Biochimica et Biophysica Acta 1823 (2012) 2217–2226

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Selenoprotein W promotes cell cycle recovery from G2 arrest through the activation of CDC25B

Yong Hwan Park, Yeong Ha Jeon, Ick Young Kim *

Laboratory of Cellular and Molecular Biochemistry, School of Life Sciences and Biotechnology, Korea University, 1, 5-Ka, Anam-Dong, Sungbuk-Ku, Seoul 136-701, Republic of Korea

ARTICLE INFO

Article history:
Received 30 July 2012
Received in revised form 4 September 2012
Accepted 5 September 2012
Available online 12 September 2012

Keywords:
Selenoprotein W
14-3-3
Cell cycle
G2 arrest
Cdk1
CDC25B

ABSTRACT

Selenoprotein W (SelW) contains a highly reactive selenocysteine (Sec; U) in the CXXU motif corresponding to the CXXC motif in thioredoxin (Trx) and thus it appears to be involved in regulating the cellular redox state. Recent reports on the interaction between SelW and 14-3-3 suggest that SelW may be redox dependently involved in the cell cycle. However, the precise function of SelW has not yet been elucidated. Here, we show that SelW is involved in the G2–M transition, especially in the recovery from G2 arrest after deoxyribonucleic acid (DNA) damage. Knockdown of SelW significantly accumulated phosphorylated cyclin-dependent kinase (Cdk1), which eventually led to a delay in recovery from G2 arrest. We also found that inactive Cdk1 is caused by the sustained inactivation of CDC25B, which removes the inhibitory phosphate from Cdk1. Our observation from this study reveals that SelW activated CDC25B by promoting the dissociation of 14-3-3 from CDC25B through the reduction of the intramolecular disulfide bond during recovery. We suggest that SelW plays an important role in the recovery from G2 arrest by determining the dissociation of 14-3-3 from CDC25B in a redox-dependent manner.

1. Introduction

Selenoproteins contain selenium in the form of Sec, and their abnormal expression is associated with various diseases, such as cancer [1]. In humans, 25 selenoproteins have been identified, some selenoproteins have been shown to display antioxidant activity [2,3]. Although their tissue distribution indicates that each selenoprotein possesses a novel function, not all selenoproteins have been fully characterized.

SelW is a low molecular weight (10 kDa) selenoprotein, and it contains a single Sec at the 13th amino acid position. SelW is expressed in several tissues and is especially abundant in the skeletal muscle and brain [4] and is known to be localized in the cytosol [5]. Because SelW contains a conserved CXXU motif, which corresponds to the CXXC motif in Trx, and a glutathione (GSH) binding site at Cys 37, it may be involved in cellular redox regulation. Previously, we suggested that SelW displays GSH-dependent antioxidant activity *in vivo*, and Sec was shown to be necessary for this activity [6]. We also reported that the sensitivity of mouse embryonic neuronal cells against oxidative stress was increased by SelW knockdown and that the expression pattern of SelW differed from that of Trx during brain development [7]. It has been suggested that SelW interacts with 14-3-3 proteins in NIH3T3 cells, which are involved in various cellular processes, including the cell cycle [8–10]. In addition, SelW may also modulate cell cycle entry [11]. Although the possibility of SelW involvement in cell cycle regulation was proposed, the precise function of SelW in the cell cycle has not been established. In this study, we found that knockdown of SelW delayed the G2–M transition in the cell cycle and caused significant retardation of recovery from G2 arrest, indicating that SelW may be involved in the G2–M transition.

The G2–M transition of the cell cycle is tightly regulated by the Cdk1/cyclin B1 complex. Cdk1 is kept in an inactive state by phosphorylation at Thr 14 and Tyr 15, which is catalyzed by the Myt1 and Wee1 kinases, respectively. At the G2–M transition, CDC25 phosphatases, which are dual-specificity phosphatases, dephosphorylate both Thr 14 and Tyr 15, resulting in the activation of the Cdk1/cyclin B1 complex and entrance into mitosis [12,13]. Under genotoxic stress conditions, cell cycle progression is halted to allow time for DNA repair, thus, resulting in the maintenance of genomic stability [14]. To prevent transmission of damaged genetic material to daughter cells, entry into mitosis is controlled. To achieve this, checkpoint kinases regulate the activity of Cdk1 [15]. However, when DNA repair is completed, cells inactivate the checkpoint kinases and resume the normal cell cycle, which is referred to as recovery [16]. Although the mechanism of recovery from G2 arrest is not well understood when compared to that of cell cycle arrest, the degradation of Wee1 kinase mediated by polo-like kinase-1 and the activation of the CDC25B isoform, not CDC25A or CDC25C, are essential for resumption of the cell cycle from G2 arrest [17]. CDC25B contains an active Cys in its catalytic site, which can form intramolecular disulfide bonds, and is regulated in a redox-dependent manner [18]. However, the exact

Abbreviations: BIAM, biotin conjugated IAM; DCFH-DA, 2', 7'–dichlorofluorescein diacetate; DTT, dithiothreitol; GSH, glutathione; IAM, iodoacetamide; IR, irradiation; NAC, N-acetyl cysteine; PTEN, phosphatase and tensin homolog; ROS, reactive oxygen species; s.d., standard deviation; Sec,U, Selenocysteine; SECIS, selenocysteine insertion sequence; Trx, thioredoxin

* Corresponding author. Tel./fax: +82 2 3290 3449.
E-mail address: ickkim@korea.ac.kr (I.Y. Kim).

0167-4889/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamcr.2012.09.001

mechanism by which the regulation of CDC25B *in vivo* is redox dependent during the cell cycle is largely unknown. In addition, because the activity of CDC25B was shown to be negatively regulated by interactions with 14-3-3 proteins [19,20], which also interact with SelW, we sought to determine whether the complex between SelW and 14-3-3 functions in regulating the activity of CDC25B during cell cycle recovery. The dissociation of 14-3-3 proteins and the reduction of intramolecular disulfide bonds are required for the activation of CDC25B. A CDC25C mutant incapable of forming intramolecular disulfide bonds bound 14-3-3 to a lesser degree than the wild type [21], suggesting that there is a relationship between the formation of intramolecular disulfide bonds and the association of 14-3-3 with CDC25B. Here, we show that SelW interacts with CDC25B and their complex formation is increased after genotoxic stress; downregulation of SelW leads to the accumulation of CDC25B with intramolecular disulfide bonds. In addition, CDC25B immunoprecipitation analysis shows that SelW may regulate the dissociation of 14-3-3 from CDC25B. Taken together, we suggest a redox mechanism of SelW by which it reactivates CDC25B in the G2–M transition, especially during the recovery process. In addition, we also found that SelW knockdown cells were more sensitive to anticancer drugs. These findings suggest that SelW might be a potential therapeutic target for cancer cells overexpressing CDC25B and may increase the efficacy of anticancer agents.

2. Materials and methods

2.1. Cell culture, transfection and synchronization

NIH3T3 cells, mouse embryonic fibroblast cells, were cultured in DMEM supplemented with 10% FBS, and MCF7 cells, human breast adenocarcinoma cells, were grown in RPMI supplemented with 10% FBS at 37 °C in 5% CO_2. For transient transfection, electroporation of NIH3T3 or MCF7 cells was performed using a Neon transfection system (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After transfection, NIH3T3 cells were subsequently incubated with thymidine (2.5 mM) for 24 h to arrest cells at the G1/S transition. The G2–M checkpoint was activated by a Cs 137 irradiator (8 Gy) or etoposide (5 μM) for 1 h at 3.5 h after release from the thymidine block. Cells were harvested at the indicated time points. For MCF7 cells, cells were subsequently incubated with thymidine (2.5 mM) for 16 h and then incubated with fresh medium for 9 h. The cells were then incubated with thymidine for 16 h. 5 h after release, the cells were treated with IR (8 Gy).

2.2. RNA interference and plasmids

siRNA corresponding to the human and mouse SelW gene was designed and synthesized by Invitrogen (Stealth RNAi). Stealth RNAi Negative Control Duplexes (Invitrogen) were used as a control in the immunoprecipitation and immunoblot analysis. The sequences used are listed in Table S1. A point mutation of SelW at Sec13 to Cys or Ser was generated by site directed mutagenesis of the cDNA by polymerase chain reaction (PCR) using the pcDNA 3.1+/SelW plasmid [6]. Mutant cDNA was subcloned into the pcDNA3.1+/HIS C vector. HA-14-3-3β was a gift from Prof. J. H. Hong (Korea University). CDC25B1-flag was a gift from Dr. K. Yamashita (Kanazawa University).

2.3. Antibodies and western blotting

Cells were lysed with lysis buffer containing 150 mM NaCl, 1% NP-40, 0.1% SDS, 50 mM Tris (pH7.4), 1 mM EDTA, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin and 3 mM DTT. Whole cell lysates were separated with 6~12% SDS PAGE. Separated proteins were transferred to a membrane, which was blocked subsequently with 5% skim milk for 30 min and then probed with antibodies specific for pan-14-3-3, CDC25B, WEE1, Cdk1, cyclin B1 (Santa Cruz, Santa Cruz, CA, USA), phospho-CDC2 (Tyr15), phospho-Chk1 (Ser317), Chk1, phospho-histone H3 (Ser10) (Cell Signaling, Danvers, MA, USA), CDC25B (Epitomics, Burlingame, CA, USA), His, HA (abm, Richmond, BC, CANADA), flag (Sigma, St. Louis, MO, USA), α-tubulin (AB Frontier, Daejeon, Korea).

For detection of SelW, cell lysates were incubated with LDS sample buffer and reducing reagent (Invitrogen) at 70 °C for 10 min. NuPAGE 12% Bis-Tris gel (Invitrogen) and SelW antibody were used. For detection of phospho-ATM and ATM, NuPAGE 3-8% Tris-Acetate gel (Invitrogen) and phospho-ATM (Ser1981) (Novus, Littleton, CO, USA), ATM (Genetex, Irvine, CA, USA) antibodies were used.

In the immunoblot for γ-H2AX, cells were washed with cold PBS and resuspended with lysis buffer containing 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA (pH 8.0) and 0.5% NP-40 and then incubated on ice for 15 min. After centrifugation at 6000×*g* for 5 min at 4 °C, the supernatant was removed and the pellet was washed twice with lysis buffer. The pellet was resuspended in 0.1 M HCl and incubated for 10 min at RT. Histone extracts were obtained by centrifugation at 6000×*g* for 5 min at 4 °C and the concentration was measured using the Bradford method. Histone extracts were boiled with sample buffer for 10 min and separated with 15% SDS PAGE and immunoblotted with an anti-γ-H2AX antibody (Epitomics).

2.4. Immunoprecipitation

Cells were lysed with immunoprecipitation buffer (50 mM Tris–HCl pH7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 5 mM EDTA, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, phosphatase inhibitor 20 mM NaF and 2 mM Na_3VO_4). For immunoprecipitation, 600~800 μg of protein was mixed with antibodies overnight. The immune complexes were then incubated with protein bead A or G for 1.5 h. Beads were washed and then mixed with 2× SDS sample buffer and boiled for 3 min. The samples were loaded on SDS-PAGE and immunoblotted with specific antibodies.

2.5. Determination of the CDC25B and SelW redox state

Analysis of the CDC25B redox state was performed as previously described with slight modifications [22]. Briefly, NIH3T3 cells were pretreated with iodoacetamide (IAM) at a final concentration of 30 mM for 20 min prior to harvest to irreversibly alkylate free thiols. Cells were washed twice with cold PBS and lysed with immunoprecipitation buffer containing IAM. Excess IAM was removed by passing lysates though Zeba Spin Desalting Columns (Thermo Scientific, Waltham, MA, USA). Lysates were incubated with 3.5 mM DTT at RT for 30 min to reduce preexisting disulfide bonds and passed through a desalting column to exchange the buffer with a reaction buffer containing 100 mM Tris–HCl (pH 6.5) and 1 mM EDTA. The lysates were then incubated with 20 μM biotin-conjugated IAM (BIAM) (Invitrogen) at 37 °C for 30 min in the dark. CDC25B was subsequently immunoprecipitated, separated by 8% SDS-PAGE and immunoblotted with HRP-conjugated streptavidin (Invitrogen) to detect BIAM-alkylated CDC25B. Membranes were stripped with a buffer containing 63 mM Tris–HCl (pH 6.5) and 2% SDS for 45 min at 55 °C with gentle shaking and then reprobed with an anti-CDC25B antibody.

2.6. Flow cytometry

For cell cycle analysis, cells were fixed overnight in ice-cold 70% ethanol. Cells were collected by centrifugation and resuspended in PBS. Resuspended cells were treated with RNaseA (50 μg/ml) for 30 min at RT. Cells were then stained with propidium iodide (50 μg/ml). Subsequently, the cell cycle distribution was analyzed using a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA). To measure the intracellular ROS, cells were incubated with 20 μM 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) for 15 min and washed twice with cold PBS and