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Delayed cell cycle progression from SEPW1 depletion is p53- and p21-dependent in MCF-7 breast cancer cells

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ABSTRACT

Selenium (Se) is an essential redox-active trace element with close connections to cancer. Most of Se's biological functions have been attributed to the antioxidant properties of Se-containing proteins. However, the relative contribution of selenoproteins and small Se compounds in cancer protection is still a matter of debate. The tumor suppressor p53 is the most frequently mutated gene in human cancer and is often referred to as the "guardian of the genome". In response to genomic stresses, p53 causes cell cycle arrest to allow time for genomic damage to be repaired before cell division or induces apoptosis to eliminate irreparably damaged cells. Selenoprotein W (SEPW1) is a highly conserved small thioredoxin-like protein required for cell cycle progression. The present work shows that SEPW1 facilitates the G1 to S-phase transition by down-regulating expression of the cyclin-dependent kinase inhibitor p21. SEPW1 controls p21 by modulating levels of the p53 transcription factor, and this is associated with changes in phosphorylation of Ser-33 in p53. More work is needed to identify the mechanism by which SEPW1 regulates phosphorylation of Ser-33 and the kinase or phosphatase enzymes involved.

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1. Introduction

The essential trace element, selenium (Se) has been associated with cancer for nearly a century [1]. Dietary Se prevents chemically induced cancers in experimental animals [2] and higher dietary Se intakes are associated with decreased risk of cancer in humans [3]. The main biologically active form of Se in mammals is selenocysteine (Sec)-containing proteins, known collectively as selenoproteins. The mammalian selenoproteome consists of 25 conserved selenoproteins [4], the most well-known of which are the glutathione peroxidases that reduce hydrogen peroxide and/or organic hydroperoxides at the expense of glutathione (GSH). Supplemental Se and over-expression of selenoproteins protects against oxidant challenges, whereas Se-depletion and genetic deletion of selenoproteins makes animals and cells more sensitive to oxidative stress. Thus, anti-oxidation is assumed to be the main biological function of selenoproteins and the anti-cancer activity of Se has been hypothesized to arise from Se's antioxidant properties [5].

Se is the only element specified in the universal genetic code and Sec has become recognized as the 21st protein amino acid [6]. Sec is synthesized from serine after aminoacylation to a unique transfer RNA, $tRNA^{Sec}$, in all three superkingdoms of life [7]. Sec is subsequently incorporated into growing polypeptide chains under control of the UGA codon (TGA in DNA), which usually codes for termination. Incorporation of Sec is directed by a specific stem-loop structure in the mRNA 3′ to the UGA codon known as a "selenocysteine insertion sequence". All of the selenoproteins for which an enzymatic activity has been identified catalyze redox reactions involving oxidation of sulfhydryl groups and/or reduction of disulfides.

The microbial selenoproteome contains over 3600 members in approximately 58 families of homologous selenoproteins [8]. The most abundant and widespread selenoprotein family is "selenoprotein W-like" [9]. Selenoprotein W (SEPW1), the mammalian archetype of the selenoprotein W-like family, is a ubiquitous 9 kDa Sec-containing protein with glutathione-dependent antioxidant activity [10]. SEPW1 occurs in humans, mice, rats, sheep, monkeys, rabbits, guinea pigs, and cattle [11]. SEPW1 is expressed in all 22 human tissues examined, with highest levels in brain, testes, and muscle. SEPW1 is one of the most highly expressed selenoproteins in humans and it is regulated at the level of mRNA stability by Se intake [12]. SEPW1 expression is proportional to dietary Se intake, increasing markedly with Se supplements and decreasing rapidly on Se-restricted diets [13]. SEPW1 mRNA expression is cell cycle-dependent in human epithelial cells and silencing of SEPW1 expression causes cells to accumulate in G0/G1 phase of the cell cycle [14]. Homozygous SEPW1-knockout mouse embryos die at the pre-implantation blastocyst stage [15].

Abbreviations: Se, selenium; SEPW1, selenoprotein W1; Cys, cysteine; Sec, selenocysteine; Ser, serine; Thr, threonine; ROS, reactive oxygen species.

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The evolutionary conservation of SEPW1 and the embryonic lethality of its deletion suggest it has an important role in cell cycle regulation.

The p53 protein is a homotetrameric transcription factor that regulates expression of a wide variety of genes through direct binding to response elements in DNA. The best understood function of p53 is to respond to an array of cellular stresses, such as DNA damage, oncogene expression, nucleotide depletion, and aberrant growth signals by inducing cell cycle arrest, DNA repair, differentiation, senescence, or apoptosis. The p53 protein senses and integrates these various stresses via a panoply of post-translational modifications, including phosphorylation, acetylation, and ubiquitination. p53 protein is expressed constitutively, but levels are normally kept low by its rapid ubiquitination by the HDM2 protein and rapid proteasomal degradation. Following DNA damage and other stresses, the human p53 N-terminal region is phosphorylated on serines 6, 9, 15, 20, 33, and 37 and threonine 18 by ATM, ATR, DNA-PK, p38 MAPK, Chk1, and Chk2. Phosphorylation of p53 disrupts its binding with HDM2, blocks ubiquitination and proteolysis, and results in a rapid increase in p53 protein levels, allowing p53 to enter the nucleus, bind to DNA and induce expression of DNA repair and cell cycle inhibitor genes. Thus, p53 facilitates the repair and survival of damaged cells or eliminates severely damaged cells from the replicative pool to protect the organism, earning it the moniker "guardian of the genome".

We investigated the role of p53 in cell cycle arrest induced by SEPW1 silencing in MCF-7 breast tumor cells. We found that total p53 and p53 phosphorylated on Ser-33 were increased in SEPW1-silenced cells. Stable knockdown of p53 with short hairpin RNA abrogated cell cycle arrest from SEPW1 silencing. p21 (Cip1/WAF1), the cyclin-dependent kinase inhibitor targeted by p53 that mediates cell cycle arrest, was increased in SEPW1-silenced cells, and silencing of p21 expression abrogated cell cycle arrest from SEPW1 depletion. Thus, cell cycle arrest from SEPW1 silencing in MCF-7 cells is mediated by p53 and p21.

2. Materials and methods

2.1. Cell culture

MCF-7 human breast adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in 1:1 DMEM/F12 (HyClone, SouthLogan, UT) supplemented with 10% FBS and 2 mM L-glutamine in the presence of 5% CO_2 in air at 37 °C. The p53-deficient MCF-7 cell line stably-transfected with shRNA targeting the p53 gene was a kind gift from Dr. Xinbin Chen at University of California at Davis and was cultured under the same conditions as the wild type MCF-7.

2.2. siRNA transfections

10^5 cells per well were reverse-transfected in six well dishes with 0.2% Lipofectamine RNAiMax reagent (Invitrogen, Carlsbad, CA) and 5 nM Silencer Select Validated siRNAs (ABI, Foster City, CA) targeting either SEPW1 (#s361), p21 (#s415), or Silencer Select negative control siRNA #1.

2.3. Cell cycle analysis

Propidium iodide staining of cellular DNA and flow cytometry analysis were performed as described before [14].

2.4. Western blots

Total cellular protein was extracted with RIPA buffer containing 1 × HALT Protease and Phosphatase Inhibitor Cocktails (Pierce, Rockford, IL) and 5 mM EDTA. Protein concentrations were determined using a standard BCA assay, and the extracts were stored at −80 °C until use. Twenty or 30 μg protein per well resolved by SDS–PAGE was transferred to Immobilon P PVDF membranes (Millipore, Billerica, MA). The membranes were blocked for 1 h in 5% milk/TBST and then probed with 1 μg/ml anti-p53, anti-beta actin (Sigma, St. Louis, MO), anti-p21, anti-phospho-Ser20-p53, or anti-phospho-Ser33-p53 (Cell Signaling Technologies Beverly, MA) antibodies overnight at 4 °C. Following 1 h incubation with the appropriate secondary antibodies, the blots were covered with Immun-Star Western C reagent (BioRad, Hercules, CA) and chemiluminescence signals were detected with a ChemiDoc XRS Imaging System (BioRad). Membranes were stripped using Restore Plus Stripping Buffer (Pierce) and re-probed when necessary. Densitometry on blot images was performed using ImageLab software (BioRad).

2.5. Immunoprecipitation and Western blotting of SEPW1

Custom rabbit polyclonal antibody (Antibodies Inc., Davis, CA) raised against full-length mutant recombinant human SEPW1 (Sec to Cys mutation introduced to allow expression in *Escherichia coli*) was purified by absorption to Protein G-Agarose and stored in PBS at −70 °C. MCF-7 cells were seeded (6×10^5 cells) in 100 mm culture plates and transfected as described above. After 72 h, cells were lysed in M-Per lysis buffer containing 1 × HALT Protease and Phosphatase Inhibitor Cocktails and 5 mM EDTA (Pierce, Rockford, IL) and 1.75 mg total protein was incubated overnight with 10 μg SEPW1 antibody and 100 μL Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) in 1:1 M-Per lysate:PBS at 4 °C with gentle shaking. The resin was collected by centrifugation, washed four times with PBS and the proteins were eluted into reducing Laemmli buffer by boiling for 5 min. Samples were analyzed by SDS–PAGE and Western blotting as described above, using the polyclonal rabbit SEPW1 antibody as the primary antibody.

2.6. Statistical analyses

Cell cycle distribution data were analyzed with Student's *t*-test with SigmaStat Software (Systat, San Jose, CA). Changes in quantitative densitometry data from Western blots were expressed as fold-change and compared to a reference value of "1" with Student's *t*-test. Only the reported contrasts were tested statistically to minimize errors from multiple hypothesis testing. A probability of $p < 0.05$ was considered significant.

3. Results

SEPW1 protein was immunoprecipitated from MCF-7 lysates, subjected to SDS–PAGE, and analyzed with Western blots to reveal a prominent immunoreactive band at approximately 9 kDa in control siRNA-treated MCF-7 (Fig. 1). In contrast, immunoprecipitates from lysates of MCF-7 cells transfected with SEPW1 siRNA yielded only a faint band of SEPW1 protein, demonstrating that the siRNA efficiently silenced expression of SEPW1 protein.

Previous work showed that depletion of SEPW1 in non-tumorigenic prostate and mammary epithelial cells causes a delay in cell cycle progression at the G1 to S-phase transition [14]. To study the mechanism of this G1 arrest, we examined the effects of SEPW1 depletion in MCF-7 breast cancer cells, which have wild-type p53 and have been used extensively in studies of p53 function and regulation. First, we confirmed that SEPW1 depletion induced the same phenotype in MCF-7 cells as was previously observed in non-tumorigenic MCF-10A cells [14]. Table 1 shows that treatment with SEPW1 siRNA caused a greater fraction of cells to be in