Direct Interaction between Nrf2 and p21<sup>Cip1/WAF1</sup> Upregulates the Nrf2-Mediated Antioxidant Response

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SUMMARY

In response to oxidative stress, Nrf2 and p21<sup>Cip1/WAF1</sup> are both upregulated to protect cells from oxidative damage. Nrf2 is constantly ubiquitinated by a Keap1 dimer that interacts with a weak-binding 29DLG motif and a strong-binding 79ETGE motif in Nrf2, resulting in degradation of Nrf2. Modification of the redox-sensitive cysteine residues on Keap1 disrupts the Keap1-29DLG binding, leading to diminished Nrf2 ubiquitination and activation of the antioxidant response. However, the underlying mechanism by which p21 protects cells from oxidative damage remains unclear. Here we present molecular and genetic evidence suggesting that the antioxidant function of p21 is mediated through activation of Nrf2 by stabilizing the Nrf2 protein. The 154KRR motif in p21 directly interacts with the 29DLG and 79ETGE motifs in Nrf2 and thus competes with Keap1 for Nrf2 binding, compromising ubiquitination of Nrf2. Furthermore, the physiological significance of our findings was demonstrated in vivo using p21-deficient mice.

INTRODUCTION

The transcription factor Nrf2 has emerged as a master regulator of an intracellular antioxidant response through transcriptional activation of an array of genes, including phase II detoxifying enzymes, antioxidants, and transporters that protect cells from toxic and carcinogenic chemicals (Kensler et al., 2007; Kobayashi and Yamamoto, 2005; Zhang, 2006). Nrf2-deficient mice are prone to chemical-induced toxicity and tumorigenesis (Aoki et al., 2001; Cho et al., 2004; Lau et al., 2008; Ramos-Gomez et al., 2001). The Nrf2 signaling pathway is negatively controlled through activation of Nrf2 under both basal and induced conditions was confirmed using p21-deficient mice, demonstrating the physiological significance of our findings.

Nrf2 is constantly ubiquitinated by a Keap1 dimer that interacts with a weak-binding 29DLG motif and a strong-binding 79ETGE motif in Nrf2, resulting in degradation of Nrf2. Modification of the redox-sensitive cysteine residues on Keap1 disrupts the Keap1-29DLG binding, leading to diminished Nrf2 ubiquitination and activation of the antioxidant response. However, the underlying mechanism by which p21 protects cells from oxidative damage remains unclear. Here we present molecular and genetic evidence suggesting that the antioxidant function of p21 is mediated through activation of Nrf2 by stabilizing the Nrf2 protein. The 154KRR motif in p21 directly interacts with the 29DLG and 79ETGE motifs in Nrf2 and thus competes with Keap1 for Nrf2 binding, compromising ubiquitination of Nrf2. Furthermore, the physiological significance of our findings was demonstrated in vivo using p21-deficient mice.
RESULTS

Nrf2 Was Required for the p21-Dependent Cellular Protection in Response to Oxidative Stress

To determine a possible functional link between p21 and Nrf2, a series of experiments were performed in mouse embryonic fibroblasts (MEFs) from Keap1- or Nrf2-deficient mice, or in HCT116 cells with or without a somatic p21 deletion. First, the protective role of p21 against the cellular stress response was verified by measuring intracellular reactive oxygen species (ROS) in HCT116-p21\(+/-\) and HCT116-p21\(-/-\) cells following H2O2 challenge. While H2O2 did not induce a measurable amount of ROS in HCT116-p21\(+/-\) cells, it increased the level of ROS in HCT116-p21\(-/-\) cells in a dose-dependent manner (Figure 1A).

To exclude the possibility that the difference in their response to H2O2 is due to clonal artifacts, p21-cDNA was reintroduced into HCT116-p21\(-/-\) cells by transient transfection. Ectopic expression of p21 rescued HCT116-p21\(-/-\) cells from H2O2-induced ROS production (Figure 1A). The residual dose-dependent increase of ROS is likely due to the fact that 100% transfection efficiency could not be achieved.

Next, the antioxidant function of Nrf2 was demonstrated in HCT116-p21\(+/-\) cells using a siRNA approach. Nrf2-siRNA significantly reduced Nrf2 protein level, compared with the control siRNA (Figure 1B). Consequently, Nrf2-siRNA-transfected cells had an elevated basal ROS level compared to the control-siRNA-transfected cells (Figure 1B).

In addition, the antioxidant response mediated by p21 and by Nrf2 was further evaluated by comparing the survival curves in response to H2O2 treatment between the pair of HCT116-p21\(+/-\)/p21\(-/-\) cells and the pair of MEF-Keap1\(+/-\)/Keap1\(-/-\) cells. The HCT116-p21\(+/-\)/p21\(-/-\) cells had a higher basal level of Nrf2 compared to the HCT116-p21\(-/-\) cells, and the basal level of Nrf2 was higher in MEF-Keap1\(-/-\) cells than in MEF-Keap1\(+/-\) cells (data not shown). Interestingly, similar toxicity curves were obtained between these two groups (see Figure S1 available...
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Direct Interaction between p21 and Nrf2

Figure 2. p21 Upregulated the Protein Level of Nrf2 and Its Downstream Genes
(A) Knockdown of p21 diminished expression of the Nrf2 target genes in MEF-Nrf2+/+ cells. MEF-Nrf2−/− cells were transfected with either control siRNA or p21-siRNA. The endogenous levels of p21 and Nrf2 proteins were detected by immunoblot analysis at 48 hr posttransfection, while the mRNA expression of Nrf2, Keap1, NQO1, and HO-1 was measured 72 hr after transfection. The error bars indicate standard deviations calculated from triplicate samples; *p < 0.05 compared with its control. (B) Knockdown of p21 had no effects in MEF-Nrf2+/+ cells as described in (A). (C) p21 upregulated the ARE-reporter gene activity. HCT116-p21−/− cells were transfected with different amounts of p21 expression vectors, along with expression vectors for the NQO1-ARE firefly luciferase and TK-renilla luciferase. The protein levels of p21 and Nrf2 were detected with anti-p21 and anti-Nrf2 antibodies (lower panels). The firefly luciferase activities were normalized to renilla luciferase activities, and the standard deviations were calculated from three independent experiments, each with duplicate samples; *p < 0.05 compared with its control.

online), indicating that p21 and Nrf2 render cells more resistant to H2O2. Next, the requirement of Nrf2 in p21-mediated cell survival in response to H2O2 was tested in both MEF-Nrf2+/+ and MEF-Nrf2−/− cells. Although overexpression of p21 protected cells from oxidative cell death in MEF-Nrf2+/+ cells, it had no effect on cell survival in MEF-Nrf2−/− cells (Figure 1C). In contrast, ectopic expression of Nrf2 had an effect on both cell types. In order to eliminate the possibility that the protection observed is due to cell-cycle arrest under conditions of p21 overexpression, a similar experiment in which p21 expression was inhibited by p21-siRNA was performed in both MEF-Nrf2+/+ and MEF-Nrf2−/− cells. Reduced p21 expression sensitized MEF-Nrf2+/+ cells to H2O2 insult, but not MEF-Nrf2−/− cells (Figure 1D). Collectively, these results demonstrate that both Nrf2 and p21 have antioxidant functions that promote survival of cells under oxidative stress. More importantly, p21-mediated protection requires Nrf2, suggesting crosstalk between p21 and the Nrf2 signaling pathway.

p21 Upregulated the Protein Level of Nrf2 and Its Downstream Genes
To further verify our hypothesis that the antioxidant function of p21 is mediated through upregulation of the Nrf2 signaling pathway, effects of p21 on the transcription of Nrf2 and Nrf2 target genes were measured by qRT-PCR in MEF-Nrf2+/+ cells. p21-siRNA reduced the expression of endogenous p21 protein approximately 60% with a concomitant decrease of the Nrf2 protein level in MEF-Nrf2+/+ cells (Figure 2A). p21-siRNA had no effect on the mRNA expression of Nrf2 or Keap1, while knockdown of p21 expression resulted in a decrease of the Nrf2-dependent transcription of NAD[P]H quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1) in MEF-Nrf2+/+ cells (Figure 2A). In contrast, p21 siRNA had no effect on transcription of NQO1 or HO-1 in MEF-Nrf2−/− cells (Figure 2B). Together, these data indicate that p21-mediated upregulation of the Nrf2 signaling pathway requires Nrf2. This notion was further verified by overexpression of p21 in HCT116-p21−/− cells. Transient expression of p21 enhanced the levels of Nrf2 protein in a p21 dose-dependent manner (Figure 2C). Consistent with the increased protein level of Nrf2, the transcriptional activity of Nrf2 was also enhanced by overexpression of p21 when a NQO1-ARE firefly luciferase reporter was used (Figure 2C). Therefore, p21 is able to activate the Nrf2 signaling pathway by enhancing Nrf2 protein levels. A similar experiment was also performed in COS-1 cells; coexpression of p21 with Nrf2 enhanced the protein level of Nrf2 in the absence or presence of exogenous Keap1, and overexpression of Keap1 decreased the protein level of Nrf2 as expected (Figure S2).

HCT116-p21−/− Cells Had Reduced Basal and Induced Levels of the Nrf2-Dependent Antioxidant Response
It has been well documented that p21 is induced in both a p53-dependent and -independent manner at the transcriptional and posttranscriptional levels in response to oxidative stress. The endogenous p21 level was measured in HCT116-p21−/− cells treated with Nrf2 activators, tert-butylhydroquinone (tBHQ), and sulforaphane (SF). Both tBHQ and SF enhanced the protein levels of Nrf2-Dependent Antioxidant Response in a dose-dependent manner (Figure 3A). Next, the Nrf2-dependent response under basal and induced conditions in HCT116-p21−/− and HCT116-p21+/+ cells was compared. First, the basal level of Nrf2 is slightly lower in HCT116-p21−/− cells (Figure 3B). tBHQ and SF enhanced the protein level of Nrf2 in both cell lines, although the Nrf2 level
Figure 3. HCT116-p21−/− Cells Had Reduced Basal and Induced Levels of the Nrf2-Dependent Antioxidant Response
(A) p21 was upregulated in response to oxidative stress. HCT116-p21+/− cells were either untreated or treated with the indicated compounds for 16 hr. Endogenous p21 and β-actin were detected with anti-p21 and anti-β-actin antibodies (left panel). The band intensity of p21 and β-actin was quantified, and the p21 protein level was normalized to the β-actin protein and plotted (right panel).
(B) The basal and induced Nrf2 protein levels were lower in HCT116-p21+/− cells. HCT116-p21+/− or HCT116-p21−/− cells were either untreated or treated with different doses of tBHQ or SF for 16 hr. Endogenous Nrf2 was detected with an anti-Nrf2 antibody. Nrf2 appears as a double band because a lower-percentage gel (7.5%) was used.
(C) Knockdown of p21 diminished the transcriptional activity of Nrf2. HCT116-p21+/− and HCT116-p21−/− cells were transfected with expression vectors for NQO1-ARE firefly luciferase and TK-renilla luciferase. The transfected cells were treated with the indicated concentration of tBHQ or SF for 16 hr prior to the measurement of luciferase activities. The standard deviations were calculated from three independent experiments, each with duplicate samples; *p < 0.05 compared with its control.
(D) mRNA expression of the Nrf2 downstream genes was reduced in HCT116-p21−/− cells. HCT116-p21+/− and HCT116-p21−/− cells were either left untreated or treated with 50 μM tBHQ or 10 μM SF for 16 hr. Relative amounts of NQO1, Gpx2, and Mrp2 mRNAs were measured by qRT-PCR. The standard deviations were calculated from triplicate samples; *p < 0.05 compared with its control.
(E) HCT116-p21−/− cells had lower NQO1 activity.

p21 Directly Interacted with Nrf2
The interaction of these two proteins was explored in vivo and in vitro. p21-Myc and HA-Nrf2 were transiently expressed in COS-1 cells. p21-Myc was detected in the HA immunoprecipitates (Figure 4A, left panel). In a reciprocal immunoprecipitation assay, HA-Nrf2 was present in the Myc immunoprecipitates (Figure 4A, right panel), indicating the presence of both p21 and Nrf2 in the same complex in vivo. To test direct binding and to identify the Nrf2-binding domain in p21, a series of GST-tagged p21 deletion mutants were purified and incubated with the 35S-labeled Nrf2 protein. Direct interaction of these two proteins was detected (Figure 4B, lanes 1–164). Based on the fact that p21 70–140 lost interaction with Nrf2, whereas

NQO1 enzyme activity in HCT116-p21+/− and HCT116-p21−/− cells was measured as the dicoumarol-inhibitable fraction of DCPIP reduction. NQO1 activity was normalized to the total protein level, and the standard deviations were calculated from triplicate samples; *p < 0.05 compared with its control.
p21 70–164 did not, it was deduced that the C-terminal domain of p21 containing 24 amino acids (from 140–164) is likely to be the interaction site for Nrf2 binding (Figure 4B). Reciprocally, GST-tagged Nrf2 was also able to pull down 35S-labeled p21 protein, but not the negative control luciferase (Figure 4C). To identify the p21-binding domain in Nrf2, an immunoprecipitation analysis was performed with cell lysates coexpressing p21-Myc and each of the several HA-Nrf2 deletion mutants. All mutants retained their ability to bind p21, including the smallest one (the N-terminal 1–115), indicating that this N-terminal domain (1–115) contains a binding site for p21 (Figure 4D). More importantly, the interaction of endogenous Nrf2 with endogenous p21 was assessed in HCT116-p21+/+ and MEF-Nrf2+/+ cells. The anti-p21 antibody, but not IgG or HA, immunoprecipitated Nrf2 in both HCT116-p21+/+ and MEF-Nrf2+/+ cells (Figure 4E and Figure S5). Furthermore, there was an increased association of p21 with Nrf2 in response to oxidative stress, likely due to increased expression of p21 and p21 in response to tBHQ treatment (Figure 4E). These results suggest that p21 upregulates the Nrf2 signaling pathway mainly through direct interaction with Nrf2 to enhance the protein level of Nrf2.

**p21 Interfered with the Keap1-Dependent Ubiquitination of Nrf2 by Competing with Keap1 for Nrf2 Binding Mainly through the 29DLG Motif**

The immunoprecipitation result indicates that the N-terminal domain (1–115) of Nrf2 interacts with p21 (Figure 4D). The N-terminal Neh2 domain of Nrf2 contains many functional motifs or residues that have been reported to be important for the function of Nrf2. In particular, it contains seven lysine residues that are required for ubiquitination and degradation of Nrf2. It also contains two Keap1-binding motifs, 29DLG and 79ETGE, that are crucial for Keap1-dependent ubiquitination and degradation of Nrf2. To further narrow down the precise amino acid residues in the Neh2 domain of Nrf2 that interact with p21, two Nrf2 mutants were generated: mDLG and mETGE in which the 29DLG or 79ETGE motif was replaced with alanine residues. Immunoprecipitation analyses were performed in COS-1 cells cotransfected with p21-Myc and Nrf2 wild-type or each of its mutants. In order to compare the binding affinity of Nrf2 to p21 with that of Nrf2 to Keap1, Nrf2 wild-type or each of its mutants was also coexpressed with the Kelch domain of Keap1 in another set of experiments. Both sets of samples were prepared and analyzed together using the immunoprecipitation/immunoblot method. Since mutation of 29DLG or 79ETGE motif was replaced with alanine residues. Immunoprecipitation analyses were performed in COS-1 cells cotransfected with p21-Myc and Nrf2 wild-type or each of its mutants. In order to compare the binding affinity of Nrf2 to p21 with that of Nrf2 to Keap1, Nrf2 wild-type or each of its mutants was also coexpressed with the Kelch domain of Keap1 in another set of experiments. Both sets of samples were prepared and analyzed together using the immunoprecipitation/immunoblot method. Since mutation of 29DLG or 79ETGE motif was replaced with alanine residues.

**Figure 4. p21 Directly Interacted with Nrf2**

(A) p21 interacted with Nrf2. COS-1 cell lysates coexpressing p21-Myc and HA-Nrf2 were immunoprecipitated with anti-HA (left panel) or anti-Myc (right panel) antibodies. The total lysates (lower two panels) and the immunoprecipitates (upper two panels) were subjected to immunoblot analysis with both anti-HA and anti-Myc antibodies for detection of Nrf2 and p21.

(B) The C-terminal domain of p21 was required for direct interaction with Nrf2. GST pull-down analyses were performed with GST-p21 fusion proteins containing different regions of p21 and the 35S-labeled Nrf2 protein.

(C) p21 directly interacted with Nrf2. The GST-Nrf2 fusion protein was used to pull down 35S-labeled p21. Luciferase was used as a negative control.

(D) The N-terminal domain of Nrf2 interacted with p21. p21 and each of the Nrf2 C-terminal deletion mutants was coexpressed in COS-1 cells. The Nrf2-containing complexes were immunoprecipitated with HA beads and blotted with anti-HA and anti-Myc antibodies for detection of Nrf2 and p21. Bands labeled with asterisks are Nrf2 and its deletion mutants.

(E) Binding of endogenous p21 to Nrf2 was enhanced in response to oxidative stress. Cell lysates from HCT116-p21+/+ cells untreated or treated with tBHQ were subjected to immunoprecipitation analysis with an anti-p21 antibody and then blotted with an anti-Nrf2 antibody (upper panel). An aliquot of total lysate was subjected to immunoblot analysis with anti-Nrf2, anti-p21, and anti-tubulin antibodies (lower three panels).
anti-Nrf2 antibody, and the immunoprecipitated proteins were subjected to immunoblot analysis with anti-Myc for detection of p21-Myc and Kelch-Myc, and anti-HA to detect HA-Nrf2 (Figure 5A, upper panels). In agreement with the two-site model reported previously, mutation of ETGE significantly diminished the Nrf2-Kelch interaction while replacement of DLG with AAA weakened Nrf2 binding slightly (Figure 5A, compare lanes 8 and 7 with lane 6). Interestingly, the association of p21 to either of the Nrf2 mutants was weaker compared to Nrf2 wild-type, indicating that both DLG and ETGE are the interacting motifs for p21 (Figure 5A, compare lanes 3 and 4 with lane 2). It seems that the binding affinities of Nrf2-p21 and Nrf2-Kelch were comparable since similar amounts of p21-Myc and Kelch-Myc were immunoprecipitated by an anti-Nrf2 antibody. In contrast, the binding was weaker for mETGE to Kelch than mETGE to p21 (Figure 5A, compare lane 8 with lane 4), indicating that p21 may readily compete with Keap1 for the DLG site (latch).

Next, a competition assay was carried out to examine if p21 is able to displace Keap1. Since ectopic coexpression of p21 changes Nrf2 levels, it is impossible to express equal amounts of Nrf2 among samples when different amounts of p21 are expressed. Therefore, the competition assay was performed in vitro. HA-Nrf2, chitin-binding domain (CBD)-tagged Keap1, or p21-Myc was individually transfected into COS-1 cells. The same amount of lysate containing HA-Nrf2 and CBD-Keap1 was mixed with an increasing amount of p21-Myc lysate. The untransfected COS-1 lysate was used to match the total volume of the mixture. Keap1-containing complexes were pulled down by chitin beads and subjected to immunoblot analysis with anti-HA and anti-CBD antibodies. Our results show that p21 is inefficient in dissociating the Nrf2-Keap1 complex (Figure S6). We reasoned that p21 is probably able to compete for DLG binding, but not for ETGE due to the strong ETGE-Kelch interaction. In the hinge...
and latch model, it was proposed that disruption of the latch (29DLG-Kelch interaction) is sufficient to compromise the Keap1-dependent ubiquitination of Nrf2. It is conceivable that p21 interferes with the Keap1-dependent ubiquitination of Nrf2 by competing with Keap1 for Nrf2 binding through the 29DLG motif. Thus, endogenous Nrf2 ubiquitination in HCT116-p21+/− and HCT116-p21−/− cells was measured under basal and stressed conditions. Cells were treated with a proteasome inhibitor, MG132, for 4 hr to block ubiquitinated Nrf2 from degradation. The results showed a reduced basal level of ubiquitin-conjugated Nrf2 in HCT116-p21−/− cells, compared to that in HCT116-p21+/− cells (Figure 5B). As expected, tBHQ was able to reduce ubiquitination of Nrf2 in both cell lines (Figure 5B). Next, the half-life of Nrf2 in HCT116-p21+/− cells was compared to that in HCT116-p21−/− cells by using cycloheximide (CHX) and immunoblot analysis. Nrf2 had a longer half-life in HCT116-p21+/− cells than in HCT116-p21−/− cells (27.9 min versus 14.4 min in the untreated condition; 45.3 min versus 23.9 min in the tBHQ-treated condition) (Figure 5C). A similar result was obtained when a pulse-chase 35S-labeling method was utilized for measurement of the half-life of Nrf2 (Figure S7). Taken together, our data indicate that p21 is able to prevent ubiquitination of Nrf2 by disrupting the latch site (29DLG-Kelch) of the Nrf2-Keap1 interaction without displacement of Keap1 from the complex.

**The C-Terminal 154KRR in p21 Was Essential for Binding and Upregulating Nrf2**

29DLG and 79ETGE motifs bind to each of the Kelch domains from the Keap1 homodimer. The Kelch domain contains six conserved Kelch repeats that form a six-bladed β-propeller structure (Li et al., 2004; Padmanabhan et al., 2006). The arginine triad (R380, R415, and R483) located in the central channel of Keap1 was found to be important for interaction with either the 29DLG motif or the 79ETGE motif (McMahon et al., 2006; Tong et al., 2006, 2007). Since p21 does not have a rigid structure, but binds to the same 29DLG or 79ETGE motif as does Kelch, it is possible that basic amino acid clusters consisting of arginine/lysine residues may be the Nrf2 interaction site in p21. In addition, the result from the GST pull-down experiment demonstrates that the 24 amino acids (140–164) in the C terminus of p21 contained the Nrf2 binding site(s) (Figure 4B). Hence, we examined the arginine/lysine clusters within the 24 amino acids. Three clusters, 140RKRR, 154KRR, and 161KRK, were found. Accordingly, three p21 mutants (mRKRR, mKRR, and mKRK) were constructed in which arginine or lysine residues were replaced with methionine residues. Next, p21 wild-type (WT) or each of its mutants was coexpressed with HA-Nrf2 in COS-1 cells for measurement of the half-life of Nrf2 (Figure S7). As expected, tBHQ was able to reduce ubiquitination of Nrf2 in both cell lines (Figure 5B). As expected, tBHQ was able to reduce ubiquitination of Nrf2 in both cell lines (Figure 5B). Next, the half-life of Nrf2 in HCT116-p21+/− cells was compared to that in HCT116-p21−/− cells by using cycloheximide (CHX) and immunoblot analysis. Nrf2 had a longer half-life in HCT116-p21+/− cells than in HCT116-p21−/− cells (27.9 min versus 14.4 min in the untreated condition; 45.3 min versus 23.9 min in the tBHQ-treated condition) (Figure 5C). A similar result was obtained when a pulse-chase 35S-labeling method was utilized for measurement of the half-life of Nrf2 (Figure S7). Taken together, our data indicate that p21 is able to prevent ubiquitination of Nrf2 by disrupting the latch site (29DLG-Kelch) of the Nrf2-Keap1 interaction without displacement of Keap1 from the complex.

**p21-Deficient Mice Had Reduced Basal and Induced Levels of Nrf2 and Nrf2 Target Genes**
p21-dependent Nrf2 upregulation under physiological conditions was confirmed using liver tissue from p21-deficient and p21 wild-type control mice that were either untreated or treated with tert-butylhydroxyanisole (BHA). BHA is metabolized into tBHQ following absorption and is a known chemical inducer of oxidative stress in animals (Hayes et al., 2000). As shown in Figure 7A, the basal Nrf2 level was slightly lower in p21−/− mice than in wild-type mice. Strikingly, the protein level of Nrf2 enhanced by BHA was substantial in wild-type mice, whereas BHA enhanced Nrf2 to a lesser extent in p21−/− mice (Figure 7A). Consistent with the Nrf2 protein level, both NQO1 and HO-1 had lower basal and induced levels in p21−/− mice, compared to p21 wild-type mice (Figure 7A). The induction of the Nrf2-dependent antioxidant response appears to be more substantial in animal models than in cultured cells. Collectively,
these results provide solid evidence that upregulation of the Nrf2 antioxidant response by p21 under both basal and induced conditions does not only occur in cell models but also under physiological conditions in animals.

**DISCUSSION**

In this report, we present our finding that p21 is able to upregulate the Nrf2 signaling pathway under both basal and induced conditions through direct interaction with Nrf2. Based on the genetic and molecular data obtained, we present a model that best explains how p21 is able to upregulate the Nrf2-dependent antioxidant response under both basal and induced conditions (Figure 7B). Keap1, an E3 ubiquitin ligase, constantly targets Nrf2 for ubiquitination and subsequent degradation under unstressed conditions. The binding of Keap1 to Nrf2 is through a hinge and latch mechanism, in which each Kelch domain from a Keap1 homodimer binds Nrf2 through two binding sites: a weak-binding DLG motif and a strong-binding ETGE motif. Binding of both sites is essential to present the seven ubiquitin-accepting lysine residues of Nrf2 in the correct orientation to accept ubiquitin, and thus targets Nrf2 for degradation. In response to oxidative stress, Keap1 is able to detect an imbalance in intracellular redox homeostasis through modification of its cysteine residues. These modifications alter its conformation and loosen the latch (DLG-Kelch), which puts p21 in a better position to compete with Keap1 for binding to the DLG motif. Binding of both sites is essential to present the seven ubiquitin-accepting lysine residues of Nrf2 in the correct orientation to accept ubiquitin, and thus targets Nrf2 for degradation. It is noteworthy that our finding of p21-mediated upregulation of the Nrf2 signaling pathway should not devalue the importance of Keap1 in sensing and regulating Nrf2. Rather, this p21-Keap1 competition model requires the
Direct Interaction between p21 and Nrf2

A

Mouse: p21 +/+ p21 +/-

Treatment: mock BHA mock BHA

Nrf2

NQO1

HO-1

GAPDH

B

Figure 7. p21-Deficient Mice Had Reduced Basal and Induced Levels of Nrf2 and Nrf2 Target Genes

(A) p21-deficient mice had reduced basal and induced levels of Nrf2 and Nrf2 target genes. Wild-type or p21-deficient mice (n = 3) were treated with 350 mg/kg BHA for 12 hr through intraperitoneal injection. Liver tissues were subjected to immunoblot analysis with anti-Nrf2, anti-NQO1, anti-HO-1, and anti-GAPDH antibodies.

(B) A model by which p21 upregulates the Nrf2-dependent antioxidant response at both basal and induced conditions. Keap1, an E3 ubiquitin ligase containing the BTB and Kelch domains, constantly targets Nrf2 for ubiquitination and subsequent degradation under basal conditions. The binding of Keap1 to Nrf2 is through a hinge and latch mechanism, in which each Kelch domain from a Keap1 homodimer binds Nrf2 through two binding sites: a weak-binding DLG motif and a strong-binding ETGE motif. Binding of both sites is essential to present the seven ubiquitin-accepting lysine residues of Nrf2 in the correct orientation to accept ubiquitin, which targets Nrf2 for degradation. In response to oxidative stress, Keap1 is able to detect an imbalance in intracellular redox homeostasis through modification of its cysteine residues. The three important cysteine residues, C151, C273, and C288, are labeled. These modifications alter conformation of Keap1 and loosen the latch (DLG motif) allowing p21 to compete with Keap1 for binding to the DLG motif. Once the latch is open, the Keap1-dependent ubiquitination of Nrf2 is compromised even though Keap1 still associates with Nrf2 through the hinge (ETGE). Thus, Nrf2 is stabilized and Nrf2-dependent cytoprotective genes are expressed under oxidative stress.

presence of Keap1 and provides solid support of the hinge and latch model. In general, our data indicate that p21 is able to upregulate the Nrf2-dependent antioxidant response more substantially under induced conditions compared to basal conditions. This is most likely due to increased p21 protein levels under induced conditions, leading to additive effects.

p21 is the founding member of the cyclin-dependent kinase inhibitor (CKI) family, which also includes p27 and p57. The N-terminal regions are well conserved among the three members, including the cyclin/CDK-binding motifs (Gartel and Tyner, 2002). In our study, the Nrf2-interacting motif is mapped to the C-terminal 154KRR. The C-terminal sequences of the CKIs are poorly conserved among the family members, implying that upregulation of the Nrf2-dependent antioxidant response is likely specific to p21. A large body of literature shows that this motif may overlap with many other p21-interacting proteins identified so far, including the PCNA-interacting region (143–160), a second cyclin-binding region (155–157), C8 α-subunit (140–164), calmodulin (145–164), c-Myc (139–164), GADD45 (139–164), MDM2 (87–164), SET (145–164), TOK1 (149–164), HPV-16E7 (139–164), and C/EBP-α (84–164) (Child and Mann, 2006; Dotto, 2000; Gartel and Tyner, 2002). The physiological roles of this pleiotropic protein mainly rely on its cellular localization and its interacting proteins, which determine the ultimate fate of cells, such as proliferation, differentiation, cell-cycle arrest, and apoptosis. Although we did not address the interplay of these putative p21-interacting proteins in this study, the physiological significance of p21 in upregulating the Nrf2-dependent antioxidant response was confirmed in animals. Both the basal and induced Nrf2-antioxidant response was reduced substantially in p21-deficient mice as compared to wild-type. Thus, our finding that p21 upregulates the Nrf2-dependent antioxidant response pathway adds another dimension to the complexity of the p21-mediated signaling network.

EXPERIMENTAL PROCEDURES

Antibodies, Transfection, Animal Treatment, Immunoblot Analysis, and Reporter Gene Assay

Anti-Nrf2, anti-Keap1, and anti-p21 (Santa Cruz); anti-HA beads (Sigma); and chitin beads and the anti-CBD (New England Biolabs) were purchased from commercial sources. Transfection of cDNA was performed with Lipofectamine Plus (Invitrogen). Nrf2-siRNA and p21-siRNA were purchased from ORIGEN. HiPerfect transfection reagent (ORIGEN) was used to deliver siRNA. Male wild-type and p21-deficient mice were purchased from the Jackson Laboratory. Mice at 6 weeks of age were randomly assigned to BHA (350 mg/kg in corn oil, i.p.) or control group (corn oil, i.p.) and euthanized at 12 hr following administration. Livers were excised and subjected to immunoblot analysis. The detailed procedures are described in the Supplemental Data. Reporter assays were performed by using the Promega Dual-Luciferase Reporter Gene Assay System according to the manufacturer’s instructions.

In Vivo Ubiquitination of Nrf2, GST Pull-Down, and Immunofluorescence Assays

To detect endogenous Nrf2 that is ubiquitin conjugated, cells were exposed to 10 μM MG132 (Sigma) for 4 hr prior to lysis. Cell lysates were subjected to immunoprecipitation with an anti-Nrf2 antibody, and precipitated proteins were immunoblotted with an anti-Ub antibody (Sun et al., 2007). GST fusion proteins were expressed in E. coli and purified using GST beads (Amersham Biosciences). The 35S-labeled Nrf2 was generated by an in vitro transcription and translation kit (Promega). Colocalization of Nrf2 with p21 was detected using double-label indirect immunofluorescence with anti-p21 and anti-Nrf2 antibodies for detection of endogenous p21 and Nrf2, with anti-Myc and anti-HA antibodies detecting ectopically expressed p21-Myc and HA-Nrf2 (Sun et al., 2007).

Cell Viability and Cell-Death Assays

Cell viability was performed by using MTT assay (Wang et al., 2007). For detection of H2O2-induced apoptotic cells, two methods were used: (1) transfected cells were treated with H2O2 and cells with the condensed chromatin were detected according to the reported method (Gong et al., 1999); and (2) apoptotic cells were detected using Annexin V-FITC apoptosis detection kit (Sigma) in combination with flow cytometry.

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ROS Detection, NQO1 Activity, and qRT-PCR
To detect ROS, cells were treated with H2O2 for 12 hr and incubated with dichlorofluorescein (DCF) (Sigma) before measuring ROS by flow cytometry. NQO1 activity was measured as the dicoumarol-inhibitable fraction of DCPIP reduction described previously (Wang et al., 2007). Total mRNA extraction and qRT-PCR analysis were performed as described in the Supplemental Data and were reported previously (Wang et al., 2008).

Protein Half-Life Analysis
The half-life of Nrf2 was measured by using both CHX/immunoblot and pulse-chase 35S-labeling methods as described in the Supplemental Data.

Statistical Analysis
Experiments were conducted in triplicate, and data are shown as mean ± SD. Statistical analysis was performed using two-tailed Student’s t tests to compare means. Significance was set at p < 0.05.

SUPPLEMENTAL DATA
Supplemental Data include seven figures and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/

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