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단 보

Reactive oxygen species-dependent down-regulation of ubiquitin C-terminal hydrolase in *Schizosaccharomyces pombe*

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*Schizosaccharomyces pombe*에서의 유비퀴틴 C-말단 가수분해효소의 활성산소종 의존성 하향조절

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ABSTRACT: The *Schizosaccharomyces pombe sdu1*$^+$ gene, belonging to the PPPDE superfamily of deubiquitinating enzyme (DUB) genes, was previously shown to encode a protein with ubiquitin C-terminal hydrolase (UCH) activity and to participate in the response against oxidative and nitrosative stresses. This work focused on the reactive oxygen species (ROS)-dependent regulation of the *S. pombe sdu1*$^+$ gene. UCH activities, encoded by the *sdu1*$^+$ gene, were attenuated in the *S. pombe* cells exposed to H_2O_2, superoxide radical-generating menadione (MD), and nitric oxide (NO)-generating sodium nitroprusside (SNP). Reduced glutathione (GSH) and its precursor *N*-acetylcysteine (NAC) were able to significantly enhance the UCH activities in the absence or presence of H_2O_2. However, the influences of both GSH and NAC on the ROS levels in the absence or presence of H_2O_2 were opposite to their effects on the UCH activities under the same conditions. The UCH activities in the Sdu1-overexpressing *S. pombe* cells were also diminished under exposure to H_2O_2, MD and SNP, but still remained to be higher than those in the vector control cells. In brief, it is proposed that the *S. pombe sdu1*$^+$ gene is regulated by ROS in a negative manner, the meaning of which largely remains elusive.

Key words: *sdu1*$^+$, *Schizosaccharomyces pombe*, deubiquitinating enzyme, reactive oxygen species, ubiquitin C-terminal hydrolase

Ubiquitination is a reversible post-translational modification which is implicated in a variety of cellular functions including diverse signal transduction cascades. It can cause significant changes in the activity, subcellular localization, protein-protein interactions or stability of the targeted protein. Deubiquitination, originally meaning the reverse of ubiquitination, also plays crucial roles in the regulation of numerous physiological pathways, including stabilization of crucial regulatory proteins (Kim *et al.*, 2003). The release of ubiquitin from attachment to other proteins and adducts is important for ubiquitin biosynthesis, proteasomal degradation and other cellular processes (Johnston *et al.*, 1999). Deubiquitinating enzymes (DUBs; also known as deubiquitylating enzymes or deubiquitinases) process ubiquitin or ubiquitin-like gene products, reverse the modification of proteins by a single ubiquitin, and remodel polyubiquitin chains on target proteins. Accordingly, DUBs promote accumulation of monomeric ubiquitin and counteract the effects of ubiquitin conjugation by removing the polyubiquitin chain from conjugated proteins before their degradation by proteasome (Rolfe *et*

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al., 1997). DUBs also play diverse regulatory roles in chromatin remodeling through histone deubiquitination, gene expression, cell cycle regulation, DNA repair, activation of kinases and other enzymes, apoptosis, microbial pathogenesis, localization and degradation of signaling intermediates, and endocytosis (Reyes-Turcu *et al.*, 2009; Ramakrishna *et al.*, 2011).

DUBs are classically divided into five families, such as ubiquitin-specific proteases (USPs, also referred to as ubiquitin processing proteases), ubiquitin C-terminal hydrolases (UCHs), ovarian tumor proteases (OTUs, also known as otubain proteases), Machado-Joseph disease proteases (MJDs, also referred to as Josephins), and JAB1/MPN/MOV34 motif proteases (JAMMs) (Komander *et al.*, 2009; Tse *et al.*, 2009). USPs, UCHs, OTUs, and MJDs are cysteine proteases, whereas JAMMs are zinc-dependent metalloproteinases (Komander *et al.*, 2009). Two additional families, such as PPPDE (after Permuted Papain fold Peptidases of DsRNA viruses and Eukaryotes) superfamily and Wss1-like metalloproteases, were afterward discovered from bioinformatics studies (Iyer, 2004).

USP and UCH are the best characterized DUBs. USPs constitutes the largest family that contains more than 60 members, and the amino acid sequences of USPs exhibit a lower level of conservation than those of UCHs (Colland, 2010). UCHs principally participate in the processing and recycling of ubiquitin, but their specific functions remain largely unknown. The three UCH isoforms, known as UCH L1, UCH L3, and UCH L5/UCH37, are distinct in their substrate specificities for ubiquitin-fusions and diubiquitin chains (Yin *et al.*, 2011). UCH L5 can process polyubiquitin chain when it is bound to proteasome, whereas both UCH L1 and UCH L3 cannot (Maiti *et al.*, 2011).

Throughout a previous work, the *sdu1*$^+$ gene, known as a member of the PPPDE peptidase superfamily of DUBs (Iyer *et al.*, 2004), was cloned from the fission yeast *Schizosaccharomyces pombe* and shown to encode an UCH activity (Kim *et al.*, 2013). Sdu1 is regarded as the first example of a PPPDE superfamily member with UCH activity. It was confirmed to participate in the response against oxidative and nitrosative stresses in *S. pombe* by modulating the reactive oxygen species (ROS) and total glutathione (GSH) levels (Kim *et al.*, 2013). In the present work, we demonstrate that the UCH activity, encoded by the *S. pombe sdu1*$^+$ gene, is negatively regulated by ROS. However, the ROS-dependent negative regulation of the *S. pombe sdu1*$^+$ gene would not be due to a general mechanism which might also be applied to all types of enzyme-coding genes.

Bovine serum albumin (BSA), dithiothreitol, hydrogen peroxide (H_2O_2), menadione (MD), sodium nitroprusside (SNP), Bradford reagent, 2',7'-dichlorodihydrofluorescein-diacetate (DCFH-DA), reduced glutathione (GSH), and *N*-acetylcysteine (NAC) were purchased from Sigma Chemical Co. Ubiquitin 7-amido-4-methylcoumarin (Ub-AMC) was from Enzo Life Sciences. Yeast extract, peptone, and agar were obtained from Amersham Life Science. All other chemicals used were of the highest grade commercially available.

S. pombe KP1 (*h*$^+$ *leu1-32 ura4-294*), a derivative of *S. pombe* heterothallic haploid strain 975*h*$^+$, was used in this work. The Sdu1-overexpressing recombinant plasmid pYSTP was previously constructed using a yeast-*E. coli* shuttle plasmid vector pRS316 (Myers *et al.*, 1986). The yeast cells were grown in yeast extract peptone dextrose medium (pH 6.5) which contains 1% yeast extract, 2% peptone, and 1% glucose. The yeast cells were incubated with shaking at 30°C, and their growth was monitored by measuring absorbance at 600 nm. Yeast cells used in the experiments were obtained preferentially from the early exponential growth phase.

The desired number of the yeast cells was harvested by centrifugation. They were re-suspended in 20 mM Tris buffer (pH 8.0) with 2 mM EDTA and disrupted using a glass bead beater. Cellular extracts were obtained after centrifugation and were used for UCH activity and protein determinations.

To determine intracellular ROS levels, the redox-sensitive fluorescent probe DCFH-DA was used as previously described (Royall and Ischiropoulos, 1993). When DCFH-DA enters the cells, its diacetate group is cleaved by an esterase, leaving a non-fluorescent molecule, which is amenable to oxidization to fluorescent dichlorofluorescein (DCF) in the presence of ROS (Kiani-Esfahani *et al.*, 2012). Yeast cells were reacted with 5 μM DCFH-DA for 30 min at 30°C. The treated cells were analyzed immediately using a microplate fluorometer (Multi-Mode Microplate Reader; Synergy^{TM} Mx, BioTek Instruments).

Ubiquitin C-terminal hydrolase (UCH) activities in cellular extracts were determined using Ub-AMC as an artificial substrate, which releases the fluorogenic AMC component by

cleaving the bond between the C-terminus of ubiquitin and AMC (Yi *et al.*, 2007). An enzymatic reaction with some modifications was carried out for 10 min at 25°C in a reaction mixture containing 50 mM HEPES (pH 7.8), 0.5 mM EDTA, 0.1 mg/ml BSA, 1 mM dithiothreitol, 0.5 μM Ub-AMC, and cellular extract. The fluorescence of an AMC moiety was monitored by spectrofluorometry at the wavelengths of 355 nm for excitation and 440 nm for emission. UCH activity assay was repeated at least three times. Protein content in cellular extracts was determined by Bradford's procedure (1976) with BSA as a standard.

The results are reported as mean ± standard deviation (SD). Statistical comparisons between experimental groups were performed using unpaired Student's *t*-test. A *P* value less than 0.05 was considered statistically significant.

To preliminarily test whether UCH activity, one of the major DUB activities, is subject to an ROS-dependent control in *S. pombe* cells, the cells in the early exponential growth phase were exposed to 100 μM H_2O_2, 10 μM MD or 10 μM SNP for 6 h. UCH activities in the cellular extracts were determined using Ub-AMC and compared. As shown in Fig. 1, the UCH activities of the *S. pombe* cells under H_2O_2, MD or SNP were significantly lower than that of the non-treated cells. In the presence of H_2O_2, the UCH activities of the *S. pombe* cells dropped to about 60% of that of the non-treated cells (Fig. 1). In brief, this finding suggests the plausible ROS-dependent down-regulation of the UCH activity in *S. pombe.*

To further verify the ROS-dependent control of the UCH activity in *S. pombe* cells, H_2O_2 was chosen to perform similar experiments using antioxidant components. Both GSH and NAC, a precursor of GSH, at the concentrations of 300 μM could enhance the UCH activities of *S. pombe* cells to 1.5- and 1.3-fold, respectively, compared with that of the non-treated cells (Fig. 2; lanes 1, 3, and 4). When the *S. pombe* cells were subjected to H_2O_2 together with GSH and NAC, they could significantly enhance the UCH activities, markedly diminished by H_2O_2, to 2.1- and 1.8-fold, respectively, compared with the cells treated with H_2O_2 only (Fig. 2; lanes 2, 5, and 6). This finding suggests that antioxidant components play up-regulating roles in the UCH activity of *S. pombe* cells, irrespective of oxidative stress-inducing substance(s).

After the *S. pombe* cells were subjected to H_2O_2 and/or antioxidant components, such as GSH and NAC, the intracellular ROS levels in the *S. pombe* cells were determined and compared to assess the ROS-dependent control of the *sdu1*$^+$ gene. The ROS levels of the *S. pombe* cells exposed to H_2O_2 only went up to about 2.4-fold, compared with that of the non-treated cells (Fig. 3; lanes 1 and 2). On the contrary, GSH

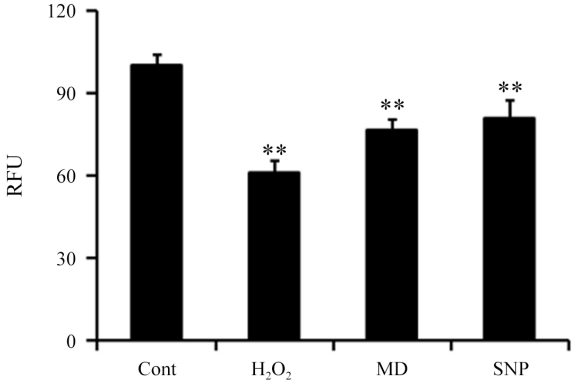

Fig. 1. Diminishment in the UCH activities of *S. pombe* cells under oxidative and nitrosative stresses. The *S. pombe* cells in the early exponential phase were subjected to hydrogen peroxide (H_2O_2, 100 μM), a superoxide generator menadione (MD, 10 μM) and nitric oxide (NO)-generating sodium nitroprusside (SNP, 10 μM) for 6 h. The UCH activities in the cellular extracts were determined using a fluorogenic substrate ubiquitin-7-amino-4-methylcoumarin (ubiquitin-AMC). Relative fluorescence unit, abbreviated as RFU, represents the specific activity of UCH. ** $P < 0.01$, compared with the non-treated control cells (Cont).

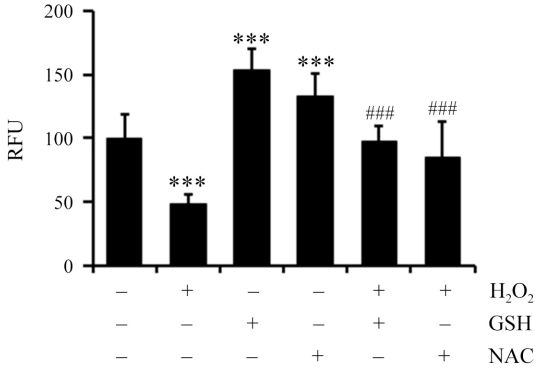

Fig. 2. Enhancing effects of glutathione (GSH) and *N*-acetylcysteine (NAC) on the UCH activity diminished in *S. pombe* cells under oxidative stress. The *S. pombe* cells in the early exponential phase were subjected to hydrogen peroxide (H_2O_2, 100 μM) in the absence or presence of GSH (300 μM) and NAC (300 μM) for 6 h. The cells were also exposed to GSH or NAC only. The UCH activities in the cellular extracts were determined using a fluorogenic substrate ubiquitin-7-amino-4-methylcoumarin (ubiquitin-AMC). Relative fluorescence unit, abbreviated as RFU, represents the specific activity of UCH. *** $P < 0.001$, compared with the non-treated control cells (Cont). ### $P < 0.001$, compared with the H_2O_2-subjected only.

or NAC only could make the ROS levels of the *S. pombe* cells insignificantly lower than that of the non-treated cells (Fig. 3; lanes 1, 3, and 4). When GSH and NAC were treated to the *S. pombe* cells together with H_2O_2, they diminished the ROS levels to 58.5% and 63.7%, respectively, of that of the cells treated with H_2O_2 only, respectively (Fig. 3; lanes 2, 5, and 6). These findings resemble a typical pattern that H_2O_2 and antioxidant components affect the ROS levels in various cell types. Here in this work, the ROS levels in Fig. 3 and the UCH activity levels in Fig. 2 were changed in the opposite direction in the presence of GSH, NAC and/or H_2O_2.

Next, changes in the UCH activities, encoded by the *sdu1*$^+$ gene, were compared in the Sdu1-overexpressing *S. pombe* cells and the vector control cells under stress conditions. As shown in the previous report (Kim *et al.*, 2013), the *S. pombe* cells harboring pYSTP were found to contain the UCH activity 3.4-fold higher than the vector control cells (Fig. 4; open and closed bars, lane 1). The UCH activities of the *S. pombe* cells harboring pYSTP were attenuated to 44.8%, 62.1%, and 58.6% of those from the non-treated cells, respectively, under exposure to 100 μM H_2O_2, 10 μM MD, and 10 μM SNP for 6 h (Fig. 4, closed bars). However, under H_2O_2, MD, and SNP, the UCH activities of the *S. pombe* cells harboring pYSTP were still maintained to be 2.6-, 2.3-, and 2.0-fold higher than those from the corresponding vector control cells, respectively (Fig. 4). These results imply that the UCH activities of the Sdu1-overexpressing *S. pombe* cells are also subject to an ROS-dependent control under stress conditions.

DUBs catalyze the hydrolysis of isopeptide or peptide linkages joining ubiquitin to substrate lysine or *N*-termini, and subsequently play a crucial role in ubiquitin signaling and employ diverse mechanisms to regulate their activities (Wolberger, 2014). DUB activities are known to be regulated by post-translational modification such as phosphorylation, ubiquitination and sumoylation, allosteric interactions and subcellular localization (Komander *et al.*, 2009). In addition, DUBs can regulate their catalytic activities by directly interacting with and co-regulating E3 ligases, altering the level of substrate ubiquitination, hydrolyzing or remodeling ubiquitinated and polyubiquitinated substrates, acting in specific locations in the cell and altering the localization of the target protein, and acting on proteasome-bound substrates to facilitate or inhibit proteolysis (Eletr and Wilkinson, 2014). One example of post-translational regulation of DUBs is that ataxin-3 directly enhances catalytic activity by ubiquitinating itself, which proposes a new function for protein ubiquitination in regulating the activities of certain DUBs (Todi *et al.*, 2009).

Until recently, a limited number of findings on the regulation of UCH activities have been revealed. UCH L1, expressed in a number of malignancies, hydrolyzes carboxy terminal esters

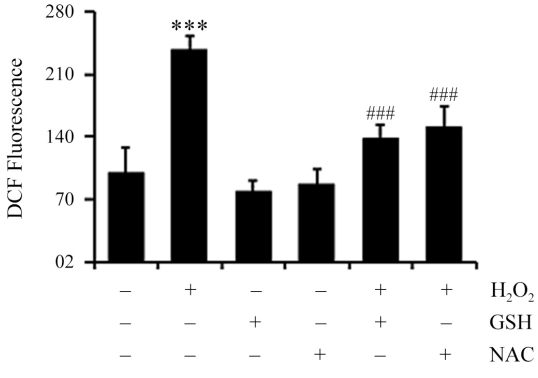

Fig. 3. Diminishing effects of glutathione (GSH) and *N*-acetylcysteine (NAC) on the reactive oxygen species (ROS) levels enhanced in *S. pombe* cells under oxidative stress. The *S. pombe* cells in the early exponential phase were subjected to hydrogen peroxide (H_2O_2, 100 μM) in the absence or presence of GSH (300 μM) and NAC (300 μM) for 6 h. The cells were also exposed to GSH or NAC only. The intracellular ROS levels were detected using DCFH-DA, and represented as relative DCF fluorescence, an arbitrary unit. *** $P < 0.001$, compared with the non-treated control cells (Cont). ### $P < 0.001$, compared with the H_2O_2-subjected only.

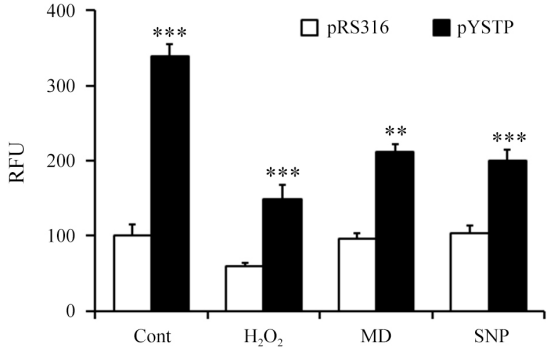

Fig. 4. Changes in the UCH activities in the *S. pombe* cells harboring pYSTP and the vector control cells under oxidative and nitrosative stresses. The *S. pombe* cells harboring pYSTP or pRS316 in the early exponential phase were subjected to hydrogen peroxide (H_2O_2, 100 μM), a superoxide generator menadione (MD, 10 μM), and nitric oxide (NO)-generating sodium nitroprusside (SNP, 10 μM) for 6 h. The UCH activities in the cellular extracts were determined using a fluorogenic substrate ubiquitin-7-amino-4-methylcoumarin (ubiquitin-AMC). Relative fluorescence unit, abbreviated as RFU, represents the specific activity of UCH. ** $P < 0.01$; *** $P < 0.001$ versus the corresponding pRS316-containing cells.

and amides of ubiquitin and possesses ubiquitin ligase activity and functions as a monoubiquitin stabilizer (Bheda *et al.*, 2009). It is produced constitutively in human neural cell lines, and upregulated following induction of neuronal differentiation, implying its participation in the nigral neuronal death and survival in Parkinson's disease (Satoh and Kuroda, 2001). When human NT2/D1 embryonal carcinoma cells are exposed to global metabolic changes due to energy withdrawal and the subsequent generation of ROS by oxygen-glucose deprivation (OGD) and reoxygenation, UCH L1 undergoes a bi-phasic change through down-regulation of its expression immediately after OGD treatment and its recovery and enhancement 6 h after OGD treatment as well as during reoxygenation, suggesting that UCH L1 has an essential role in maintaining cell homeostasis under oxidative stress conditions (Shen *et al.*, 2006). UCH L1, which is involved in the degradation of unwanted, misfolded or damaged proteins within cells, is overexpressed in > 50% of lung cancers, and its overexpression also in chronic smokers may represent an early event in the complex transformation from normal epithelium to overt malignancy (Carolan *et al.*, 2006). In the lung cancer cell line H1299, UCH L1 activity is anti-proliferative and its expression is induced as a response to tumor growth (Liu *et al.*, 2003). Although some DUBs with cysteine protease activity was suggested to be sensitive to redox regulation by ROS or RNS (Komander *et al.*, 2009), it has not been clarified. However, the detailed examples of ROS-dependent control of DUBs have been rarely reported. At the level of activity control, USP1, a key regulator of genomic stability, is reversibly inactivated through the oxidation of catalytic cysteine residue by ROS, proposing that DUBs of the cysteine protease family acts as ROS sensors and that ROS-mediated DUB inactivation is a crucial mechanism for fine-tuning stress-activated signaling pathways (Cotto-Rios *et al.*, 2012).

In this communication, we demonstrate that UCH activity, possibly encoded by the *sdu1*$^{+}$ gene belonging to the PPPDE peptidase superfamily which has not been extensively studied, is regulated by ROS in a negative manner. Further studies at protein and mRNA levels would offer the detailed knowledge on the ROS-dependent regulation of UCHs and its functional significance.

적 요

탈유비퀴틴 효소 중 PPPDE 상과에 속하는 *Schizosaccharomyces pombe*의 *sdu1*$^{+}$ 유전자가 유비퀴틴 C-말단 가수분해 효소 활성을 갖는 단백질을 인코딩하고, 산화적 및 일산화질소 스트레스 방어에 관여함이 이전에 밝혀진 바 있다. 예비적인 본 연구는 정상적인 및 과잉발현의 조건에서 *S. pombe* 유비퀴틴 C-말단 가수분해 효소 활성의 활성산소종 의존성 조절에 초점을 맞추었다. 과산화수소, 수퍼옥사이드 라디칼 생성하는 메나디온 및 일산화질소 생성하는 sodium nitroprusside (SNP)에 노출시킨 *S. pombe* 세포에서 유비퀴틴 C-말단 가수분해 효소 활성이 감소되었다. 환원형 글루타치온과 그 전구체인 *N*-acetylcysteine은 과산화수소의 존재 유무에 상관없이 유비퀴틴 C-말단 가수분해 효소 활성을 현저하게 증강시켰다. 그러나, 과산화수소의 부재 시 혹은 존재 시 활성산소종에 미치는 글루타치온과 *N*-acetylcysteine의 영향은 같은 조건 하에서의 유비퀴틴 C-말단 가수분해 효소 활성 패턴과 상반되었다. 과잉발현의 유비퀴틴 C-말단 가수분해 효소 활성을 보이는 재조합 플라즈미드 pYSTP를 보유하는 *S. pombe* 세포에서 유비퀴틴 C-말단 가수분해 효소 활성도 과산화수소, 메나디온 및 SNP에의 노출되는 조건에서 감소되었지만, 벡터 대조 세포에서 보다는 높게 유지되었다. 요약하면, *S. pombe* 유비퀴틴 C-말단 가수분해 효소 활성은 활성산소종에 의하여 하향조절 되지만, 그 의의는 현재로썬 알려지고 있지 않은 상태이다.

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