Deletion of the Selenocysteine tRNA Gene in Macrophages and Liver Results in Compensatory Gene Induction of Cytoprotective Enzymes by Nrf2*

Received for publication, October 9, 2007, and in revised form, November 26, 2007 Published, JBC Papers in Press, November 26, 2007, DOI 10.1074/jbc.M708352200

Takafumi Suzuki†1, Vincent P. Kelly†1,2, Hozumi Motohashi§, Osamu Nakajima1, Satoru Takahashi†, Susumu Nishimura†, and Masayuki Yamamoto†§¶†1

From the †Exploratory Research for Advanced Technology Environmental Response Project and Center for Tsukuba Advanced Research Alliance and the §Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8577, Japan, the ¶Research Laboratory for Molecular Genetics, Yamagata University, Yamagata 990-9585, Japan, and the ‡Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, 2-1 Seiryo-cho, Aoba-ku, Sendai 980-8575, Japan

The selenocysteine tRNA (tRNA^Sec) molecule is the sight of synthesis for the amino acid selenocysteine and the adaptor for its translational insertion into selenoprotein enzymes, the majority of which contribute to cellular redox homeostasis. To examine the consequences of selenoprotein depletion on the oxidative environment of the cell, we generated a conditional knock-out mouse for the tRNA^Sec gene (Trsp). Deletion of Trsp in either macrophages or liver elevated oxidative stress and activated the transcriptional induction of cytoprotective antioxidant and detoxification enzyme genes, including glutathione S-transferase P1 and NAD(P)H:quinone oxidoreductase 1, and other well known target genes of the transcription factor Nrf2 (NF-E2-related factor 2). Simultaneous disruption of Trsp and Nrf2 severely compromised the cytoprotective response. Double knock-out macrophages displayed reduced viability, elevated oxidative stress, and increased susceptibility to hydrogen peroxide treatment compared with deletion of either gene alone. Mice carrying a liver-specific deletion of Trsp on an Nrf2-null background experienced hepatocellular apoptosis and displayed a severely reduced survival rate compared with loss of Trsp alone. Our results thus demonstrate that reduced selenoprotein activity is counterbalanced by an Nrf2-mediated cytoprotective response, which is essential for maintaining cellular redox homeostasis and viability.

Selenium is an essential micronutrient for the health and well being of all higher animals and many other forms of life. Deficiencies have been associated with increased incidences of infertility, cardiovascular disease, asthma, rheumatoid arthritis, immune incompetence, and cancer (1). Many of the protective properties associated with selenium may be attributed to the activities of the selenoproteins (2). These enzymes (numbering 25 in humans) contain selenium in the form of selenocysteine, an amino acid that is essential for their catalytic activity (3). The ribosomal incorporation of selenocysteine relies upon a number of cis- and trans-acting factors unique to this group of enzymes, including a specific selenocysteine tRNA molecule (tRNA^Sec) (4).

Genetic targeting of the tRNA^Sec gene (Trsp) in mice has shown that selenoproteins are necessary in embryonic development and for liver function because loss of Trsp in whole animals is lethal at embryonic day 4.5 (5), and specific removal from liver leads to death between 1 and 3 months of age (6). Cells in culture also display an absolute requirement for selenium and can neither grow nor survive in its absence (7). Interestingly, cells grown under selenium-free conditions may be viably maintained by the addition of the lipid-soluble antioxidant α-tocopherol, or other vitamin E-related analogs, to the medium (8). This suggests that at least one major functional role of the selenoproteins is to help maintain the redox status of the cell. Member proteins known to perform such a function include glutathione peroxidase (GPx)4 enzymes; selenoproteins P and W, which are capable of reducing hydrogen and lipid peroxides (9–11); selenoprotein R, a methionine-sulfoxide reductase (12); and the thioredoxin reductase enzymes, which maintain thioredoxin in a reduced and active state (9).

The important link between selenoproteins and redox homeostasis has been highlighted by studies in selenium-deficient animals, where a compensatory induction of various antioxidant-and xenobioto-metabolizing enzymes, such as glutathione S-transferase (GST), NAD(P)H:quinone oxidoreductase 1

4 The abbreviations used are: GPx, glutathione peroxidase; GST, glutathione S-transferase; NQO1, NAD(P)H:quinone oxidoreductase 1; HO-1, heme oxygenase 1; ARE, antioxidant-responsive element; ES, embryonic stem; ROS, reactive oxygen species; H2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; TUNEL, terminal nucleotidyltransferase dUTP nick end labeling; RT, reverse transcription; GCLC, glutamate-cysteine ligase, catalytic subunit; FACS, fluorescence-activated cell sorter; LysM, lysozyme M; Alb, albumin.
Antioxidant Gene Induction in Trsp-null Macrophages and Liver

(NQO1), and heme oxygenase 1 (HO-1), was observed (13–16). Similarly, mice that harbor a liver-specific deletion of the Trsp gene were shown to have increased expression of a GST Mu class member (6). It is proposed that the induction of cytoprotective enzymes is an attempt to re-establish the normal redox environment of the cell and to protect against the harmful metabolites that arise from oxidative damage.

Understanding this cytoprotective response has particular relevance to human health because of the widespread usage of 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors, collectively referred to as “statins.” In addition to functioning as anti-cholesterol agents, statin drugs directly inhibit selenoprotein production by blocking isopentenyl pyrophosphate formation, and, as a consequence, the isopentenylation of tRNA^{Sec} at position 37, a necessary base modification for efficient translation of the selenocysteine codon (17, 18). Statins are, in general, well tolerated by patients, but a sizable number of patients report muscle-related complaints, myopathies (19), the major-well tolerated by patients, but a sizable number of patients report muscle-related complaints, myopathies (19), the major-well tolerated by patients, but a sizable number of patients report muscle-related complaints, myopathies (19), the major-

Nrf2

MATERIALS AND METHODS

Construction of the Targeting Vector and Embryonic Stem Cell Screening—The backbone of the conditional targeting vector (see Fig. 1A) consisted of a Sall-PuVII fragment (7931 bp) retrieved from a 129/Sv genomic library as described previously (5). A single loxp element was cloned into the SnaBl site positioned upstream of the tRNA^{Sec} promoter elements, and a floxed pMC/neomycin-pMC/thymidine kinase (positive negative selection) cassette was inserted into the Hpal site immediately downstream of the tRNA^{Sec} transcription unit. Finally, a diphtheria toxin (DT3) cassette was placed downstream of the long-arm homology region. The targeting vector was linearized with PacI for electroporation into embryonic stem (ES) cells. Candidate homologous recombinant clones were screened by PCR using the KDFP1 and KDRP1 primer pair, followed by Southern blot hybridization using a 285-bp Swal-SspI short-arm internal probe as described previously (24). To confirm correct homologous recombination of the targeting vector, Southern blot analysis was performed using the Swal-SspI probe and a long-arm external PuVII-PvuI probe (1191 bp).

Generation and Genotyping of Floxed Trsp Mice—Homologous recombinant ES cell clones carrying a floxed allele were injected into C57BL/6 blastocytes and transferred to pseudo-pregnant females. The resulting high percentage chimeras (90% or greater based on coat color) were mated with wild-type C57BL/6 mice, and tail DNA samples from the F1 offspring were analyzed for germ line transmission. PCR genotyping of tail DNA samples was performed for 35 cycles (5 s at 94 °C, 30 s at 65 °C, and 30 s at 72 °C) using a mixture of three primers; the forward SeC1F primer (5′-GGC TGA CCT ACA GTT TCA GAG GCA C-3′) in conjunction with the reverse SeC1R primer (5′-ACC AAC TCC CCT TGA GTT TAG ACG C-3′) was used to identify the wild-type allele (439 bp) and targeted allele (494 bp), whereas SeC1F in combination with the reverse SeC2R primer (5′-GAC TGA GCC GGA GTG ACG AAA AAA TGA AC-3′) was used to confirm the presence of the Trsp-null allele (465 bp).

Deletion of the floxed Trsp gene in macrophages was performed by crossing heterozygous animals with a knock-in line of mice that have the cDNA for Cre recombinase inserted into the lysozyme M locus (25). The presence of Cre recombinase was determined using the CreF and CreR primers, whereas the neomycin phosphotransferase gene was detected using the Neo5-Prime and Neo3-Prime pair described previously (24).

Primer Extension Assay—Quantification of tRNA^{Sec} and the 18 S ribosomal RNA molecules was performed by primer extension as described previously (24, 26).

Culture of Mouse Primary Peritoneal Macrophages—Macrophages were isolated by lavage from mice that had received a 2-mI intraperitoneal injection of 4% thioglycolate broth 4 days before. The cells were transferred onto 6-well plates at a density of 7.5 × 10^5 cells/ml and maintained in RPMI 1640 medium containing 2 mM L-glutamine, 2 g/liter sodium bicarbonate, 1 mM sodium pyruvate, 10 mM HEPES, penicillin (10 units)/streptomycin (0.1 mg/ml), and 10% fetal calf serum in a humidified atmosphere of 5% CO_2 at 37 °C.

Cell Viability Assays—Cell viability after H_2O_2 treatment was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (27). Briefly, macrophage cells were plated at a density of 1 × 10^5 cells/ml in a 6-well culture plate. Cells were washed with phosphate-buffered saline and changed to untreated medium (control) or medium containing 30 or 100 μM H_2O_2. After an overnight incubation, the cytotoxic effect of H_2O_2 was determined using a cell-counting assay kit (Dojin Laboratories), a modified version of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The absorbance of the medium was measured at 450 nm.
Measurement of Reactive Oxygen Species—Intracellular reactive oxygen species (ROS) were measured using the membrane-permeable fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCFDA; Molecular Probes). Macrophages were incubated with H$_2$DCFDA at a final concentration of 5 μM in phosphate-buffered saline for 15 min at 37 °C, washed once with phosphate-buffered saline, and placed at 37 °C. After 30 min of incubation, the cells were washed and resuspended in phosphate-buffered saline for flow cytometric analysis using a FACScan (BD Biosciences) set to excitation and emission wavelengths of 475 and 525 nm, respectively. For each analysis, 10,000 events were recorded.

Aptoptotic Assay—Cellular apoptosis was examined by flow cytometry using fluorescein isothiocyanate-conjugated annexin V and propidium iodide staining as described previously (28).

**TUNEL Assay**—Liver and lung tissues were fixed in 4% paraformaldehyde for 2 h at 4 °C and embedded in OCT compound. Sections were stained with fluorescein isothiocyanate-conjugated TUNEL reaction mixture (TaKaRa) for 1 h at room temperature.

**Real-time Reverse Transcription (RT)-PCR and RNA Blot Analyses**—The RNA transcripts for GSTP1, GCLC, HO-1, and NQO1 were quantified by real-time RT-PCR. Briefly, total RNA was isolated from peritoneal macrophages using the ISOGEN method (Nippon Gene Co., Ltd.). cDNA was prepared using random hexamer primers, and real-time RT-PCR was performed using an Applied Biosystems 7700 sequence detector as described previously (23). The primer sequences used are shown in Table 1. For RNA blot analysis, NQO1, GSTP1, and HO-1 probes were hybridized as described previously (22).

**Immunoblot Analyses**—GPx1 protein was detected by immunoblot analysis using a 1:2000 dilution of the rabbit GPx1 antisera prepared previously (24). Nuclear extracts of peritoneal macrophages were prepared by sucrose gradient and probed with anti-Nrf2 antibody (29) and anti-lamin B antibody (Santa Cruz Biotechnology, Inc.) at dilutions of 1:2000 and 1:1000, respectively.

**RESULTS**

Generation of the Trsp Conditional Knock-out Mice—To clarify how Nrf2 contributes to cellular protection against oxidative stress in the absence of selenoprotein production, we created a conditional knock-out mouse line for the Trsp gene. The conditional targeting vector was made using a SalI-PvuII conditional targeting vector, the wild-type allele (WT) or analyzed by PCR (E) using the SeC1F and SeC1R primer pair to detect the wild-type allele (WT: 439 bp) and floxed allele (494 bp) and the SeC1F and SeC1R primer pair to detect the null allele (465 bp).

**RESULTS**

**FIGURE 1. Creating the Trsp conditional knock-out mouse.** A, the targeting strategy for ES cells homologous recombination, not drawn to scale, showing the targeting vector, the wild-type Trsp locus, and the targeted allele. Functional elements are shown as open boxes. Overhead arrows depict the orientation of transcriptional units. The positive negative selection (PNS) cassette and the tRNA<sup>sec</sup> transcript unit are shown flanked by lox<sup>P</sup> elements, designated by open arrowheads. Southern blotting probes are shown as black bars. Only relevant restriction sites are shown. B, BamHI; H, HindIII; N, Nott; P, PvuII; R, Rcal; Sa, Sall; Sn, SnaBl; X, Xhol. B and C, Southern hybridization analyses of ES cell lines C83, C157, and C227. DNA digested with BamHI or Rcal was hybridized to a short-arm internal probe (B), whereas DNA digested with Nott and Xbal was probed using a long-arm external probe (C). Lines C83 and C227 were confirmed as true homologous recombinants, and chimeric mice from C227 transmitted the conditional allele to the germ line. D and E, deletion of the floxed Trsp allele in whole animals by Cre-mediated recombination. Genomic DNA was isolated from Trsp<sup>wt</sup>/− and Trsp<sup>fl</sup>/− ES cells and from tail samples of Trsp<sup>fl</sup>/−, Trsp<sup>fl</sup>/−<sup>Ayu1-Cre</sup>, and Trsp<sup>fl</sup>/−<sup>TdR</sup> mice. The DNA samples were digested with Rcal and hybridized with the short-arm internal probe (D) or analyzed by PCR (E) using the SeC1F and SeC1R primer pair to detect the wild-type allele (WT: 439 bp) and floxed allele (494 bp) and the SeC1F and SeC1R primer pair to detect the null allele (465 bp).
Heterozygous mutant mice with the conditional Trsp allele (Trsp\(^{+/+}\)) were crossed with a transgenic mouse strain capable of ubiquitous expression of Cre recombinase under the control of the Ayu1 promoter (Ayu1-Cre) to yield Trsp\(^{+/+}\):Ayu1-Cre mice. Functionality of the loxP elements \textit{in vivo} was demonstrated by generating heterozygous null mutant mice (Trsp\(^{+/-}\):Ayu1-Cre) and heterozygous mutant mice with the floxed and null alleles (Trsp\(^{+/del}\)). We analyzed the recombination status of the Trsp allele in these mice by Southern blotting (Fig. 1D) and PCR (Fig. 1E). The results clearly showed that the loxP elements could faithfully direct the removal of the sandwiched region.

**Deletion of Trsp in Peritoneal Macrophages and Effect on tRNA\(_{Sec}\) Production**—We first examined tRNA\(_{Sec}\) function in macrophages. Preliminary studies showed that adherent myelomonocytic cultures (derived from peritoneal exudates 4 days post-thioglycolate administration) almost exclusively contained fully differentiated macrophages, as determined by non-specific esterase staining (data not shown). Cultures of exudates isolated earlier than this time point (days 1–3 post-thioglycolate administration) contained higher amounts of monocytic cells, in agreement with a previous report (30). Therefore, all subsequent studies were performed on macrophage cultures that had been retrieved from mice 4 days after receiving thioglycolate.

A mouse line with the cDNA for Cre recombinase inserted into the lysozyme M locus (LysM-Cre) has been described, allowing the removal of an appropriately floxed gene in non-myelomonocytic cells or the product of a fraction of macrophages, as determined by non-specific esterase staining (data not shown). Cultures of exudates isolated earlier than this time point (days 1–3 post-thioglycolate administration) contained higher amounts of monocytic cells, in agreement with a previous report (30). Therefore, all subsequent studies were performed on macrophage cultures that had been retrieved from mice 4 days after receiving thioglycolate.

A mouse line with the cDNA for Cre recombinase inserted into the lysozyme M locus (LysM-Cre) has been described, allowing the removal of an appropriately floxed gene in non-myelomonocytic cells or the product of a fraction of macrophages, as determined by non-specific esterase staining (data not shown). Cultures of exudates isolated earlier than this time point (days 1–3 post-thioglycolate administration) contained higher amounts of monocytic cells, in agreement with a previous report (30). Therefore, all subsequent studies were performed on macrophage cultures that had been retrieved from mice 4 days after receiving thioglycolate.

A mouse line with the cDNA for Cre recombinase inserted into the lysozyme M locus (LysM-Cre) has been described, allowing the removal of an appropriately floxed gene in non-myelomonocytic cells or the product of a fraction of macrophages, as determined by non-specific esterase staining (data not shown). Cultures of exudates isolated earlier than this time point (days 1–3 post-thioglycolate administration) contained higher amounts of monocytic cells, in agreement with a previous report (30). Therefore, all subsequent studies were performed on macrophage cultures that had been retrieved from mice 4 days after receiving thioglycolate.

A mouse line with the cDNA for Cre recombinase inserted into the lysozyme M locus (LysM-Cre) has been described, allowing the removal of an appropriately floxed gene in non-myelomonocytic cells or the product of a fraction of macrophages, as determined by non-specific esterase staining (data not shown). Cultures of exudates isolated earlier than this time point (days 1–3 post-thioglycolate administration) contained higher amounts of monocytic cells, in agreement with a previous report (30). Therefore, all subsequent studies were performed on macrophage cultures that had been retrieved from mice 4 days after receiving thioglycolate.

A mouse line with the cDNA for Cre recombinase inserted into the lysozyme M locus (LysM-Cre) has been described, allowing the removal of an appropriately floxed gene in non-myelomonocytic cells or the product of a fraction of macrophages, as determined by non-specific esterase staining (data not shown). Cultures of exudates isolated earlier than this time point (days 1–3 post-thioglycolate administration) contained higher amounts of monocytic cells, in agreement with a previous report (30). Therefore, all subsequent studies were performed on macrophage cultures that had been retrieved from mice 4 days after receiving thioglycolate.

**Figure 2. Deletion of Trsp in peritoneal macrophages and effect on tRNA\(_{Sec}\) production**—Peritoneal macrophages isolated from Trsp\(^{+/+}\), Trsp\(^{+-}\):LysM-Cre, and Trsp\(^{+/del}\):LysM-Cre mice were cultured for 1, 3, or 5 days and analyzed by PCR to establish the recombination status of the Trsp locus (A) or by primer extension analysis to examine tRNA\(_{Sec}\) levels (B, upper panel) relative to the 18 S ribosomal subunit (lower panel).

Deletion of Trsp in peritoneal macrophages and effect on tRNA\(_{Sec}\) production was reported for mature macrophages and an 83–98% deletion rate for granulocytes (25). PCR analysis was used to examine the recombination status of the Trsp locus in macrophages from wild-type, Trsp\(^{+/+}\):LysM-Cre, and Trsp\(^{+/del}\):LysM-Cre mice that had been maintained in culture for 1, 3, and 5 days (Fig. 2A). Cre recombinase expression from the lysozyme M locus is maximally induced upon maturation of myelomonocytic cells (31) in the same manner as the endogenous lysozyme M gene (32). Following 1 day of culture, the floxed Trsp allele of Trsp\(^{+/-}\):LysM-Cre and Trsp\(^{+/del}\):LysM-Cre macrophages had almost fully recombined, resulting in heterogeneous and homozygous null mutants of the Trsp locus, respectively. A marginal proportion of PCR product indicated the presence of residual floxed Trsp allele. It is not known if this fraction represents the PCR product from a contaminating population of non-myelomonocytic cells or the product of a fraction of macrophages that failed to undergo complete loxP recombination.

Primer extension analysis showed that the cellular levels of tRNA\(_{Sec}\) reflected the genomic status of the Trsp allele (Fig. 2B). In wild-type macrophages, the levels of tRNA\(_{Sec}\) did not change during the 5 days of culture (compare lanes 1, 4, and 7). Heterozygous cells had lower levels of tRNA\(_{Sec}\) compared with wild-type cells (~60%), which did not change over the 5-day period (compare lanes 2, 5, and 8). A small but observable quantity of tRNA\(_{Sec}\) was detectable in macrophages from Trsp\(^{+/del}\):LysM-Cre mice that had been cultured for 1 day, but thereafter, levels became too low to detect (compare lanes 3, 6, and 9). The rapid decline observed in tRNA\(_{Sec}\) levels is consistent with results from Chinese hamster ovary cells showing that the turnover of the tRNA\(_{Sec}\) molecule is rapid relative to the total tRNA population, decreasing by 61% within 10 h of treatment with actinomycin D (33).

Trsp\(^{+/del}\):LysM-Cre Macrophages Show a Parallel Decline in tRNA\(_{Sec}\) and Selenoprotein GPx1—On the basis of studies in selenium-deficient animals, it was expected that Trsp deletion in myelomonocytic cells might interfere with the phagocytic function of neutrophils and macrophages (34) and the biosynthesis of leukotriene B\(_4\) by macrophages (35). We found that Trsp\(^{+/del}\) mice carrying the LysM-Cre gene (thereby presumably null mutants in myelomonocytic lineages) displayed no obvious phenotypic abnormalities under pathogen-free conditions. Therefore, we carried out immunoblot analysis of whole cell extract from peritoneal macrophages for the GPx1 selenoprotein (Fig. 3A). In wild-type and Trsp\(^{+/+}\):LysM-Cre macrophages, the production of GPx1 protein was maintained over the 5 days in culture (compare lanes 1–4 with lanes 7–10 and 13–16). By contrast, in Trsp\(^{+/del}\):LysM-Cre macrophages, loss of tRNA\(_{Sec}\) resulted in a gradual decrease in GPx1 over time, being almost undetectable by day 5 (compare lanes 5 and 6 with lanes 11 and 12 and lanes 17 and 18).

**Deletion of Trsp in Primary Peritoneal Macrophages Results in Increased Oxidative Stress and Induction of Antioxidant Enzymes**—A major function for mammalian selenoproteins is their direct or indirect involvement in antioxidant defense pathways. Therefore, we examined the intracellular ROS levels...
Antioxidant Gene Induction in Trsp-null Macrophages and Liver

These data reveal that deletion of Trsp in primary peritoneal macrophages causes an increase in intracellular oxidative stress.

Previous reports have shown that transcriptional expression of the GST and NQO1 genes is induced under selenium-deficient conditions (13, 14, 16). Deletion of the Trsp gene is functionally equivalent to selenium deficiency because it also leads to loss of selenoprotein members. Therefore, we examined the gene expression of a number of xenobiotic and antioxidant enzyme species known to be up-regulated by various oxidative insults using real-time RT-PCR analysis (Fig. 3C). The expression of NQO1, GSTP1, and HO-1 genes increased linearly in macrophages from Trspfl/flo::LysM-Cre mice from 1 to 5 days in culture concurrent with loss of GPx1 expression. GCLC levels also increased but displayed an inexplicable decrease on day 5 relative to day 3. These increases were statistically significant (data not shown). These results support our contention that, during selenium deficiency, the transcription of cytoprotective enzymes is induced as a mechanism to compensate for loss of protective selenoprotein activities and to facilitate the repair of cellular damage.

Induction of Antioxidant Genes in Trsp-null Macrophages Relies on Nrf2 Activation—It has been shown that the transcription of each cytoprotective enzyme described above responds to oxidative stress (36, 37). The above observations, together with our observation on Nrf2 knock-out (22) and Trsp knock-down mice (24), drew us to hypothesize that Nrf2 may be central to the cytoprotective response caused by and compensating for loss of selenoproteins. To examine this possibility, we generated peritoneal macrophages from Trsp+/+, Trspflo::LysM-Cre, and Trspflo/flo::LysM-Cre mice on either a wild-type (Nrf2+/+) or Nrf2-null (Nrf2−/−) background. Macrophages were cultured for 5 days, and the expression levels of NQO1, GSTP1, GCLC, and HO-1 were examined by real-time RT-PCR (Fig. 4A). In the absence of Nrf2 alone, macrophages displayed a modest decrease in the RNA transcript of cytoprotective enzymes compared with wild-type levels, indicating that Nrf2 is partially responsible for basal expression from the corresponding genes. Strikingly, deletion of Nrf2 in Trsp-null macrophages completely impaired the induction of NQO1, GSTP1 and GCLC, verifying the role of this transcription factor in responding to loss of selenoproteins. Intriguingly, the induction of HO-1 in Trsp-null macrophages was not impaired by loss of Nrf2 but rather was further enhanced.

The transcriptional activity of Nrf2 is controlled by its interaction with the cytoplasmic regulatory protein Keap1 (kelch-like ECH-associated protein 1) (38). Exposure of cells to electrophiles or ROS causes Nrf2 to be liberated from Keap1 regulation, leading to its translocation to and accumulation in the nucleus. Therefore, to determine the activation status of Nrf2, nuclei were extracted from peritoneal macrophages of Trsp+/+ and Trspflo/flo::LysM-Cre mice that had been cultured for 5 days, and the levels of Nrf2 protein were examined by immunoblot analysis. Nrf2 protein was found to markedly accumulate in the nuclei of Trspflo/flo::LysM-Cre macrophages compared with wild-type macrophages (Fig. 4B), demonstrating that deletion of Trsp leads to the activation of Nrf2.
Antioxidant Gene Induction in Trsp-null Macrophages and Liver

FIGURE 4. Effect of Nrf2 deletion on antioxidant gene expression and ROS production in Trsp null macrophages. A, peritoneal macrophages from Trsp+/+ and Trspfl/−;LysM-Cre mice on a wild-type background (Nrf2+/+) or crossed onto an Nrf2-null background (Nrf2−/−) were cultured for 5 days, and the expression levels of NQO1, GSTP1, GCLC, and HO-1 were examined by real-time RT-PCR with the 18S ribosomal subunit as an internal control. B, nuclei were extracted from the peritoneal macrophages of Trsp+/+ and Trspfl/−;LysM-Cre mice that had been cultured for 5 days, and the levels of Nrf2 protein and the nuclear protein lamin B were examined by Western blotting. C, macrophages were isolated from the peritoneal cavities of Trsp+/+ and Trspfl/−;LysM-Cre mice on a wild-type (Nrf2+/+) or Nrf2-null (Nrf2−/−) background, and ROS production was evaluated on day 5 by FACS analysis using the redox-sensitive dye H2DCFDA (upper panel). A graphical representation of the results is also shown (lower panel).

As noted above, the induction of Nrf2-regulated cytoprotective enzymes is presumably a cellular response to compensate for an absence of important selenoprotein activities, in particular the antioxidant function that this group of enzymes performs. This possibility was explored by comparing ROS levels in macrophages isolated from mice on a wild-type, a singular, and a double knock-out mutant background using the redox-sensitive dye H2DCFDA (Fig. 4C). Notably, the absence of Nrf2 or Trsp alone gave rise to an increase in cellular ROS production compared with wild-type cells, demonstrating the functional overlap between Nrf2-regulated genes and selenoproteins. Corroborating this conclusion, simultaneous deletion of Trsp and Nrf2, genetically annotated as Nrf2−/−;Trspfl/−;LysM-Cre macrophages, accentuated the production of ROS in comparison with loss of either gene alone.

Removal of the Nrf2 Gene from Trsp-null Macrophages Increases Sensitivity to Hydrogen Peroxide Treatment—Given that the absence of Trsp and Nrf2 (either alone or in combination) leads to heightened production of intracellular ROS, it is conceivable that such cells no longer retain the ability to protect themselves from oxidative metabolites. We examined this possibility by determining the resistance of wild-type, Trsp-null, Nrf2-null, and double Trsp/Nrf2-null macrophages to overnight treatment with 30 or 100 μM H2O2, viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Note that Nrf2−/− macrophages were markedly weaker than wild-type macrophages upon treatment with H2O2. * and **, statistically significant differences (p < 0.01 and 0.05, respectively). B, FACS analysis of macrophages with the genotypes described above was performed using fluorescein isothiocyanate-conjugated annexin V and propidium iodide (PI) to distinguish between apoptotic and necrotic cell death. Note that apoptotic cell death (open bars) increased compared with live cells (gray bars) in the Trspfl/−;LysM-Cre macrophages.

FIGURE 5. Sensitivity of Trsp and Nrf2 single and double knock-out macrophages to hydrogen peroxide treatment and apoptosis. A, Trsp+/+ and Trspfl/−;LysM-Cre macrophages on a wild-type (Nrf2+/+) or Nrf2-null (Nrf2−/−) background were cultured for 5 days. Following 1 day in culture, Trsp+/+ and Trspfl/−;LysM-Cre macrophages were cultured for 5 days. After an overnight challenge with 30 or 100 μM H2O2, viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assay (Fig. 5A). Following 1 day in culture, Trsp single knock-out, Nrf2 single knock-out, and Trsp/Nrf2 double mutant knock-out macrophages did not display any decrease in viability (data not shown), as GPx1 levels, and presumably the levels of other selenoproteins, had not yet decreased substantially as shown earlier (Fig. 3A). On day 5 of culture, wild-type macrophages appeared to be insensitive to H2O2 (Fig. 5A, first through third bars). Trsp-null macrophages showed a marginal change in sensitivity to H2O2 treatment (fourth through sixth bars), whereas loss of Nrf2 resulted in increased sensitivity to H2O2 (compare the eighth and ninth bars with the seventh bar). Even in the absence of H2O2, treatment, the simultaneous deletion of Trsp and Nrf2 caused a substantial decline in cell viability after 5 days compared with loss of either gene alone (compare the tenth bar with the fourth and seventh bars), attesting to the importance of
Antioxidant Gene Induction in Trsp-null Macrophages and Liver

Nrf2 Protects Trsp-null Macrophages from Apoptotic Death—The notable intracellular increase in ROS levels in Trsp::Nrf2 double mutant macrophages (Fig. 4C) and the pronounced reduction in viability after 5 days in culture (Fig. 5A) led us to ask whether loss of selenoprotein activity gives rise to apoptotic cell death. To distinguish between non-apoptotic, apoptotic, or necrotic cell death, the binding of fluorescein isothiocyanate-conjugated annexin V to phosphatidylserine (which redistributes from the cytoplasmic face of the plasma membrane to the outer leaflet during apoptosis) and propidium iodide staining were examined by flow cytometry (Fig. 5B, left panels). The results showed that loss of Trsp caused an increase in the apoptotic cell population from 6.2% (seen in wild-type cells) to 31% (summarized in right panels). Simultaneous deletion of Trsp and Nrf2 greatly exacerbated the effect, with 53% of the cell population presenting the annexin V apoptotic marker. These results indicate that Nrf2 protects Trsp-null macrophages from apoptotic death.

Nrf2 Activates Compensatory Induction of Cytoprotective Enzymes in Trsp-null Mouse Liver—Up to this point, our studies had focused on the compensatory gene induction by Nrf2 at the cellular level through the use of Trsp-null macrophages. However, the Nrf2 cytoprotective response is not unique to macrophages and may also be important to the protection of many animal tissues under conditions of selenium deficiency, injury, or inflammation. Therefore, we decided to examine Nrf2-mediated compensatory gene induction in whole animals following a decrease in selenoprotein production. To this end, the Trsp gene was specifically removed from liver using albumin-Cre transgenic mice (39), in which the cDNA for Cre recombinase is under the control of the liver-specific albumin promoter (Alb-Cre). In accordance with our expectations, immunoblot analysis failed to detect GPx1 protein in the livers of Trsp<sup>fl/fl</sup>::Alb-Cre mice (Fig. 6A), and similar to what was observed in macrophages, the RNA transcripts for NQO1, GSTP1, and HO-1 were all increased in abundance, as determined by RNA blotting (Fig. 6B). Supporting the observation made in macrophages, immunoblot analyses showed nuclear accumulation of Nrf2 protein in the livers of Trsp<sup>fl/fl</sup>::Alb-Cre mice (Fig. 6C). Upon removal of Nrf2 from Trsp<sup>fl/fl</sup>::Alb-Cre mice, compensatory gene induction was severely inhibited, as measured by RT-PCR analysis of liver RNA (Fig. 6D). These results strongly argue that the Nrf2-mediated induction of cytoprotective enzymes is a compensatory response that is common to various Trsp-null mouse tissues.

Removal of the Nrf2 Gene from Trsp-null Hepatocytes Dramatically Increases Mouse Mortality and Results in Liver Degeneration—We observed that Trsp<sup>fl/fl</sup>::Alb-Cre mice died between 1 and 3 months of age, which is consistent with published results (6). Strikingly however, a Kaplan-Meyer survival assessment revealed that Nrf2<sup>−/−</sup>:Trsp<sup>fl/fl</sup>::Alb-Cre double knock-out mice all died within 7 weeks of birth, with the highest rate of mortality occurring by 3 weeks of age (Fig. 7A). These results affirm the requirement for the compensatory cytoprotective response evoked by Nrf2 in the absence of selenoprotein activity and concur with the observation that full recombination of the floxed Trsp gene in Alb-Cre transgenic animals occurs over a period of 3–4 weeks postpartum (6).

There were no significant differences in the blood biochemistries of single Trsp-null or wild-type mice, as reported previously (6). By contrast, the levels of serum alanine aminotransferase, a protein released into the blood when the liver is damaged, were significantly higher in Trsp::Nrf2 double knock-out mice at 3 weeks of age compared with those in wild-type, Trsp<sup>fl/fl</sup>::Alb-Cre, and Nrf2<sup>−/−</sup>:Alb-Cre mice (Fig. 7B), signifying severe hepatic degeneration. Curiously, the livers of Trsp::Nrf2 double knock-out mice had a distinctive pale-white color (Fig. 7C), characterized by massive cell death and permeation of inflammatory cells into the liver (Fig. 7D). The discoloration phenotype and associated pathologies (Fig. 7, C–E) were not observed in Nrf2-null mice (F–H) or Trsp-null mice (data not shown). Higher magnification of liver sections showed that many of the hepatocytes in Trsp::Nrf2 double knock-out mice had succumbed to necrosis and apoptosis (Fig. 7E), a phenotype not observed in Nrf2-null liver (Fig. 7H). In contrast to the lack of apoptotic cell death in the livers of wild-type and single mutant mice (Fig. 7, I–K), the apoptotic susceptibility of Trsp::Nrf2 double knock-out hepatocytes was clearly distinguishable by TUNEL assay (Fig. 7L). These results thus demonstrate that removal of the Nrf2 gene from Trsp-null hepatocytes dramatically increases mouse mortality and provokes liver degeneration.
Nrf2

sent results from three to five animals. The gene regulatory regions of
polys, and the metabolism of the pro-oxidant heme, respectively (reviewed in Ref. 40). The gene regulatory regions of
 confer cells a strong defense against oxidative damage

FIGURE 7. Effect of removing the Nrf2 gene from mice with Trsp-null livers on animal survival, hepatic function, and hepatocyte viability. A, survival was monitored over a 24-week period for Nrf2+/+::Trsp+/+ (n = 20), Nrf2+/+::TrspflAlb-Cre (n = 53), Nrf2−/−::Trsp+/+ (n = 20), and Nrf2−/−::TrspflAlb-Cre (n = 24) mice. B, the serum alanine aminotransferase (ALT) activity of mice at 3 weeks of age was examined. The data represent results from three to five animals. C and F, shown are whole livers from Nrf2−/−::TrspflAlb-Cre and Nrf2−/−::TrspflAlb-Cre mice, respectively, and low (D and G) and high (E and H) magnifications of hematoxylin/eosin-stained liver sections. Arrowheads indicate necrotic hepatocytes displaying swollen cytoplasmic vacuolation and apoptotic cells with condensed nuclei. Permeation of many inflammatory cells can be seen in the livers of Nrf2−/−::TrspflAlb-Cre mice. I-L, the TUNEL staining of liver sections from mice of each genotype shows TUNEL-positive cells (green) in the livers of Nrf2−/−::TrspflAlb-Cre mice.

FIGURE 8. Model for compensatory gene induction by Nrf2 in the absence of selenoprotein production. A, selenoproteins constitutively maintain redox balance. The Nrf2 gene battery is an inducible protective system and usually under repression during normal conditions. B, selenoprotein production is restricted when selenium is withdrawn from the diet. Under these circumstances, enzymes of the Nrf2 gene battery are induced in an attempt to restore normal redox conditions and to repair oxidative damage. Relatively weak challenges to the cellular redox balance may be taken care of by selenoprotein members, some of which are inducible by Nrf2. However, those challenges, which threaten the integrity of the cell, such as hyperoxia and chemically induced oxidative stress, signal activation of Nrf2, resulting in a gauged induction of a broad range of antioxidant and cytoprotective enzymes. SeCys, selenocysteine; 2GSH, reduced glutathione; Trx-S2, oxidized thioredoxin; Trx-(SH)2, reduced thioredoxin; GR, glutathione reductase; PRX, peroxiredoxin; TrxR, thioredoxin reductase; IVR, intervening region; Ub, ubiquitin; 7Ks, α-helix containing seven lysines.

In general, with the exception of GPx2 and thioredoxin reductase, which have recently been shown to be direct targets of Nrf2 (42, 43), the selenoprotein enzymes appear to be unresponsive to external oxidative insults, and their activities are limited to a “housekeeping” role. The dependence of selenoproteins on an exogenous supply of selenium and the requirement for protein and RNA components unique to their translation run contrary to the criteria for a rapid and independent response to oxidative insults. Such a role is conceptually supplied by the Nrf2 system.

Recently, a mechanism to explain Nrf2 activation under oxidative/electrophilic stress conditions has been proposed and is referred to as the two-site substrate recognition and hinge-latch model (44–46). As depicted in Fig. 8, Nrf2 is tethered via its DLG and ETGE motifs to a homodimer of the Keap1 protein. Between the DLG and ETGE motifs is a central α-helical region containing lysine residues that act as ubiquitination sites, thereby marking Nrf2 for proteasomal degradation under basal non-stressed conditions. The high affinity ETGE motif acts as a hinge to allow efficient recruitment of Nrf2 to the Cul3-based ubiquitin-protein isopeptide ligase. On the other hand, the DLG motif functions as a latch by locking and unlocking the lysines that are recognized by the catalytic pocket of the ubiquitin carrier protein enzyme. Electrophiles and oxidants appear to bind to the reactive cysteine residues in the BTB (Broad

DISCUSSION

It has been widely accepted that selenoproteins contribute to the control of cellular redox homeostasis. In this study, we have demonstrated for the first time that the loss of selenoprotein activity is counterbalanced by activation of the Nrf2-Keap1 pathway by cytotoxic reactive and antioxidant enzyme induction, which provides protection against oxidative damage and apoptosis on a cellular and organismal level. The pronounced induction of GCLC, NQO1, GSTP1, and HO-1 upon deletion of the Trsp gene would be expected to confer cells a strong defense against oxidative damage through their known catalytic activities, which include the production of glutathione, the reduction of reactive quinones, the glutathione conjugation of acrolein and various base propanols, and the metabolism of the pro-oxidant heme, respectively (reviewed in Ref. 40). The gene regulatory regions of these enzymes contain an ARE, defining them as a part of the Nrf2 gene battery (41). It is highly conceivable that all Nrf2 target genes participating in redox homeostasis are up-regulated by deletion of the Trsp gene.
Antioxidant Gene Induction in Trsp-null Macrophages and Liver

Complex/Tramtrack/Bric-a-Brac) and intervening region domains of Keap1 and hamper the affinity or alter the binding conformation of the DLG motif to Keap1 (46). By these means, Nrf2 is stabilized and is no longer a target for ubiquitination. Therefore, the Nrf2-Keap1 system ensures a response that is rapid (immediate liberation of Nrf2 from Keap1 repression), amplifiable (Nrf2 self-induction), broad (through the presence of an ARE within numerous gene promoter regions), and modifiable (by Nrf2-interacting proteins, phosphorylation, and ubiquitination) (47).

The observations of this study and those above are presented in Fig. 8, in which the selenoproteins are shown as homeostatic mediators of redox levels in the cell. By contrast, the Nrf2 gene battery is involved in fine-tuning the redox balance and eradicating oxidative damage by enhancing the synthesis of glutathione and thioredoxin and the induction of antioxidant and cytoprotective enzymes. We contend that, in animals, the functional importance of the Nrf2-Keap1 regulatory pathway lies in its unique inducible capacity to absorb the overflow of endogenous oxidative metabolites that have escaped eradication by conventional, non-inducible, stress response systems. Clearly, the Nrf2 response has a vital role in the absence of selenoproteins because simultaneous deletion of Nrf2 and Trsp in liver greatly accelerated tissue degeneration and mortality in mice compared with deletion of Trsp alone.

In cultures of primary Trsp-null macrophages, the expression levels of the ARE-regulated genes increased in a graded and sustained manner over time. Such an effect suggests that the level of ARE-regulated gene induction is set to reflect the severity of the insult. Furthermore, it indicates that, despite the induction of ARE-driven antioxidant genes, the endogenous stimulation of Nrf2 activation could not be eradicated in selenoprotein-deficient cells. In this regard, treatment of Trsp-null macrophages with the soluble antioxidant N-acetyl-L-cysteine, which has the ability to increase intracellular glutathione, failed to repress the induction of ARE-regulated genes (data not shown). It would therefore appear that ARE-regulated genes are unable to fully replace the selenoproteins in their capacity as antioxidant and redox regulatory enzymes. The distinctive cytoprotective properties of selenoproteins and the Nrf2 gene battery were also evident upon H$_2$O$_2$ treatment, where Nrf2-null macrophages appeared to be more sensitive than Trsp knock-out macrophages, and the loss in viability of double knock-out cells did not appear to be cumulative. Notably, homozygous Trsp knockout-out mice fail to develop in utero from an early stage (5), whereas homozygous Nrf2 knock-out mice are viable and fertile, albeit sensitive to oxidative insults (22, 48, 49).

The identity of the stimulating molecule(s) leading to the activation of Nrf2 in cells devoid of tRNAs$_{Sec}$ production remains unknown. Numerous studies have documented the ability of various synthetic and naturally derived chemicals to promote Nrf2 activation. An essential attribute of these inducers is the ability to react with thiol/disulfide groups and, accordingly, the cysteine residues of Keap1 by alkylation, oxidation, reduction, or thiol interchange (50–52). Probable endogenous stimuli for Nrf2 activation include H$_2$O$_2$, hydroperoxides, tyrosine breakdown products, and 4-hydroxynonenal (53) and the recently discovered electrophilic product from the reaction of nitric oxide with cGMP, denoted 8-nitro-cGMP (54).

A wide range of muscle-related side effects have been described for statins, including myalgia, myositis, myopathy, and, in severe cases, rhabdomyolysis (18). RNA blot experiments have suggested that the levels of Nrf2 are extremely low in muscle (55), and literature searches failed to identify Nrf2 activation or canonical Nrf2 target gene induction in skeletal muscle, raising the interesting although unsubstantiated possibility that the absence of a muscular cytoprotective response contributes to statin-associated myopathies. Our previous observation that Nrf2 expression is absent in the pregastrular stages of mouse embryonic development (24) likewise raises the question of whether this could be a contributing factor in congenital abnormalities following exposure of women to statins during the first trimester of pregnancy. Conversely, numerous publications attest to the multiple protective roles of Nrf2 against oxidative insults, inflammation, and harmful xenobiotics, suggesting that activation of Nrf2 may partly account for the beneficial “pleiotropic” (cholesterol-independent) effects of statins, such as protection from vascular inflammation and ischemic stroke (17, 56).

The induction profile of the HO-1 gene in Trsp-null cells is clearly distinct from that of the other ARE-regulated genes because simultaneous knock-out of Nrf2 caused a further enhancement of transcription. The result reflects those of our earlier study in Trsp knockdown day 7.5 embryos, where we observed an induction of HO-1 gene expression despite the absence of Nrf2 expression (24). HO-1 is an early indicator of the inflammatory immune response, being induced by lipopolysaccharide and various pro-inflammatory cytokines, such as tumor necrosis factor-α and interleukin-1β, in addition to its own substrate heme (57, 58). Therefore, it is likely that the induction of HO-1 in Trsp-null embryos and cells is indicative of a pro-inflammatory environment brought about by the absence of appropriate antioxidant enzyme activities.

It has been shown that the induction of the Nrf2 gene battery in whole animals by exogenous synthetic and naturally occurring chemicals protects against chemically induced carcinogenesis (59) and the oxidative damage arising from hypoxic conditions (60, 61). Here, we have shown, that in the absence of any exogenous stimuli, Nrf2 is activated by a perturbation in the intracellular redox status and that, by way of target gene induction, Nrf2 functions to reduce ROS production and help sustain viability. Our results suggest that, in addition to its role in detoxification processes, the Nrf2 gene battery is responsible for fine-tuning the cellular redox levels, which could be perturbed following inadequate consumption of selenium or from long-term exposure to cholesterol-lowering statin drugs.

Acknowledgments—We thank Drs. Ken Itoh, Makoto Kobayashi, Tania O’Connor, Jon Maher, and Fumiki Katsuoka for advice and discussion. We also thank the members of the Tsukuba Research Institute of Banyu Pharmaceutical Co. for generous help.

REFERENCES
1. Rayman, M. P. (2000) Lancet 356, 233–241
2. Hatfield, D. L., and Gladyshev, V. N. (2002) Mol. Cell. Biol. 22, 3565–3576
Antioxidant Gene Induction in Trsp-null Macrophages and Liver

9. Behne, D., and Kyriakopoulos, A. (2001)
10. Jeong, D. W., Yoo, M. H., Kim, T. S., Kim, J. H., and Kim, I. Y. (2002)
11. Saito, Y., Yoshida, Y., Akazawa, T., Takahashi, K., and Niki, E. (2003)
12. Kryukov, G. V., Castellano, S., Novoselov, S. V., Lobanov, A. V., Zehtab, V., and Hayashi, M., and Yamamoto, M. (2005)
13. Mostert, V., Hill, K. E., Ferris, C. D., and Burk, R. F. (2003)
14. Christensen, M. J., Nelson, B. L., and Wray, C. D. (1994)
15. Mosmann, T. (1983)
16. Carlson, B. A., Novoselov, S. V., Kumaraswamy, E., Lee, B. J., Anver, M. R., Gladyshev, V. N., and Hatfield, D. L. (2004)
17. Clausen, B. E., Burkhardt, C., Reith, W., Renkawitz, R., and Förster, I.
18. Kelly, V. P., Suzuki, T., Nakajima, O., Arai, T., Tamai, Y., Takahashi, S., Nakajima, O., M, Sato, H., Yanagawa, T., Katoh, Y., Bannai, Y., and Yamamoto, M. (2000)
19. Itoh, K., Wakabayashi, N., Katoh, Y., Iishi, T., Igarashi, K., Engel, J. D., and Yamamoto, M. (1999)
20. Sinzinger, H., Lupattelli, G., Chehne, F., Oguogho, A., and Furberg, C. D. (2001)
21. McWalter, G. K., Higgins, L. G., McLellan, L. I., Henderson, C. J., Song, L., Thornley, P. J., Itoh, K., Yamamoto, M., and Hayes, J. D. (2004)
22. Mosspoon, B., and Behl, C. (2004)
23. Katsuoka, F., Motohoshi, H., Engel, J. D., and Yamamoto, M. (2005)
24. Kelly, G. J., Berry, M. J., Moustafta, M. E., Carlson, B. A., Hatfield, D. L., and Faust, J. R. (2000)
25. Thompson, P. D., Clarkson, P., and Karas, R. H. (2003)
26. Sinzinger, H., Lupattelli, G., Chehne, F., Oguogho, A., and Furberg, C. D. (2001)
27. Clausen, B. E., Burkehardt, C., Reith, W., Renkawitz, R., and Förster, I. (1999)
28. Moustafta, M., Carlson, B. A., El-Saadani, M. A., Kryukov, G. V., Sun, Q.-L., Harney, J. W., Hill, K. E., Combs, G. F., Feigenbaum, L., Mansur, D. B., Burk, R. F., Berry, M. J., Diamond, A. M., Lee, B. J., Gladyshev, V. N., and Hatfield, D. L. (2001)
29. Mosmann, T. (1983)
30. Engel, V. M., Ramaekers, F. C., Schutte, B., and Reutelingsperger, C. P. (1996)
31. Suzuki, T., Takagi, Y., Osanai, H., Li, L., Takeuchi, M., Katoh, Y., Kobayashi, S., and Yamamoto, M. (2005)
32. Faust, N., Varas, F., Kelly, L. M., Heck, S., and Graf, T. (2000)
33. Cross, M., Mangelsdorf, I., Wedel, A., and Renkawitz, R. (1988)