Enhanced Redox Factor 1 (REF1)-modulated p53 Stabilization and JNK1 Dissociation in Response to Selenomethionine

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Abstract. *Aim: p53 is reportedly activated without any genotoxicity through redox modulation of redox factor 1 (REF1). REF1 is documented to modulate the redox status under selenomethionine (SeMet). In this study, we investigated the mechanism of p53 stabilization by SeMet. Materials and Methods: We mainly used ubiquitination assay and immunoprecipitation to determine the potential role of REF1 and c-jun N-terminal kinase 1 (JNK) in modulation of p53 stabilization by SeMet. Results: The amount of ubiquitinated p53 decreased significantly under SeMet treatment, suggesting that SeMet might inhibit the proteasome-dependent degradation of p53. In addition, we observed that JNK was considerably associated with p53 in REF1 siRNA-treated cells, implying a possible role for SeMet-induced REF1 activity in modulation of the interaction between JNK and p53 via changes in p53 redox status. Conclusion: Our results suggest that the alternate mechanism of p53 stabilization by SeMet might provide an important clue in elucidating the molecular mechanism of chemopreventative compounds against various oxidative stresses.*

Selenium has been identified as a potent chemopreventive agent and was reported to protect against the development of cancer in human populations (1, 2). Several organic and inorganic selenium compounds were screened for their chemopreventive properties against cancer (3). One of the several chemical forms of selenium, selenomethionine (SeMet), is the major component of dietary selenium. The recommended daily allowance (RDA) of dietary selenium by the U.S. Food and Drug Administration is specified to be 50 μg per day, and the RDA can be exceeded by four-fold with no toxicity. Studies using animal carcinogenesis models have found strong evidence that a monomethyl selenium metabolite pool, perhaps methylselenol, is a selenium species with chemopreventive activity, although the molecular mechanisms of SeMet have not yet been clearly established.

A possible role for the activation of the tumor suppressor p53 in chemoprevention by SeMet has been suggested by previous studies from our group (4, 5). Moreover, it was suggested that p53 might be activated by redox modulation of redox factor 1 (APE1/REF1) in response to SeMet (5). APE/REF1, also known as HAP-1, is a bifunctional enzyme with two separate catalytic domains: one domain has thiol reductase activity, while the other is an apurinic/apyrimidinic (AP) endonuclease (6). Furthermore, REF1 was shown to be sensitive to changes in redox status and facilitate DNA binding and activation of transcription factors including activator protein-1 (AP-1), cAMP response element binding protein (CREP), activating transcription factor (ATF), and p53 *via* the reduction of a cysteine residue (7).

On the other hand, p53 has been studied for its role in mechanisms of basal control of protein levels because of its protective function against genotoxic stresses. In its latent (inactive) form, p53 is constitutively unstable and adopts a conformation in which the extreme *C*-terminal domains hinder the interactions of the DNA-binding domain with its target (8). Activation of p53 proceeds through coordinated steps, including escape from proteasome-dependent degradation, release of the negative inhibition carried by the *C*-terminus, and conversion of the conformation of the DNA-binding domain into a form with high affinity for target DNA (9).

Negative regulators of p53 containing murine double minute-2 (MDM2), c-JUN *N*-terminal kinase 1 (JNK1), constitutively photomorphogenic 1 (COP1), and p53 induced RING-H2 protein (PIRH2), all of which bind and change the conformation of p53, have been reported to degrade p53 through proteasomes (10-14). One of the major negative regulators, JNK1, is known to bind to residues 97-116 in p53 and target p53 for degradation by proteasome with inactive

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forms in unstressed cells. Complexes between p53 and JNK1 are preferentially found in the G_0/G_1 phase of the cell cycle, in contrast with MDM2-p53 complexes, which are mostly detectable in the S and G_2/M phases. It has been proposed that JNK1 might essentially act as a regulator of basal p53 protein level in unstressed cells (14, 15).

In the present study, we investigated the mechanism of p53 activation in response to non-genotoxic antioxidant SeMet on the JNK-mediated ubiquitination pathway. REF1-specific small interference RNA (siRNA) was used to determine the role of REF1 in the enhancement of p53 stabilization under antioxidant SeMet.

Materials and Methods

Cell lines and treatment. Human colon cancer RKO cells exhibiting wild-type *p53* were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) plus antibiotics. RKO cells were treated with SeMet (10, 20, and 40 μM) for 16 h at 37˚C and with 5% CO_2.

siRNA transfection of JNK1 and REF1. siRNA duplexes targeting JNK1 and REF1 were designed and synthesized by Dharmacon (Chicago, IL, USA). The transfection of RKO was accomplished using Oligofectamine reagent (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's instructions with single-strand sense and antisense RNA oligonucleotides (human REF1 sense RNA: 5'-AUAUUGCUUCGGUGGGUGA-3'; human REF1 antisense RNA: 5'-UCACCCACCGAAGCAAUAUdTdT-3'). For each well, 5 μl of 5 nmol of siJNK1 was diluted with 170 μl of Opti-MEM (Invitrogen, Carlsbad, CA, USA). Then, 4 μl of Oligofectamine (Invitrogen, Carlsbad, CA, USA) was diluted and incubated for 10 min in 15 μl of serum-free RPMI-1640 medium. Both solutions were combined and stored at room temperature for 20 min. After 4 h of transfection, RKO cells were grown in RPMI-1640 containing 10% FBS before harvesting the cells.

Immunoblotting. All immunoblots were prepared as previously described by Jung *et al*. (16). The antibodies used were as follows: p53 (DO-1; Santa Cruz Biotechnology, CA, USA); ubiquitin (LabFrontier, Seoul, South Korea); JNK1 (C-17; Santa Cruz Biotechnology); REF1 (BD Pharmingen, San Diego, CA, USA); Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (LabFrontier). Horseradish peroxidase-conjugated secondary antibodies (Sigma, St. Louis, MO, USA) and enhanced chemiluminescence (Pierce, Rockford, IL, USA) were used for detection.

Immunoprecipitation. The cells were lysed in immunoprecipitation buffer [1% Nonidet P 40, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1 mM Dithiothreitol (DTT), 0.5 mM ethylenediaminetetraacetic acid (EDTA)] containing protease inhibitors (Roche Molecular Biochemicals, Mannheim, Germany) for 30 min at 4˚C. The samples were sonicated for 5 s. Equal amounts of soluble protein extracts were incubated with 20 μl protein A/G agarose (Santa Cruz Biotechnology) for 1 h at 4˚C with shaking. Supernatants were aliquoted for immunoprecipitation with 1 μg of either a mouse antibody to p53 (DO-1; Santa Cruz Biotechnology) or a mouse antibody to ubiquitin (LabFrontier) for 6 h at 4˚C. After incubation for 6 h with the primary antibody, the immune complexes were collected with 20 μl protein A/G agarose beads (Santa Cruz Biotechnology) overnight and washed three times with inmmunoprecipitation (IP) buffer. The immunoprecipitated proteins were resuspended in 4× sample buffer, resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred to membranes before immunoblotting with the appropriate antibodies.

In vivo ubiquitination assay. For the purposes of an *in vivo* ubiquitination assay, RKO cells were treated with proteasome inhibitor MG132 (25 μM) for 8 h before harvesting the cells. Then, the cells were lysed using modified RIPA buffer [1% Nonidet P 40, 0.1% SDS, 50 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA] containing MG132 (25 μM) and protease inhibitors (Complete™; Roche Molecular Biochemicals, Mannheim, Germany). The lysates were immunoprecipitated with a mouse antibody to p53 (DO-1; Santa Cruz Biotechnology) and analyzed using an antibody to JNK1 (C-17; Santa Cruz Biotechnology).

Detection of intracellular reactive oxygen species (ROS). At the end of experimentation, the cells were washed with PBS and further treated with general oxidative stress indicator CM-H_2DCFDA at 5 μM with incubation at 37˚C for 30 min. The cells were then washed with PBS and immediately observed under a Leica DMIRB fluorescence microscope (Wetzlar, Germany). The fluorescence intensity was measured using NIS-Elements BR 3.0 software (Nikon, Japan) from 10 random confocal images of each group.

Results

Enhanced p53 protein stabilization in response to SeMet. To determine whether p53 accumulation was likely to be post-translational, the interaction of p53 and ubiquitin in the presence and absence of the proteasome inhibitor MG132 (25 μM, 8 h) and in response to SeMet was studied in human colon cancer RKO cells exhibiting wild-type *p53*. The study was conducted using a ubiquitination assay (Figure 1). Our data showed that the interaction of *p53* and ubiquitin was considerably reduced after treatment of the cells with SeMet in the presence of MG132. This implied that SeMet inhibited p53 degradation *via* the proteasome-mediated ubiquitination pathway.

Decreased JNK-dependent p53 ubiquitination in response to SeMet. We investigated the contribution of JNK1 to p53 ubiquitination *via* the proteasome-dependent degradation pathway, in response to SeMet. To do so, JNK1-specific siRNA was introduced into the cells. Our data showed that the quantity of ubiquitinated p53 was significantly lower in the JNK siRNA-treated cells under the proteasome inhibitor MG132 (25 μM, 8 h), in contrast with the mock-treated cells (Figure 2), suggesting that enhanced stabilization of p53 in response to SeMet might be modulated by JNK1.

REF1-modulated dissociation of JNK and p53 interaction after treatment with SeMet. In order to investigate the role of redox factor REF1 in p53 stabilization after treatment of the

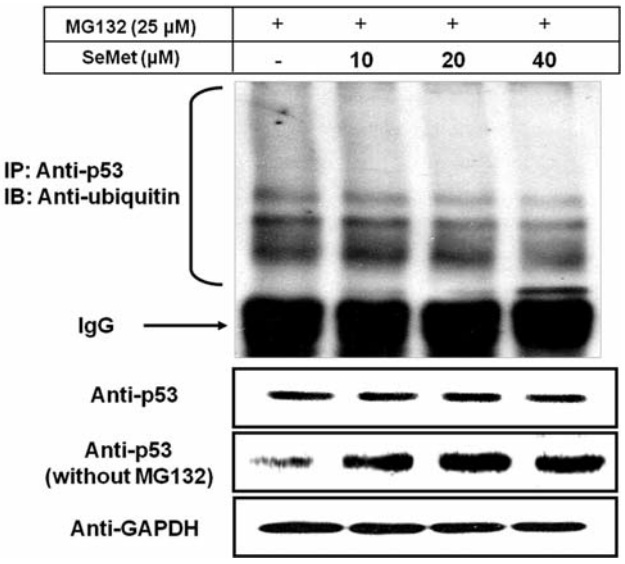

Figure 1. *A decrease in proteasome-mediated p53 degradation in response to selenomethionine (SeMet). The ubiquitination of p53 immunoprecipitated from p53 wild-type human colon cancer RKO cells was detected after treatment of the cells with the proteasome inhibitor (25 μM, MG132) for 8 h (lanes 1, 2, 3 and 4), and without treatment with the proteasome inhibitor (lanes 5, 6, 7 and 8), as indicated. An unsaturated amount of anti-full-length p53 antibody (1 μg) was used to ensure similar total amounts of immunoprecipitated p53. Ubiquitinated p53 and total p53 are indicated. Ubiquitinated p53 decreased in response to SeMet after treatment with proteasome inhibitor MG132.*

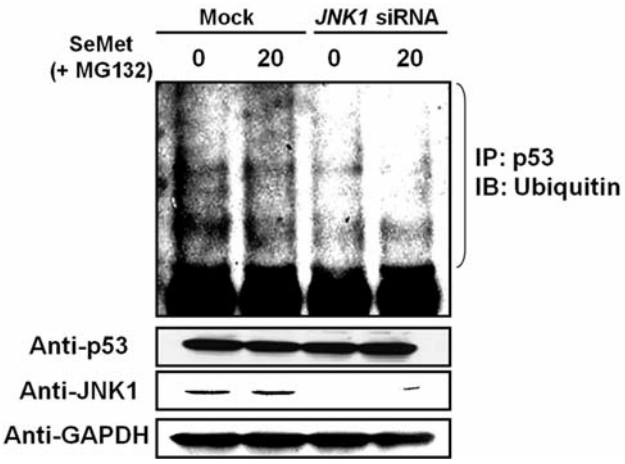

Figure 2. *Inhibition of c-jun N-terminal kinase 1 (JNK1)-dependent ubiquitination of p53 by selenomethionine (SeMet). RKO cells were transfected with JNK1 siRNA for 24 h before treatment with 20 μM SeMet for 16 h. The total cell lysates were harvested after treatment of the cells with 25 μM MG132 for 8 h for an in vivo ubiquitination assay. Antibody to p53 was employed to immunoprecipitate using total protein extracts. Ubiquitination of p53 was monitored using an antibody to ubiquitin. JNK1 siRNA-treated cells exhibited a significantly lower level of p53 ubiquitination.*

cells with SeMet (20 μM), we examined the interaction of JNK and p53 in the REF1 siRNA-treated cells and mock-treated cells using immunoprecipitation. Our data showed that JNK-p53 interaction was enhanced in REF1 down-regulated cells treated with SeMet in contrast to the mock-treated cells (Figure 3A). The protein expression level of p53 and JNK in whole-cell lysates was detected by western blot analysis. Our results showed a decrease in p53 levels in REF1 siRNA-treated cells in response to SeMet. Meanwhile, the JNK level in whole lysates was not altered by REF1 down-regulation (Figure 3B). These data suggest that the enhancement of p53 protein stability in response to SeMet might be modulated *via* dissociation of JNK and p53 by REF1, which plays a role in the regulation of the cellular redox status.

To clarify whether REF1 was involved in the antioxidant activity of SeMet, we stained REF1 siRNA-treated cells with cell-permeable dye CM-H_2DCFDA, which itself is a non-fluorescent dye. Upon entering the cells, the dye is cleaved by intracellular esterases and oxidized to fluorescent compounds by intracellular pro-oxidants. The fluorescent intensity is directly proportional to the level of ROS present in the cells. As shown in Figure 3C, confocal microscopic analysis showed that the fluorescence intensity of the cells was significantly higher upon treatment with REF1 siRNA compared to the cells treated with a control siRNA, implying that REF1 activity might be a critical factor in the modulation of the intracellular redox status in response to SeMet. The data were quantified from the imaging analysis (Figure 3D) using the Quantity One Basic software (Bio-Rad, Hercules, CA, USA).

Discussion

Over the past 10 years, p53 has been shown to be activated by a wide range of DNA-damaging agents and non-genotoxic agents (17). The induction of *p53* in response to various stimuli consists of two inter-connected processes: stabilization of the protein (by escape from constitutive, proteasome-dependent degradation), and activation (by conversion from the latent into the active form, with high affinity for specific DNA sequences in the regulatory regions of target genes) (18, 19). Through these processes, activated p53 is augmented by anticancer or cancer-preventive mechanisms, thereby protecting cells from DNA damage.

The antioxidant selenium compound SeMet has been documented to be relatively non-toxic and non-DNA damaging, unlike the prototype forms including sodium selenite (3, 20, 21). This property has been considered to play

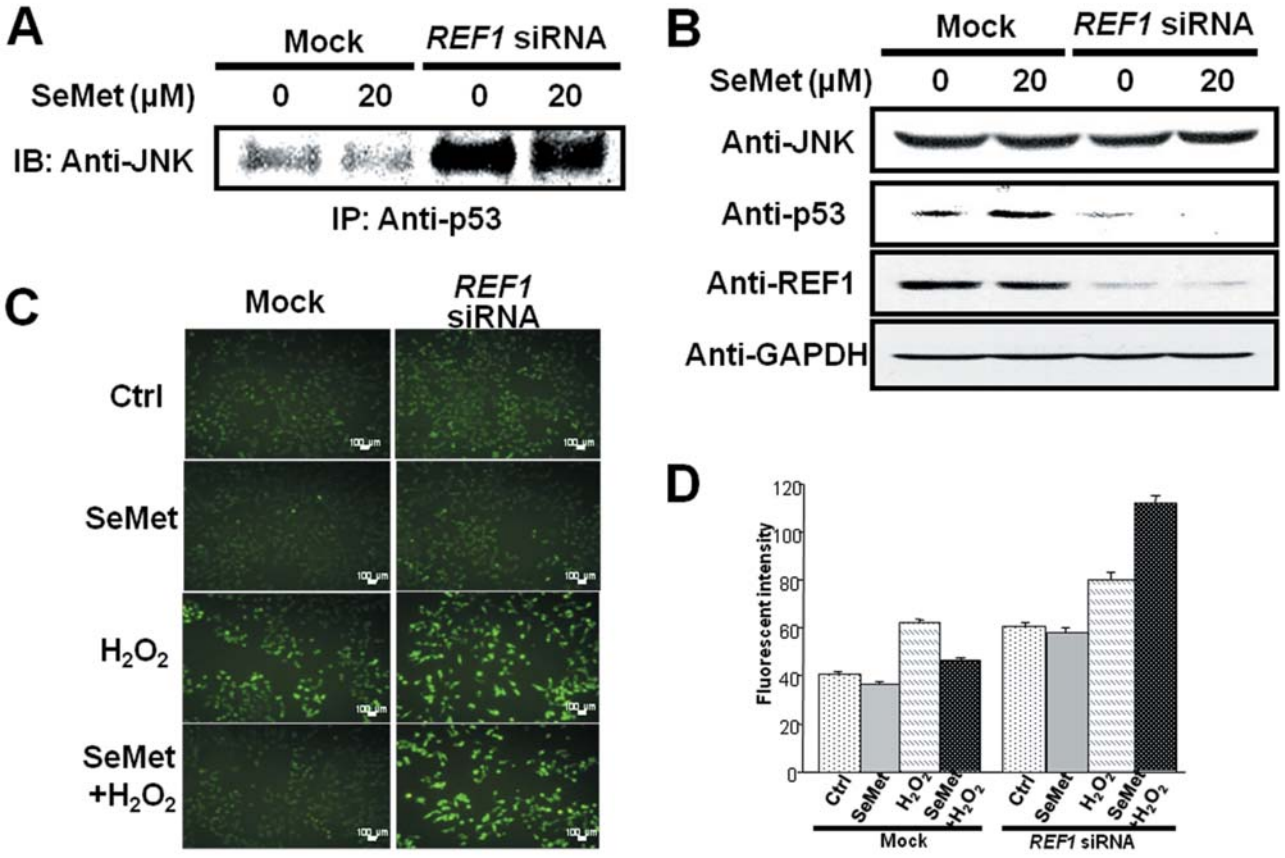

Figure 3. *Redox factor 1 (REF1)-modulated dissociation of c-jun N-terminal kinase 1 (JNK) and p53 under selenomethionine (SeMet) treatment. A: RKO cells were transfected with mock or REF1 siRNA for 24 h before 20 μM SeMet treatment for 16 h. Total protein extracts were immunoprecipitated (IP) using antibodies to p53 (DO-1). The immunoprecipitates were analyzed by western blot with antibody to JNK1. In response to SeMet, REF1 siRNA-treated cells exhibited considerably enhanced interaction of JNK1 and p53. B: The total protein extracts were also used to determine JNK1, p53, and REF1 levels using western blotting. Detection of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. The total p53 (DO-1) levels were decreased in REF1-down-regulated cells, both with and without SeMet, in contrast to JNK1 levels. C: Cells treated with REF1-specific siRNA were stained with a cell-permeable redox sensitive dye (CM-H2DCFDA) in the presence of 10 μM H_2O_2 and in the absence of H_2O_2, indicating the level of intracellular ROS. REF1 siRNA inhibited the activity of REF1 as well as the repression of its protein expression. D: Quantification of the average fluorescence intensity of cells stained with CM-H_2DCFDA (scale bar represents 100 μm, x50 magnification).*

a potential role in chemopreventive nutrition. Recently, Taylor and colleagues suggested that the beneficial effects of Se, vitamin E, and beta-carotene on total and gastric cancer mortality were still evident up to 10 years after the supplementation and were consistently greater in participants younger than 55 years (22-24). This general population nutrition intervention trial, conducted in Linxian, China, where are nutritional deficiencies and high cancer rates, indicated that the basal level of minerals including Se is critical in cancer prevention. Although one recent clinical trial did not show any effect of SeMet in preventing prostate cancer (25), in other recent clinical trials of chemopreventive compounds, the baseline nutrient status was recommended to be in the suboptimal range based on the U-shaped dose-response, where the region of optimal nutrient status lies between two suboptimal (low and high) regions and the extreme values of deficiency and toxicity (24, 26). Furthermore, the need for an enhanced mechanistic studies to better-understand the outcome of animal data and clinical studies such as the SELECT study remains an urgent priority (27).

Our group has reported that SeMet might transcriptionally activate p53 and induce sequence-specific DNA binding by p53 without DNA damage through a redox mechanism that requires the redox factor REF1 (5). The direct reduction/oxidation of p53 was shown to be induced by a SeMet-dependent response that was blocked by a dominant-negative REF1 (5). However, the mechanism of p53 protein stabilization in the presence of SeMet has not yet been established.

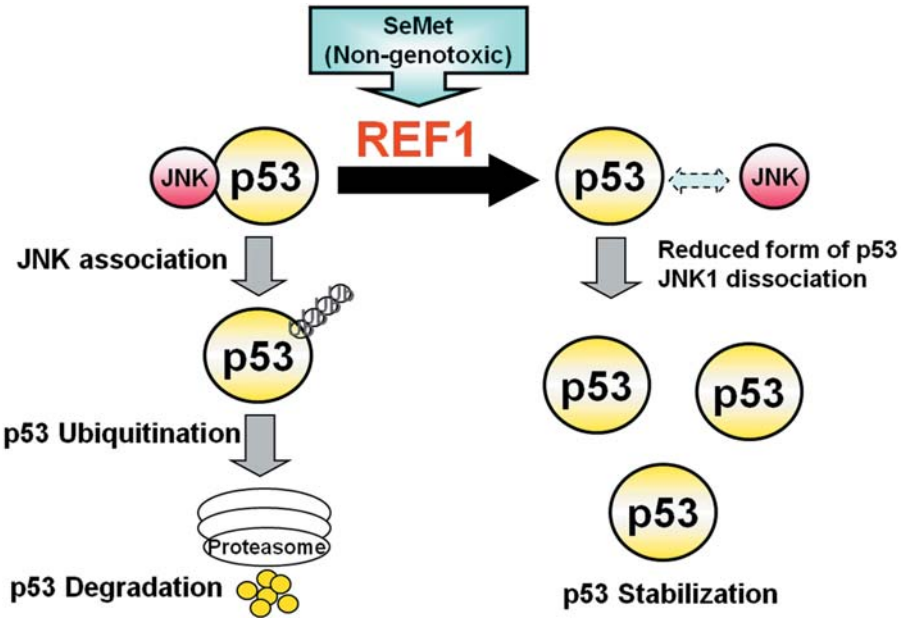

Figure 4. *Suggested model for the competitive role of redox factor 1 (REF1) and c-jun N-terminal kinase 1 (JNK1) in the increased stabilization of p53 by a non-genotoxic antioxidant, selenomethionine (SeMet). Several reports have shown the mechanisms of p53 protein degradation including ubiquitination, Small ubiquitin-related modifier (SUMO)-ylation, and acetylation (34, 35). JNK1 is known to be a major E3-ubiquitin ligase responsible for the constitutive instability of p53 through its binding to p53 for degradation by the 26S proteasome (17). In this report, we suggest that p53 stabilization might be enhanced without induction of genotoxicity in response to the antioxidant SeMet through its dissociation from the negative modulator JNK1 by REF1, which modulates the redox status of proteins.*

Here, we investigated whether a proteasome-mediated pathway is involved in p53 protein stabilization by SeMet. Using *in vivo* ubiquitination assays with the treatment of proteasome-specific inhibitor MG132, we showed that the ubiquitination of p53 was inhibited by SeMet (Figure 1). The ubiquitination of p53 was decreased in JNK siRNA-treated cells, suggesting that JNK-mediated p53 ubiquitination might be modulated by the SeMet treatment (Figure 2). Our data showed that the inhibition of p53 ubiquitination is also induced under non-genotoxic low-dose treatment of organic methylseleninic acid (MSeA), implying that the stabilization of endogenous p53 might be modulated by organic selenium compounds (data not shown). The involvement of other known factors such as constitutively photomorphogenic 1 (COP1) and PIRH2 in p53 stabilization must be explored in further studies. Our data further demonstrated that SeMet did not induce the phosphorylation of JNK and p53, although JNK is activated by stress conditions and can thus induce p53 stabilization, unlike in unstressed cells (data not shown). However, a recent study has suggested that selenium might repress the activation of JNK and thereby protect cells from DNA damage (28). Here, we also report that the phosphorylation of JNK was induced under UV irradiation but inhibited by SeMet (data not shown). The modulation of p53 and JNK under genotoxic stress needs to be clarified in future studies.

On the other hand, MDM2 is well-documented as an E3 ligase for ubiquitination of p53 (10, 11). The phosphorylation of MDM2, which requires the export of p53 to the cytosol, was not altered in the presence of SeMet (data not shown). Indeed, complexes between p53 and JNK1 have been documented to preferentially form in the G_0/G_1 phase of the cell cycle, in contrast with MDM2-p53 complexes, which are mostly detectable in the S and G_2/M phases. In a previous study conducted in our group, SeMet did not induce any cell cycle alteration or cell viability (4), suggesting that the association of JNK and p53 might be important in the normal cell cycle.

Recently, redox regulation was underlined as a critical factor in the optimum functioning of a number of transcription factors. Prominently, REF1 was demonstrated to play an important role in maintaining basal p53 levels by inducing dissociation of MDM2 from p53 in response to genotoxic insult (17). Our data showed that the interaction of p53 and JNK was decreased in response to SeMet (Figure 3A). Indeed, the complex of p53 and JNK was noticeably associated with redox factor REF1-down-regulated cells treated with SeMet, as compared to the mock-treated cells (Figure 3A). This implied that REF1 is involved in p53 stabilization through dissociation of the p53 and JNK complex under SeMet treatment. Recently, our group

suggested that the reduction of cysteine residues in the p53 protein might be induced in response to SeMet that was otherwise blocked by a dominant-negative REF1 (5). The dissociation of JNK and p53, which was modulated by REF1, implies that the change in conformation of the p53 protein to its reduced form might affect its stability.

Whether administered by diet or applied to tissue culture cells, SeMet undergoes a trans-sulfuration reaction through a selenohomocysteine intermediate to form selenocysteine, which is then incorporated specifically by a selenocystyl-tRNA into cellular selenoproteins, including thioredoxin reductase and glutathione peroxidase (29). Selenium increases the specific activity of these enzymes and promotes a cellular reducing environment. A number of cellular proteins, including REF1 and p53, are substrates of the thioredoxin reductase system (30-32) and have been shown to interact under reducing conditions. Furthermore, REF1 was shown to modulate p53 function through binding in the *C*-terminus of p53 and activating its DNA-binding capacity. This is in spite of its thiol-reducing properties that facilitate DNA binding without forming stable interactions with p53 *in vitro* (30, 33). Our data show that REF1 plays a critical role in the functioning of SeMet as an antioxidant with ROS measurement induced by treatment with hydrogen peroxide. The fluorescent intensity of REF1 siRNA-treated cells was considerably increased by CM-H2DCFDA, an indicator of ROS production, suggesting that the redox sensitizer REF1 might be modulated by SeMet (Figure 3C).

In summary, we here suggest, to our knowledge for the first time, that the stabilization of p53 *via* escape from the JNK-modulated ubiquitination pathway is an alternate chemopreventive mechanism of SeMet (Figure 4). In addition, this study found evidence that the redox factor REF1, activated by SeMet, might be involved in the dissociation of the p53-JNK complex; the structural relationship between the redox proteins and p53 remains to be investigated in future studies. This alterative mechanism of p53 stabilization in response to SeMet might provide an important clue for the development of chemopreventive strategies against various oxidative stresses.

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