Delayed Cell Cycle Progression in Selenoprotein W-depleted Cells Is Regulated by a Mitogen-activated Protein Kinase Kinase 4-p38/c-Jun NH₂-terminal Kinase-p53 Pathway

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Background: Selenium may prevent or help cause cancers, but the mechanisms are unclear.

Results: Silencing MKK4, JNK2, p38γ, or p38δ rescued G₁ arrest from SEPW1 depletion.

Conclusion: G₁ arrest from SEPW1 silencing is mediated by a MKK4-p38γ/p38δ/JNK2-p53 signaling pathway.

Significance: The function of SEPW1 in cell cycle regulation is important to understanding the role of selenium in carcinogenesis.

Selenoprotein W (SEPW1) is a ubiquitous, highly conserved thioredoxin-like protein whose depletion causes a transient p53- and p21Cip1-dependent G₁-phase cell cycle arrest in breast and prostate epithelial cells. SEPW1 depletion increases phosphorylation of Ser-33 in p53, which is associated with decreased p53 ubiquitination and stabilization of p53. We report here that delayed cell cycle progression, Ser-33 phosphorylation, and nuclear accumulation from SEPW1 depletion require mitogen-activated protein kinase kinase 4 (MKK4). Silencing MKK4 rescued G₁ arrest, Ser-33 phosphorylation, and nuclear accumulation of p53 induced by SEPW1 depletion, but silencing MKK3, MKK6, or MKK7 did not. SEPW1 silencing did not change the phosphorylation state of MKK4 but increased total MKK4 protein. Silencing p38γ, p38δ, or JNK2 partially rescued G₁ arrest from SEPW1 silencing, suggesting they signal downstream from MKK4. These results imply that SEPW1 silencing increases MKK4, which activates p38γ, p38δ, and JNK2 to phosphorylate p53 on Ser-33 and cause a transient G₁ arrest.

Selenium is an essential trace element that has been associated with cancer for more than a century. Nevertheless, there remains considerable uncertainty whether selenium protects against cancer or actually promotes cancer development. There are three families of closely related MAP kinases: the extracellular-receptor kinases (ERKs) that include ERK1, ERK2, ERK3/ERK4, and ERK5 and two families that respond to cellular stress and inflammatory signals, the so-called “stress-activated” MAP kinases; the c-Jun NH₂-terminal kinases (JNKs) that include JNK1, JNK2, and JNK3; p38 MAP kinases p38α, p38β, p38γ, and p38δ. Upon activation, MAPKs phosphorylate other protein kinases and transcription factors that control a wide variety of cellular processes involved with proliferation, differentiation, development, cell division, apoptosis, cell motility, and survival. MAP kinases play a prominent role in controlling the phosphorylation and thus activation of p53 (4).

JNK-activating kinase 1 (JNK1)/stress-activated protein kinase ERK kinase 1 (SEK1)/mitogen-activated protein kinase kinase 4, hereinafter referred to as MKK4, is a stress-activated MAP2K that phosphorylates and thereby activates MAP

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kinases in response to various environmental stresses or mito-
genic stimuli. MKK4 is phosphorylated and thus activated by the MAP3Ks: ASK1, MEKK1, MEKK4, TAK1, TPL2, and by the mixed-lineage kinase MLK3 (5–7). In addition to JNK1, JNK2, and JNK3, MKK4 phosphorlates and thereby activates p38 stress-activated MAP kinases (8), but MKK4 is not known to activate the extracellular signal-regulated kinases ERK1 or ERK2. MKK4 is a candidate tumor suppressor protein that mediates survival signals in T cell development (9) and liver organogenesis (10). MKK4 inhibits cell proliferation during replicative senescence (11) and in prostate tumor xenografts by causing a transient delay in G1- to S-phase progression (8). On the other hand, there are also reports of a pro-oncogenic role of MKK4 in breast and pancreatic cancers (5).

We screened MAP kinase pathways using siRNA to identify events upstream of p53 in the pathway controlling cell cycle arrest from SEPW1 silencing. We found evidence suggesting SEPW1 depletion increases MKK4, which activates p38γ, p38δ, and JNK2 to phosphorylate p53 on Ser-33 and cause a transient G1 arrest.

EXPERIMENTAL PROCEDURES

Cell Culture—RWPE-1 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in KSFM (Invitrogen) supplemented with 50 μg/ml bovine pituitary extract and 5 ng/ml epidermal growth factor at 37 °C under an atmosphere of 95% humidified air, 5%CO2.

siRNA Transfections—105 cells per well were reverse-transfected for 24 h in 6-well dishes with 0.2% Lipofectamine RNAiMax reagent (Invitrogen) and 5 nm Silencer Select siRNAs (ABI, Foster City, CA) targeting SEPW1 (s361, s363), JNK1 (s11152, s11153), JNK2 (s229708, s11159), JNK3 (s11161, s11163), p38α (s3585, s3586), p38β (s11155, s11157), p38γ (s12468, s12467), p38δ (s11165, s11166), MKK3 (s11173, s11175), MKK4 (s12701, s12703), MKK6 (s11180, s11181), MKK7 (s11182, s11183), MEKK1 (s8668), MEKK3 (s8675), ASK1 (s8676), TAK1 (s13767), TPL2 (s3383), MLK3 (s8670) or Silencer Select negative control siRNA #1. Transfection medium was replaced with fresh medium the next day, and cells were harvested 72 h post-transfection. Silencing efficiencies of the siRNAs were assessed with Western blots (supplemental Figs. S1–S5). Unless stated otherwise, data identified only as “SEPW1 siRNA” refers to siRNA s361.

Cell Cycle Analysis—Cells were fixed using 70% ethanol in PBS, washed with PBS, and then stained with a solution of propidium iodide, Triton X-100, and RNase A in PBS. At least 20,000 events per sample were collected on a FACSCalibur flow cytometer (BD Biosciences). Data analysis was conducted with ModFit LT 3.0 software (Verity Software, Topsham, ME). Singlelets were manually gated on a FL-2 width versus FL-2 area plot, and cell cycle phase percentages were calculated by the software using the diploid model. Representative pseudo-color density plots of events with each siRNA treatment are provided (supplemental Figs. S6–S8).

Western Blots—Western blots were obtained as described before (3) using antibodies targeting the following proteins: MKK3, MKK4, MKK6, MKK7, phospho-Ser-257/Thr-261 MKK4, phospho-Ser-80 MKK4, phospho-Ser-33 p53, p38α, p38β, p38γ, p38δ, JNK1, JNK2, JNK3 (Cell Signaling Technology, Beverly, MA), p35, β-actin, and β-tubulin (Sigma). Densitometry was performed with ImageLab software (Bio-Rad), and chemiluminescence of protein bands was normalized to the average chemiluminescence of the immunoblot before statistical analysis.

Indirect Immunofluorescence Microscopy—Three days after siRNA transfection, cells grown on coverslips were fixed using 3.7% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked in 0.2% gelatin, incubated with 20 μg/ml anti-p53 antibodies followed by 5 μg/ml Texas Red goat anti-mouse IgG (Sigma) and 100 ng/ml 4’,6-diamidino-2-phenylindole (DAPI, Sigma), all diluted in PBS. Coverslips were mounted using SlowFade anti-fade reagent (Invitrogen), and images were collected on an Axiovert 40 CFL microscope (Carl Zeiss, Jena, Germany) using a Spot RT3 camera (Diagnostic Instruments, Sterling Heights, MI) at equal exposure times.

MKK4 Stability Determination—Cells were grown in medium containing 60 μg/ml cycloheximide (Sigma) to inhibit protein synthesis, and lysates were collected at several time points up to 24 h. The amount of MKK4 protein at each time point was estimated from Western blots.

Statistical Analysis—Cell cycle phase distributions and Western blot densitometry measurements from different siRNA treatments were compared using two-way analysis of variance or paired t tests with SigmaStat 2.03 (Systat, San Jose, CA) and Excel (Microsoft, Redmond, WA) software. Unless stated otherwise, estimates of experimental variability are expressed as S.E. For data sets including several independent experiments, S.E. were calculated from pooled standard deviations (12). Between-group comparisons were analyzed with Tukey’s test for cell cycle data or with Fisher’s LSD test for quantitative densitometry data. A probability of <0.05 was considered significant.

RESULTS

Silencing p38γ, p38δ, or JNK2 Rescues G1 Arrest from SEPW1 Depletion—Because Ser-33 phosphorylation is associated with G1 arrest from SEPW1 silencing and Ser-33 is known to be phosphorylated by JNK and p38 (13, 14), we used an siRNA screen to test if silencing p38α, p38β, p38γ, p38δ, JNK1, JNK2, or JNK3 could rescue the G1 arrest from SEPW1 silencing. Silencing expression of SEPW1 alone caused RWPE-1 cells to accumulate in the G0/G1 phase of the cell cycle, indicative of a transient G1-phase arrest (Table 1). Silencing p38γ, p38δ, or JNK2 in SEPW1-depleted cells inhibited the increase in the G0/G1 fraction by 33, 63, or 60%, respectively (p < 0.05, two-way ANOVA with Tukey’s test). Silencing p38α or JNK1 partially rescued the G1 arrest in some experiments but did not reach statistical significance, suggesting they may also have some role in this pathway. On the other hand, silencing p38β or JNK3 did not appear to have an effect in any experiment, suggesting these MAP kinases are not involved.

Silencing p38γ, p38δ, or JNK2 Reverses Phosphorylation of Ser-33—Because G1 arrest from SEPW1 silencing was rescued by silencing p38γ, p38δ, or JNK2, we tested if silencing p38γ, p38δ, or JNK2 also reversed Ser-33 phosphorylation. Each MAPK was silenced with two different siRNA sequences, and
the data were pooled to calculate the mean Ser(P)-33-p53 resulting from silencing each MAPK. Silencing p38γ, p38δ, or JNK2 significantly decreased Ser(P)-33-p53 (*p < 0.05, n = 3, two-way ANOVA with Fisher’s LSD test, Fig. 1), suggesting that p38γ, p38δ, and JNK2 are involved in phosphorylating Ser-33 in SEPW1-depleted cells.

Silencing MKK4 Blocks p53 Activation—Because both JNK and p38 seemed to be involved in G1 arrest from SEPW1 silencing, we focused our search for upstream signaling components on the four MAP2Ks known to activate both pathways: MKK3, MKK4, MKK6, and MKK7. When expression of MKK3, MKK6, or MKK7 was silenced, the G1 arrest from SEPW1 depletion was not rescued (Table 2). In contrast, silencing MKK4 completely rescued G1 arrest from SEPW1 depletion, showing that MKK4 is required for transmitting the signal leading to G1 arrest. This is consistent with the role of MKK4 in inhibiting G1- to S-phase progression during senescence (11) and in xenografts (8). To ensure that rescue by MKK4 siRNA was not due to off-target effects, we confirmed the results with two SEPW1 siRNAs and two MKK4 siRNAs, tested in all four combinations (Table 3). Both SEPW1 siRNAs caused a statistically significant accumulation of cells in G1/G0 phase, and both MKK4 siRNAs rescued the delay in cell cycle progression induced by either of the two SEPW1 siRNAs (p < 0.05, two-way ANOVA with Tukey’s test, Table 3). Thus, the rescue did not depend on the sequence of the siRNAs or on which SEPW1 siRNA was tested with which MKK4 siRNA.

Silencing MKK4 Blocks p53 Activation—Silencing SEPW1 with either of two siRNAs caused marked increases in Ser(P)-33-p53 (Fig. 2). When MKK4 was also silenced with either of two different siRNAs, the increases in Ser(P)-33-p53 were largely blocked (*p < 0.05, n = 2, two-way ANOVA with Fisher’s LSD test), confirming MKK4 is upstream of p53 in the pathway leading to G1 arrest. Activation of p53 is a complex function of numerous post-translational modifications culminating in accumulation of p53 in the nucleus, a necessary step before p53 can act as a transcription factor. Therefore, nuclear accumulation is an integrated measure of p53 activation that reflects the net effect of many different modes of regulation (15). Silencing SEPW1 caused a large shift in the subcellular distribution of p53 (Fig. 3), increasing the fraction of cells with nuclei stained positive for p53 from 4 to 85% (*p < 0.0001, χ² test). Simultaneous silencing of MKK4 substantially prevented nuclear accumulation of p53 in response to SEPW1 depletion. Thus, MKK4 expression is required for activation and nuclear accumulation of p53 when SEPW1 is silenced.

### TABLE 1

| Effect of silencing MAP kinase isoforms on delayed G1 to S-phase progression due to SEPW1 depletion |
|---------------------------------------------------------------|
| Seventy-two hours after transient transfection, cells were fixed and stained with propidium iodide, and their DNA contents were determined by flow cytometry: 20,000 events per sample were collected. Data were analyzed with ModFit LT 3.0. The experiments were conducted at least five times using two different siRNAs targeting each MAPK, and the data shown are the means ± S.E. calculated from pooled standard deviations. Cell cycle phase data for individual siRNAs targeting each MAPK are listed in supplemental Table S2. Representative dot plots of flow cytometry files are shown in supplemental Fig. S8. |

| siRNA treatment                  | G0 and G1 | S phase | G2 and M |
|---------------------------------|-----------|---------|----------|
| No transfection                 | 61.9 ± 0.2| 30.2 ± 0.3| 7.9 ± 0.2 |
| Non-targeting control siRNA     | 63.4 ± 0.4 |27.8 ± 0.3 | 23.5 ± 0.4 |
| SEPW1 siRNA                     | 74.6 ± 0.2 | 20.2 ± 0.2 | 5.2 ± 0.2 |
| p38δ siRNA and SEPW1 siRNA     | 72.8 ± 0.3 | 21.1 ± 0.3 | 6.1 ± 0.2 |
| p38γ siRNA and SEPW1 siRNA     | 75.1 ± 0.3 | 20.5 ± 0.2 | 4.3 ± 0.1 |
| p38δ siRNA and SEPW1 siRNA     | 70.9 ± 0.3 | 23.5 ± 0.4 | 5.6 ± 0.2 |
| p38δ siRNA and SEPW1 siRNA     | 67.5 ± 0.3 | 25.6 ± 0.2 | 6.9 ± 0.3 |
| JNK1 siRNA and SEPW1 siRNA     | 78.5 ± 0.2 | 16.0 ± 0.4 | 5.5 ± 0.3 |
| JNK2 siRNA and SEPW1 siRNA     | 67.9 ± 0.3 | 26.4 ± 0.3 | 5.7 ± 0.2 |
| JNK3 siRNA and SEPW1 siRNA     | 76.1 ± 0.5 | 19.1 ± 0.4 | 4.8 ± 0.3 |

* Significantly different from SEPW1 siRNA alone.
* Significantly different from non-targeting control siRNA.

### FIGURE 1

**Effect of silencing SEPW1 and MAPks on p53 Ser-33 phosphorylation.** RWPE-1 cells were transfected with a single siRNA targeting only SEPW1, with two siRNAs targeting SEPW1 and a MAPK or a non-targeting control siRNA. Lysates were collected at 72 h post-transfection and analyzed with Western blots using antibodies specific for Ser(P)-33-p53, total p53, JNK2, p38γ, and p38δ. β-Tubulin served as a loading control. The experiment was repeated three times using SEPW1 siRNA s361 and two different siRNA sequences targeting each MAPK. A representative Western blot image from a single experiment is shown. Band intensities were estimated by quantitative densitometry. Error bars represent S.E. Asterisks indicate MAPK siRNAs that significantly decreased Ser(P)-33-p53 compared with SEPW1 siRNA alone (*p < 0.05, two-way ANOVA with Fisher’s LSD Test). -ctrl, non-targeting control siRNA; No TF, non-transfected cells.
SEPW1 Depletion and MKK4-p38/JNK-p53-dependent G₁ Arrest

TABLE 2
Effect of MAP2K silencing on delayed G₁ to S-phase progression due to SEPW1 depletion

| siRNA treatment              | G₀ and G₁ | S phase | G₂ and M |
|------------------------------|-----------|---------|---------|
| No transfection              | 58.0 ± 0.7 | 34.3 ± 1.0 | 7.7 ± 0.3 |
| Non-targeting control siRNA  | 63.7 ± 0.3ᵃ | 29.9 ± 0.4ᵇ | 6.4 ± 0.2 |
| SEPW1 siRNA s361             | 76.3 ± 0.2ᵇ | 18.7 ± 0.2ᵇ | 4.9 ± 0.1 |
| MKK3 siRNA and SEPW1 siRNA  | 80.5 ± 0.1ᵇ | 14.0 ± 0.1ᵃᵇ | 5.5 ± 0.1 |
| MKK4 siRNA and SEPW1 siRNAᵃ | 62.6 ± 0.9ᵇ | 29.0 ± 1.0ᵇ | 8.3 ± 0.2ᵃ |
| MKK6 siRNA and SEPW1 siRNAᵇ | 74.4 ± 0.4ᵃᵇ | 21.2 ± 0.5ᵇ | 4.4 ± 0.2 |
| MKK7 siRNA and SEPW1 siRNA   | 74.0 ± 0.3ᵇ | 18.8 ± 0.2ᵇ | 7.2 ± 0.5ᵃ |

ᵃ Significantly different from SEPW1 siRNA alone.
ᵇ Significantly different from non-targeting control siRNA.
* The experiment was repeated six times.

TABLE 3
Rescue of SEPW1 depletion-induced G₁ arrest by MKK4 silencing is independent of siRNA sequences

| siRNA treatment              | G₀ and G₁ | S phase | G₂ and M |
|------------------------------|-----------|---------|---------|
| No transfection              | 59.0 ± 0.5 | 34.3 ± 0.6 | 6.7 ± 1.1 |
| Non-targeting control siRNA  | 63.0 ± 1.5ᵃ | 30.4 ± 1.6ᵃᵇ | 6.6 ± 0.1ᵃ |
| SEPW1 siRNA s361             | 77.3 ± 0.2ᵇ | 18.1 ± 0.3ᵇ | 4.6 ± 0.3ᵇ |
| s361 and MKK4 siRNA s12701   | 70.5 ± 0.7ᵇ | 24.6 ± 0.8ᵇ | 5.0 ± 0.3 |
| s361 and MKK4 siRNA s12703   | 60.3 ± 0.4ᵇ | 29.5 ± 0.5ᵇ | 10.2 ± 0.3ᵇ |
| s363 and MKK4 siRNA s12701   | 66.2 ± 0.2ᵇ | 26.3 ± 0.2ᵇ | 7.4 ± 0.1ᵇ |
| s363 and MKK4 siRNA s12703   | 62.1 ± 2.0ᵇ | 31.1 ± 2.7ᵇ | 6.8 ± 0.7 |
| s363 and MKK4 siRNA a        | 66.1 ± 0.8ᵃ | 27.4 ± 0.9ᵇ | 6.5 ± 0.8 |

ᵃ Significantly different from the respective SEPW1 siRNA alone.
ᵇ Significantly different from non-targeting control siRNA.

Silencing Upstream MAP3Ks Does Not Block G₁ Arrest from SEPW1 Depletion—In the standard model of MAP kinase cascades, MAP2Ks such as MKK4 are activated via phosphorylation of serine and threonine residues by MAP3Ks. Therefore, we undertook to identify a MAP3K upstream of MKK4 that was involved in propagating the signal from SEPW1 silencing. We tested siRNAs targeting ASK1, MEKK1, MEKK4, TAK1, TPL2, and MLK3 for their ability to block cell cycle arrest from SEPW1 silencing. None of the six MAP3K siRNAs significantly blocked G₁ arrest from SEPW1 silencing, suggesting these MAP3Ks may not be involved in signaling G₁ arrest from SEPW1 silencing (Table 4). However, we only tested one siRNA for each MAP3K, and we did not verify protein knockdown. Thus, we cannot exclude involvement of any of these MAP3Ks.

MKK4 Is Neither Activated nor Inhibited by SEPW1 Silencing—Because six MAP3Ks upstream of MKK4 seemed not to be involved in signaling G₁ arrest from SEPW1 silencing, we assessed the phosphorylation state of MKK4 in SEPW1-silenced RWPE-1 cells. Antibodies specific for activated MKK4 phosphorylated on Ser-257 and Thr-261 were used to detect activation of MKK4. There was no significant difference between cells transfected with non-targeting control siRNA or SEPW1 siRNA 72 h post-transfection (p = 0.682, n = 4, paired t test, Fig. 4), indicating that SEPW1 silencing does not activate MKK4. MKK4 can also be negatively regulated by phosphorylation of Ser-80 (16), suggesting the possibility that cell cycle arrest from SEPW1 silencing might be mediated via de-repression of inhibited MKK4. However, phosphospecific antibodies directed toward this form of MKK4 did not detect any change in Ser-80 phosphorylation (p = 0.923, n = 5, paired t test, Fig. 4). Therefore, we found no evidence that the activity of MKK4 was altered MKK4 stability cannot explain increased MKK4 protein in SEPW1-depleted cells.

Total MKK4 Protein Is Increased by SEPW1 Silencing—Although the phosphorylation status of MKK4 was not affected, Western blots with pan-specific MKK4 antibodies showed that total MKK4 protein was higher in SEPW1-depleted cells (p = 0.009, paired t test, Fig. 4). To begin to investigate how MKK4 protein is increased by SEPW1 depletion, we re-examined microarray data from a previously published experiment (1). In that work we knocked down SEPW1 with three different siRNAs targeting different sequences in the SEPW1 mRNA and analyzed the effects with whole-genome microarrays. None of the three SEPW1 siRNAs caused a significant change in the amount of RNA hybridizing to the two probe sets for MKK4 (p > 0.13, supplemental Table S3), suggesting increased MKK4 protein was not due to altered levels of MKK4 mRNA. MKK4 is a stable protein in PC3, LNCaP, and SKOV3 prostate cancer cell lines (17). We found that MKK4 is also stable in RWPE-1 prostate cells; the slope of a semi-logarithmic plot of MKK4 protein versus time was not significantly different from zero (−0.0041 ± 0.0036 h⁻¹, p = 0.27), suggesting that altered MKK4 stability cannot explain increased MKK4 protein in SEPW1-depleted cells.

72734 JOURNAL OF BIOLOGICAL CHEMISTRY
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This research has identified a non-canonical signaling pathway from MKK4 to p53 that induces a transient G1-phase arrest in response to SEPW1 depletion (Fig. 5). Because it activates the JNK enzymes, MKK4 is well known as a mediator of p53-dependent apoptosis (18–20) but has a less recognized role in cell cycle arrest. However, it is known that a MKK4-JNK pathway blocks cell cycle progression in response to UV radiation (21), and MKK4 suppresses metastasis of prostate cancer xenografts through a transient G1 arrest (8) similar to the phenotype of SEPW1-silenced cells. Even though JNKs play a critical role in death receptor-initiated as well as mitochondrial apoptotic pathways (40) and our data suggest SEPW1 silencing activates JNK2, we did not observe significant numbers of SEPW1-depleted cells with sub-2n DNA content, indicative of cells actively undergoing apoptosis (supplemental Figs. S6–S8), nor did we see large amounts of the cellular debris commonly found in the culture medium when cells undergo apoptosis. Our findings mirror a previous report of SEPW1 siRNA decreasing the fraction of multiple myeloma endothelial cells in S-phase without an increase in sub-2n DNA content or apoptosis (41).

Silencing any of the six MAP3Ks that are most well known to activate MKK4 did not decrease G1 arrest from SEPW1 silencing, suggesting that these MAP3Ks may not be involved. However, we only screened each MAP3K with a single siRNA, and we did not verify silencing efficiencies, so these negative results must be viewed with caution. MKK4 is also reported to be activated by other MAP3Ks such as: p21 protein (Cdc42/Rac)-activated kinase 1 (22), germinal center kinase-like kinase (23), apoptosis signal-regulating kinase 2 (24), mixed-lineage kinase 2 (25), and MAPK/ERK kinase kinase 2 (26). Thus, we cannot exclude the possibility that MAP3Ks may be involved. However, because G1 arrest was not associated with changes in the phosphorylation state of MKK4, we did not investigate MAP3Ks further. It is also possible that MKK4 was phosphorylated early in the 72-h course of the experiment and activated p38 and JNK, but was subsequently dephosphorylated. Because activation of MAPK cascades can result in self-sustaining physiological states, transient activation of MKK4 might have been sufficient.

Control of this pathway is unusual in that MKK4 appears to be regulated by changes in protein level. Although phosphorylation is the best-known mechanism regulating MAP kinases,
**SEPW1 Depletion and MKK4-p38/JNK-p53-dependent G₁ Arrest**

**TABLE 4**

| siRNA treatment | G₀ and G₁ (± S.E.) | S phase | G₁ and M (± S.E.) |
|-----------------|-------------------|--------|------------------|
| No transfection | 61.5 ± 0.6        | 29.1 ± 0.4 | 8.7 ± 0.4 |
| Non-targeting control siRNA | 61.6 ± 0.5*       | 31.1 ± 0.5* | 7.4 ± 0.4* |
| SEPW1 siRNA s361 | 73.2 ± 0.7*       | 22.2 ± 0.5b | 4.6 ± 0.4b |
| SEPW1 siRNA and MEKK1 siRNA | 70.7 ± 0.2*       | 24.4 ± 0.2b | 4.9 ± 0.1b |
| SEPW1 siRNA and MEKK4 siRNA | 86.1 ± 0.6b       | 7.9 ± 0.2b  | 5.9 ± 0.4b |
| SEPW1 siRNA and ASK1 siRNA | 72.9 ± 0.5b       | 23.4 ± 0.4b | 3.7 ± 0.2b |
| SEPW1 siRNA and TAK1 siRNA | 76.9 ± 0.1b       | 17.8 ± 0.1b | 5.3 ± 0.1b |
| SEPW1 siRNA and TPL2 siRNA | 70.0 ± 0.1b       | 22.5 ± 0.5b | 7.5 ± 0.4* |
| SEPW1 siRNA and MLK3 siRNA | 71.5 ± 0.7b       | 23.7 ± 0.5b | 4.8 ± 0.2b |

* Significantly different from SEPW1 siRNA alone.
* Significantly different from non-targeting control siRNA.

**FIGURE 4.** Effect of silencing SEPW1 on MKK4 phosphorylation and total MKK4 protein. RWPE-1 cells were transfected with SEPW1 siRNA s361 or a non-targeting control siRNA. Lysates were collected at 72 h post-transfection and analyzed with Western blots using antibodies specific for Ser(P)-257/Thr(P)-261-MKK4, Ser(P)-80-MKK4, and total MKK4. UV-treated cell lysate was included as a positive control for activated MKK4 (Ser(P)-257/Thr(P)-261-MKK4, Ser(P)-80-MKK4, and total MKK4. UV-treated cell lysate was included as a positive control for activated MKK4 (Ser(P)-257/Thr(P)-261-MKK4, Ser(P)-80-MKK4, and total MKK4. β-Actin served as a loading control. A representative Western blot image from a single experiment is shown. Band intensities were estimated by quantitative densitometry. The bar graphs summarize the results of at least four experiments. Error bars represent S.E. Asterisk indicates total MKK4 protein was significantly increased compared with the negative control (p = 0.009, n = 9, two-tailed paired t test). -cntrl, non-targeting control siRNA; No TF, non-transfected cells.

**FIGURE 5.** Schematic diagram of proposed pathway for delayed cell cycle progression from SEPW1 silencing.

Prolonged stresses can also up-regulate members of the MAPK network at the protein expression level, as has been shown for the MAP3K, ASK1 (27). MKK4 is reported to be a stable protein in at least three prostate cancer cell lines (17), and we also could not detect a statistically significant change in MKK4 protein in RWPE-1 cells during 24 h of treatment with cycloheximide. Thus, altered stability cannot explain the increase of MKK4 in SEPW1-silenced cells. Regulation at the transcriptional or post-transcriptional levels also seems unlikely as there was no change in MKK4 mRNA transcript levels measured by hybridization to DNA oligonucleotide arrays (supplemental Table S3). Because we tested three different SEPW1 siRNAs and the arrays contained two MKK4-specific probe sets covering 22 sequences in MKK4 mRNA, this is a fairly robust negative result. MKK4 is known to be regulated by changes in translational efficiency due to altered polysome formation (17). Thus, it seems plausible to speculate that SEPW1 silencing may increase MKK4 protein by up-regulating the rate of MKK4 synthesis. However, our data do not directly address whether increased MKK4 protein actually drives the G₁ arrest from SEPW1 silencing. It is also possible that the basal activity of MKK4 is adequate to activate the downstream MAP kinases and that increased MKK4 protein is not essential. Although MKK4 is clearly required for the SEPW1 depletion-induced G₁ arrest, more research is needed to clarify whether the increase of MKK4 is essential or incidental.

p53 is expressed constitutively and degraded rapidly so that only low levels of p53 protein are present in unstressed cells. When cells are stressed by DNA damage, ionizing radiation, ultraviolet radiation, heat, osmotic shock, etc., a variety of protein kinases phosphorylate Ser and Thr residues in the N-ter-
minal region of p53. These phosphorylation events disrupt the interaction of p53 with proteins (e.g. HDM2, JNK1/2) that target p53 for proteasomal degradation. As a result, p53 rapidly increases, forms a homotetramer, and accumulates in the nucleus where it activates expression of p53-responsive genes, such as the cyclin-dependent kinase inhibitor p21^{Cip1}. MAPKs play an important role in p53 phosphorylation (4). A common event in many p53 activation pathways is phosphorylation of Ser-15 and/or Ser-20 (28). We showed in our previous work that silencing SEPW1 did not affect phosphorylation of p53 on Ser-15 or Ser-20 but increased phosphorylation of Ser-33 (2, 3).

The JNK enzymes regulate p53 in at least three ways. Murine JNK1, JNK2, and JNK3 associate with p53 in vivo and phosphorylate p53 on Ser-34 (Ser-33 in human p53) in vitro (13). In non-stressed cells, association with JNK1 or JNK2 targets p53 for ubiquitination and degradation (38). Phosphorylation of p53 on Thr-81 by JNK1 or JNK2 during translation blocks ubiquitination of p53 that would otherwise prevent its tetramerization and activation (39). Thus, JNK enzymes can either activate or suppress p53 function depending on the conditions. Although usually considered a JNK-specific MAP2K, MKK4 also activates p38 MAP kinase in a cell type- and context-dependent manner (29). While best known as a mediator of inflammatory responses in immune cells, p38 has a well recognized role in cell cycle regulation at both the G1/S and G2/M transitions (30). p38 responses in immune cells, p38 has a prominent role in controlling N-terminal phosphorylation and, therefore, activation of p53. p38 can phosphorylate p53 on Ser-15, Ser-33, Ser-46, and Ser-392 (36) and promotes phosphorylation of Ser-20 by its downstream target MAPKAPK2 (37). Phosphorylation of p53 on Ser-33 by p38 without phosphorylation of Ser-15 or Ser-20 is known to be sufficient to induce p53-dependent G1 arrest; osmotic shock induces p38-dependent G1 arrest mediated through p53, which is phosphorylated on Ser-33 without phosphorylation at Ser-15 or Ser-20 (14).

Additionally, a similar p38-p53-p21 pathway regulates G1 arrest when centrosomes are compromised and is associated with p38-directed phosphorylation of Ser-33 on p53 (33). However, in contrast to our findings, both of these studies attributed activation of p38 to MKK3/6, not MKK4.

Silencing JNK2, p38, and p38 each significantly rescued the G1 arrest and Ser-33 phosphorylation induced by SEPW1 depletion, suggesting that all three are involved in this pathway. In each case the G1 arrest reversal was only partial, suggesting the signal downstream from MKK4 may pass partly through JNK2, partly through p38, and partly through p38 (Fig. 5). This is similar to an ASK1-MKK4-p38/JNK pathway in human omental fat tissue that signals through MKK4 and then bifurcates to activate both the p38 and JNK pathways (42). Moreover, ASK1 was reported to be regulated at the level of protein expression and not by phosphorylation, similar to our finding for MKK4. Interpretation of our results is complicated by the intricate web of regulatory interactions between JNK and p38 (43, 44). Signaling through the JNK and p38 pathways is highly synchronized (45–47). Moreover, JNK and p38 have overlapping and redundant functions in addition to sharing several upstream regulators that activate both pathways (43). Thus, the overall outcome of silencing one MAPK isoform is the net effect of decreased Ser-33 phosphorylation, altered phosphorylation of other sites in p53 (4, 48), decreased activation of downstream pathways, and the responses of myriad compensatory mechanisms. It should be noted the evidence supporting involvement of p38 lies weakened by the fact siRNA s12467 targeting p38 failed to rescue G1 arrest from SEPW1 depletion (supplemental Table S2). Nevertheless, siRNA s12467 strongly reversed Ser-33 phosphorylation (Fig. 1), and the overall rescue from silencing p38 was significant in the ANOVA when cell cycle data from both p38 siRNAs were included (Table 1). Further investigation will be required to sort out the individual contributions of JNK and p38 isoforms in p53 activation and G1 arrest induced by SEPW1 depletion.

The best characterized molecular interaction of SEPW1 is with 14-3-3 proteins (49). SEPW1 binds 14-3-3 in a redox-dependent manner with high specificity (50). 14-3-3 proteins regulate the G1- to S-phase transition by binding MDMX (51), E2F1 (52), and p53 (53). 14-3-3 proteins are known to regulate some components of the MAPK network, including several MAP3Ks (54, 55), MAPK7 (56), and MLK3 (57), and some p38 and JNK pathways are also regulated by 14-3-3 proteins (58–60). Binding sites for 14-3-3 proteins are found in many ribosomal proteins (61), and 14-3-3 proteins have been shown to regulate the yeast proteome by interacting with proteins involved in protein synthesis (62). In higher eukaryotes, 14-3-3 proteins interact with several components of the translational machinery, including eukaryotic initiation factor 4B (63), eukaryotic translation initiation factor 2 α-subunit (64), enhancer of mRNA-decapping protein 3 (65), and a specific nucleotide motif in the 3′-UTR of some mRNAs (66). Thus, there are abundant potential mechanisms by which SEPW1 could plausibly regulate MKK4 synthesis and cell cycle progression via its interactions with 14-3-3 proteins. Future research is needed to identify the molecular mechanisms and protein interactions linking SEPW1 to cell cycle progression.

Our results imply that SEPW1 normally suppresses MKK4 that would otherwise activate p38 and JNK to phosphorylate and activate p53 that would block cell cycle progression. This raises the question, What purpose is served by this? Because the Sec at the active site makes SEPW1 exquisitely sensitive to oxidation (selenoproteins react at least 100 times faster with H2O2 than the corresponding Cys mutants (67)), SEPW1 could plausibly serve as a kind of sensor of oxidative stress. In this scenario oxidation of Sec would cause SEPW1 to stop suppressing MKK4, thus allowing MKK4 to increase and activate p53 to shut down cell cycle progression until the damage was repaired. Consequently, SEPW1 would serve as a kind of “safety fuse” that “blows” if the stress on SEPW1 becomes too high and shuts cell cycle progression down to protect the cell. It is also conceivable that SEPW1 is required for cell cycle entry because it is involved in transmitting a growth signal from an unidentified upstream activator. Further investigation is required to discriminate between these possibilities.

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AUGUST 10, 2012 VOLUME 287 NUMBER 33 JOURNAL OF BIOLOGICAL CHEMISTRY 27377
SEPW1 Depletion and MKK4-p38/JNK-p53-dependent G0 Arrest

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