-containing molecules (130 and 160%, respectively), of which difference might be PCs, whereas those in control cells were not changed over the entire experimental period (Fig. 4, B and C). The overexpressations of the nonprotein thiols (mainly GSH and PCs) are well consistent with the notion that these molecules are actively involved in the cellular defense mechanism against Cd^{2+} (3, 11–14).

We also analyzed inorganic sulfide from the cells because certain yeasts and plants showed enhanced Cd^{2+} resistance when they produce higher level of sulfide (54–56). As expected, Cd^{2+}-treated cells produced almost three times more sulfide than the control cells, indicating there might be different mechanisms for the detoxification of Cd^{2+} between the two yeasts (Fig. 4D, see “Discussion”).

**DISCUSSION**

To understand systematically the cellular responses of a species to the heavy metal stress, we profiled quantitatively the *S. pombe* proteome exposed to Cd^{2+}. Our quantitative proteomic approach consists of three key steps (Scheme 1): i) introduction of AACT using a stable isotope-labeled amino acid (Leu-d3) in the culturing medium, ii) 1D SDS-PAGE and subsequent microcapillary LC separation, and iii) identification and concurrent quantification of differentially expressed proteins under the Cd^{2+} stress through MS/MS. In general, our method allows for high-throughput analysis of multiple time-resolved changes in the *S. pombe* proteome from Cd^{2+} stress. There are several advantages using our in vivo AACT quantitative proteomic method: i) it does not require any additional chemical modification steps because the labeled amino acid is naturally incorporated into cellular proteins during cell growth, ii) there is a greater flexibility to select an amino acid to label proteins, which is limited to only cysteine residue in the case of the ICAT method (27), iii) it increases specificity and accuracy in assigning the labeled peptide, which commonly lack in uniform labeling methods due to the variable number of nitrogen atoms in labeled peptides (28), iv) the increased sequence coverage brought by larger label distribution has lead to identifications of isoforms in a same enzyme/protein family, and v) proteins with extreme pl or molecular masses were able to be quantitated and identified. In addition, equal numbers of cells are mixed in our experiment instead of equal quantities of proteins as required in other methods, resulting in further elimination of artifacts, which commonly occur during sample preparation processes. In comparison to 2D-PAGE-based quantitative approaches, which have been commonly used in comparative proteomics, the protein separation through 1D SDS-PAGE allows us both to significantly reduce the experimental time and to recover an intact protein profile regardless their pl values (Supplementary Fig. S1B). To compensate the insufficient resolving power of 1D SDS-PAGE, on-line capillary LC-MS/MS was used to increase the resolution and identification efficiency of peptide mixtures (Figs. 2 and 3). Up to 35 proteins from a single band...
failure occurred due to low expression level of the enzyme, we analyzed 20 proteins from a 2D gel, which are positioned around a possible location of the cysteine synthase (43 kDa and 7.63 pl). However, none of them matched to the enzyme, suggesting its extremely low expression level (data not shown). We further measured the activity of cysteine synthase in the cells before and after Cd$^{2+}$ treatment. The enzyme activity in the Cd$^{2+}$-treated cells was in fact lower than that in the control cells (Fig. 4A). However, the amounts of free cysteine in both cells were similar and not different from the values in the available literatures (35, 53). It might be possible that the low activity of the enzyme in *S. pombe* is still sufficient to supply the normal physiological level of cysteine. We also cannot rule out the possibility that *S. pombe* uses unidentified pathways to synthesize cysteine. With respect to how *S. pombe* cells produce the enhanced amounts of both GSH and PCs without increasing the synthesis of cysteine, this yeast species might reprogram its metabolism in the presence of Cd$^{2+}$, repressing the synthesis of some abundant cysteine-rich proteins and concomitantly replacing with low-cysteine isoforms and using the accumulated cysteine to synthesize both thiol molecules, as seen in *S. cerevisiae* (58). The Cd$^{2+}$-treated cells produced almost three times higher sulfide than the control cells (Fig. 4D). The increased thiol-containing molecules can initially accommodate the intracellular Cd$^{2+}$ ions and then incorporate inorganic sulfide to form nanocrystalline CdS-thiol complexes. All of the three components, GSH, PCs, and inorganic sulfide, seem to be essential for the high Cd$^{2+}$ tolerance in *S. pombe* because its mutants, which lack any component of the three, showed much more sensitivity to Cd$^{2+}$ than wild-type cells (59–61).

Our quantitative analysis data revealed 28 up-regulated proteins either to detoxify ROS produced by Cd$^{2+}$ or to repair the cellular components damaged directly by Cd$^{2+}$ or its derived ROS (Supplementary Fig. S2A and Table II). Our data also showed a similarity in the responses for the detoxification of ROS and for the repair of the damaged cellular components in the both yeast species (37). Both mitochondrial and cytoplasmic superoxide dismutases and a catalase were up-regulated in the Cd$^{2+}$ -treated cells for the direct removal of ROS. Glutaredoxin, thioredoxin, thioredoxin reductase, thioredoxin peroxidase, GST, and peroxisomal membrane protein pmp20 were significantly up-regulated in the cells treated with Cd$^{2+}$ for the repair of the cellular macromolecules damaged by ROS. In addition to the proteins, of which functions are clearly known in either Cd$^{2+}$ or oxidative stress, many heat shock proteins were up-regulated, whose functions are possibly related to a wide range of stress conditions (Table II). Furthermore, we identified as many as five hypothetical proteins among the up-regulated proteins, whose functions are not yet assigned and might be related to the detoxification of Cd$^{2+}$ (Supplementary Fig. S2A).

In summary, for the first time, we report here a global overview of the cellular responses to Cd$^{2+}$ at the proteomic
levels in the fission yeast \textit{S. pombe}. Our experiments unambiguously revealed 106 up-regulated proteins on exposure to Cd\(^{2+}\), which are the detoxification proteins for ROS, the repair proteins for the cellular constituents damaged by either Cd\(^{2+}\) or ROS, and heat shock proteins. Some of these identifications were also validated using various biochemical assays. \textit{S. pombe} cells produced significantly higher inorganic sulfide as a direct mechanism for scavenging free Cd\(^{2+}\) ions as a nanocrystalline complex, CdS-GSH/PCs.

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