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Cytoplasmic localization and ubiquitination of p21^{Cip1} by reactive oxygen species

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Abstract

Reactive oxygen species were previously shown to trigger p21^{Cip1} protein degradation through a proteasome-dependent pathway, however the detailed mechanism of degradation remains to be elucidated. In this report, we showed that p21^{Cip1} was degraded at an early phase after low dose H_2O_2 treatment of a variety of cell types and that preincubation of cells with the antioxidant, *N*-acetylcysteine, prolonged p21^{Cip1} half-life. A mutant p21^{Cip1} in which all six lysines were changed to arginines was protected against H_2O_2 treatment. Direct interaction between p21^{Cip1} and Skp2 was elevated in the H_2O_2-treated cells. Disruption of the two nuclear export signal (NES) sequences in p21^{Cip1}, or treatment with leptomycin B blocked H_2O_2-induced p21^{Cip1} degradation. Altogether, these results demonstrate that reactive oxygen species induce p21^{Cip1} degradation through an NES-, Skp2-, and ubiquitin-dependent pathway.

Keywords: Nuclear export; Ubiquitination; Degradation; Reactive oxygen species; Cyclin-dependent kinase inhibitor

The cyclin-dependent kinase (CDK) inhibitor, p21^{Cip1}, is involved in cell proliferation, differentiation, senescence, and apoptosis. p21^{Cip1} was initially identified as a component of a quaternary complex containing CDK, cyclin, and proliferating cell nuclear antigen (PCNA). In addition to CDK regulation, p21^{Cip1} prevents DNA replication by inhibiting PCNA. The regulation of p21^{Cip1} is quite complex [1]. While transcriptional regulation by p53-dependent and p53-independent mechanisms is well established, recent studies suggest that p21^{Cip1} can be regulated by post-translational mechanisms. There appear to be multiple pathways regulating p21^{Cip1} degradation. Proteasomal degradation processes regulate p21^{Cip1} protein levels [2], and Skp2-containing SCF (Skp1, Cullin, and F-box protein) complexes contribute to the ubiquitination of p21^{Cip1} [3,4]. Other reports suggest that p21^{Cip1} turnover does not require ubiquitination on the internal lysine [5], and instead requires N-terminal ubiquitination [6] or direct p21^{Cip1}–proteasome interactions [7].

Reactive oxygen species (ROS) are important chemical mediators involved in a number of cellular processes. ROS are produced by UV and ionizing radiation exposure, normal oxidative phosphorylation, and pathological conditions like ischemia and inflammation. ROS are also generated during cell signaling processes following cytokine, growth factor, and receptor agonist stimulation [8]. Depending on the level of ROS exposure and cellular context, cells exhibit a wide range of adaptive cellular responses that range from growth stimulation to transient growth arrest, permanent growth arrest, apoptosis, and necrosis [9]. Cellular levels of ROS are tightly regulated throughout the cell cycle [10,11]. ROS can inactivate many protein tyrosine phosphatases, activate some kinases and transcription factors [8], and modulate a ubiquitin ligase activity that controls cyclin levels [11], thus altering the cell cycle. Interestingly, ROS also trigger proteasome-dependent degradation of p21^{Cip1} in GM00637 human fibroblast cells and cystic fibrosis lung epithelial cells [12,13].

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In this report, we show that ROS induce nucleocytoplasmic translocation of $p21^{Cip1}$ via two nuclear export signal (NES) sequences that are necessary for its degradation. We find that ROS increases $p21^{Cip1}$-Skp2 binding, which in turn elevates $p21^{Cip1}$ ubiquitination and subsequent proteasome-mediated degradation. These findings suggest that altering the intracellular redox state may affect the cell cycle.

Materials and methods

Reagents. H_2O_2, *ert*-butyl hydroperoxide, DNase-free RNase, and protein A-agarose were purchased from Sigma. MG-132, lactacystin, and the synthetic substrate *N*-benzyloxycarbonyl-valyl-alanyl-aspartyl-fluoromethylketone (Z-VAD) were purchased from EMD Biosciences. Nickel-affinity agarose from QIAGEN, leptomycin B from LC Laboratories, and the DNA dye 4′6-diamidino-2-phenylindole (DAPI) from Roche were used. The antibodies against $p21^{Cip1}$ (C19 and F5), ubiquitin, β-actin, and Skp2 were all obtained from Santa Cruz Biotechnology. Antibodies against hemagglutinin (HA; H6908, Sigma; 12CA5, Roche) and Myc (Invitrogen) were used.

Cell culture. Human lung IMR-90 fibroblasts from ATCC and human dermal fibroblasts (HDF) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 10% fetal bovine serum (Invitrogen), 20 mM Hepes, and antibiotics (Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO_2. MCF-7 (human breast cancer), HeLa (human cervical cancer), HaCaT (transformed human keratinocyte), Chang liver, Cos-7 (monkey kidney fibroblast), BT-20 (human breast cancer), Saos-2 (human osteosarcoma), HEK293T (human embryonic kidney), NIH 3T3 (mouse fibroblast), CHO (Chinese hamster ovary), and *ts*20TG^R cells (gift from Harvey Ozer, UMDNJ) were cultured in DMEM supplemented with 5% fetal bovine serum, 5% bovine calf serum (HyClone), 20 mM Hepes, and antibiotics at 37 °C. HL-60 (human promyelocytic leukemia) cells were cultured in RPMI 1640 (Invitrogen) supplemented with 5% fetal bovine serum, 5% bovine calf serum, and antibiotics. HeLa and HEK293T cells were transfected with various plasmids using calcium phosphate or Lipofectamine (Invitrogen).

Construction of plasmids. N-terminally HA epitope-tagged human $p21^{Cip1}$ cDNA was generated by PCR and subcloned into the BamHI and XhoI sites of pcDNA3 (Invitrogen). The mutant $p21^{Cip1}$ construct with multiple substitutions of six leucines in two NESs to alanine (amino acids 71, 73, 76, 78, 113, 115) named L6A and the deletion $p21^{Cip1}$ construct of nuclear localization signal (NLS) (amino acids 140–155) named ΔNLS were made using the QuikChange site-directed mutagenesis kit (Stratagene). Human ubiquitin cDNA was inserted into the pCMV (Clontech) vector with N-terminal 6× His-tag. The $p21^{Cip1}$-K6R mutant was a generous gift from Rati Fotedar (Institut de Biologie Structurale J-P Ebel). A plasmid encoding Myc epitope-tagged Skp2 was kindly provided by Yeon-Soo Seo (KAIST).

Immunoprecipitation and immunoblotting. Cells were washed with phosphate-buffered saline (PBS), lysed in a lysis buffer (20 mM Hepes [pH 7.2], 0.15 M NaCl, 0.5% NP-40, 10% glycerol, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg of leupeptin), and centrifuged to remove insoluble debris. Cell lysates were incubated with antibodies bound to protein A-agarose for 4 h at 4 °C. The immobilized proteins were collected by centrifugation, washed with lysis buffer three times, and subjected to SDS–PAGE. Proteins were transferred onto a nitrocellulose membrane (Schleicher and Schuell) that was blocked with 5% skim milk, washed briefly, and incubated with specific antibodies. Blots were washed three times with TTBS buffer (20 mM Tris [pH 7.4], 150 mM NaCl, and 0.05% Tween 20) and incubated with horseradish peroxidase-conjugated anti-mouse (Pierce), anti-rabbit (Pierce), or anti-goat IgG (Sigma) antibody, and then developed with a chemiluminescence detection system (Pierce).

Immunohistochemistry. Cells were plated onto glass cover slips at a density of 7×10^3 cells/ml in six-well dishes. At 36 h after transfection, cells were washed three times with PBS, fixed in 4% paraformaldehyde dissolved in PBS for 30 min, permeabilized with 0.2% Triton X-100, and incubated with a specific antibody for 1 h followed by tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibody (Sigma) plus DAPI. Cover slips were mounted in 10% glycerol and examined under a fluorescence microscope (Axioskop; Zeiss).

Results

Downregulation of endogenous $p21^{Cip1}$ protein by oxidative stimulation

We found that low (200–500 μM) but not high (>1 mM) doses of H_2O_2 induced a rapid reduction (within 30 min following stimulation) in endogenous $p21^{Cip1}$ protein levels in HeLa cells (Fig. 1A and B). Within 4 h, $p21^{Cip1}$ levels returned to basal level (Fig. 1B). The low doses of H_2O_2 did not induce cell death, but slightly increased cell number (data not shown), a finding consistent with previous reports [14–16]. We examined the effects of H_2O_2 on $p21^{Cip1}$ protein levels in a variety of cell types: IMR-90, HDF, MCF-7, HaCaT, Chang liver, Cos-7, BT-20, HL-60, Saos-2, and NIH 3T3 (Fig. 1C). The decrease in $p21^{Cip1}$ protein levels was observed after H_2O_2 treatment of all cell types and only showed minor variation from cell type to cell type. The decrease was independent of p53, since it was evident in cells with both functional p53 (IMR-90, HDF, and MCF-7) and defective p53 (HeLa, HaCaT, and Cos-7). Oxidative stimulation with *tert*-butyl hydroperoxide produced a similar decrease in $p21^{Cip1}$ levels in HeLa cells (data not shown). To test whether oxidative stimulation regulates the half-life of $p21^{Cip1}$ protein, we examined $p21^{Cip1}$ levels following inhibition of protein synthesis with cycloheximide (Fig. 1D and E). The half-life of $p21^{Cip1}$ protein was shorter after H_2O_2 exposure than in mock-treated cells. In contrast, pretreatment with the antioxidant, N-acetylcysteine, prolonged the $p21^{Cip1}$ half-life, confirming the redox dependence of $p21^{Cip1}$ stability.

Ubiquitin-dependent degradation of $p21^{Cip1}$ by oxidative stimulation

$p21^{Cip1}$ protein levels are regulated by the proteasome pathway [2]. Treating HeLa cells with the proteasome inhibitors, MG-132 or lactacystin, but not the caspase inhibitor, Z-VAD, prevented the decrease in $p21^{Cip1}$ protein levels after H_2O_2 exposure (Fig. 2A), indicating that H_2O_2 triggers proteasome-dependent degradation of $p21^{Cip1}$. Targeted proteolysis by the proteasome that occurs following ubiquitination is responsible for regulating diverse biological systems. To determine whether oxidative stimulation induces $p21^{Cip1}$ ubiquitination, we analyzed high-molecular-weight ubiquitin conjugates of $p21^{Cip1}$ after treating cells that coexpress HA-tagged $p21^{Cip1}$ and His-tagged ubiquitin with H_2O_2. Cells were pretreated with MG-132 to prevent degradation of ubiquitin-conjugated $p21^{Cip1}$. Immunoprecipitation with anti-HA

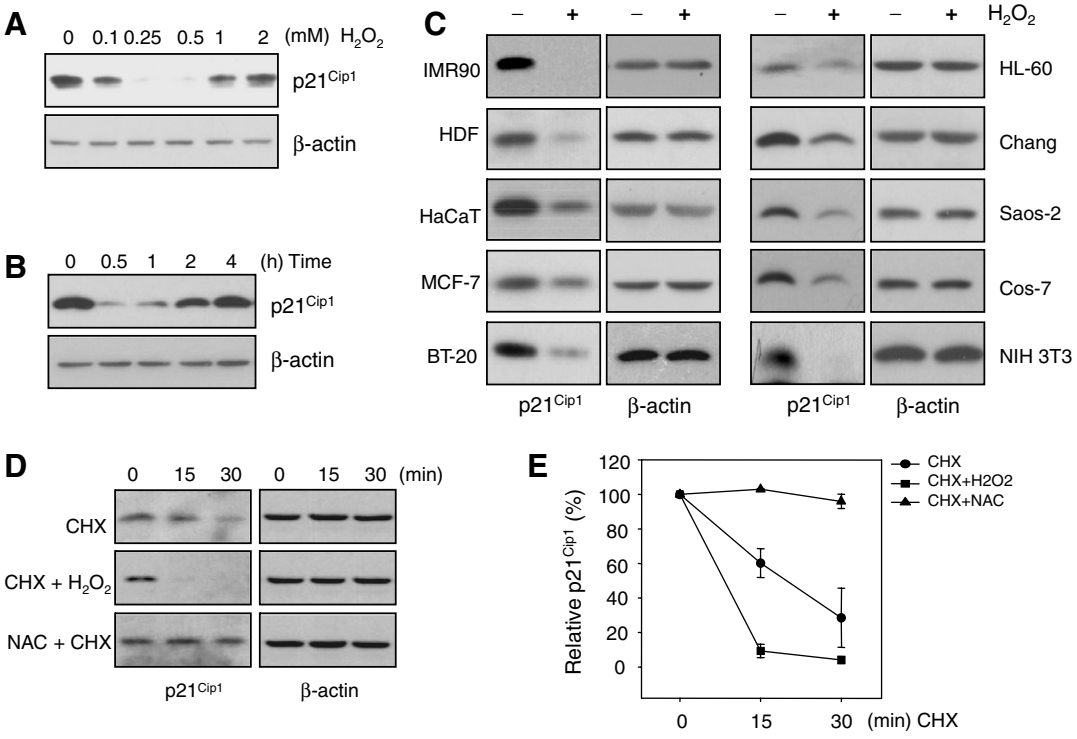

Fig. 1. Downregulation of endogenous $p21^{Cip1}$ by oxidative stimulation. (A) $p21^{Cip1}$ levels after treating HeLa cells with various concentrations of H_2O_2. $p21^{Cip1}$ levels were determined 30 min after H_2O_2 treatment. Cell lysates were immunoblotted with anti-$p21^{Cip1}$ antibody and normalized with anti-β-actin antibody. (B) Time course of $p21^{Cip1}$ levels after treatment of HeLa cells with 0.2 mM H_2O_2. (C) $p21^{Cip1}$ downregulation in a variety of cell types. $p21^{Cip1}$ levels in cells were examined before and after 30 min treatment with 0.2 mM H_2O_2. (D) Effect of N-acetylcysteine (NAC) on $p21^{Cip1}$ protein half-life. HeLa cells were pretreated with 5 mM NAC for 2 h prior to cycloheximide (CHX) treatment. Cell lysates prepared at different times after CHX treatment were analyzed by immunoblotting. Cells treated with CHX alone (upper panels) or co-treated with 0.2 mM H_2O_2 (middle panel) served as controls. (E) Quantification of $p21^{Cip1}$ band intensity obtained from (D) by densitometry. All signals are normalized to the signal at time 0 (100%).

antibody and ubiquitin immunoblots demonstrated the high-molecular-weight bands of $p21^{Cip1}$ that represent ubiquitinated $p21^{Cip1}$ increased in the H_2O_2-treated cells (Fig. 2B, left panel). Trapping the His-tagged ubiquitin by Ni^{2+}-affinity agarose and $p21^{Cip1}$ immunoblots also demonstrated intense bands representing ubiquitinated $p21^{Cip1}$ when cells were treated with H_2O_2 (Fig. 2B, right panel). These results indicate that proteasome-mediated degradation of $p21^{Cip1}$ is dependent on ubiquitination.

To confirm ubiquitination at the lysine residues of $p21^{Cip1}$ in response to H_2O_2 exposure, we used a $p21^{Cip1}$ expression vector in which all six lysines were mutated to arginine ($p21^{Cip1}$-K6R). While ectopically expressed wild-type $p21^{Cip1}$ ($p21^{Cip1}$-WT) was degraded by H_2O_2 exposure, the $p21^{Cip1}$-K6R mutant was not degraded (Fig. 2C), demonstrating direct ubiquitination at internal lysine residues in response to oxidative stimulation.

Ubiquitination is mediated by a conserved pathway consisting of a ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2), and an ubiquitin protein ligase (E3). *ts*20TG^R cells [17] grown at 34 °C express wild-type E1, while cells grown at the restrictive temperature (39 °C) express inactive E1, resulting in disabled ubiquitination. H_2O_2-induced degradation of endogenous $p21^{Cip1}$ was observed in *ts*20TG^R cells maintained at 34 °C but not at 39 °C (Fig. 2D). We thus conclude that a ubiquitin-dependent pathway regulates H_2O_2-induced $p21^{Cip1}$ degradation.

The SCF complex formed by Skp1, Cullin, F-box protein, and Rbx/Roc1 is an E3 responsible for the ubiquitination of several cell cycle proteins, including $p27^{Kip1}$, $p21^{Cip1}$, p130, and cyclin E [2]. We tested whether the F-box protein, Skp2, directly associates with $p21^{Cip1}$ after H_2O_2 exposure. Endogenous $p21^{Cip1}$ was immunoprecipitated from the Skp2-overexpressing cells both before and after H_2O_2 exposure, and the immunoprecipitates were analyzed for the presence of associated Skp2. Skp2 was associated with $p21^{Cip1}$, and this association increased dramatically after H_2O_2 exposure (Fig. 2E), suggesting that SCF^{Skp2} participates in $p21^{Cip1}$ degradation in response to oxidative stimulation.

Nucleocytoplasmic translocation is required for ROS-induced $p21^{Cip1}$ degradation

To determine whether H_2O_2 triggers $p21^{Cip1}$ degradation by affecting its localization, we used immunostaining to analyze the subcompartmental localization of $p21^{Cip1}$ after H_2O_2 exposure. Ectopic $p21^{Cip1}$ expressed in HeLa and HEK293T cells was primarily present in the nucleus (Fig. 3, leftmost panels), but was in both the cytoplasm and nucleus at even lower concentrations after H_2O_2

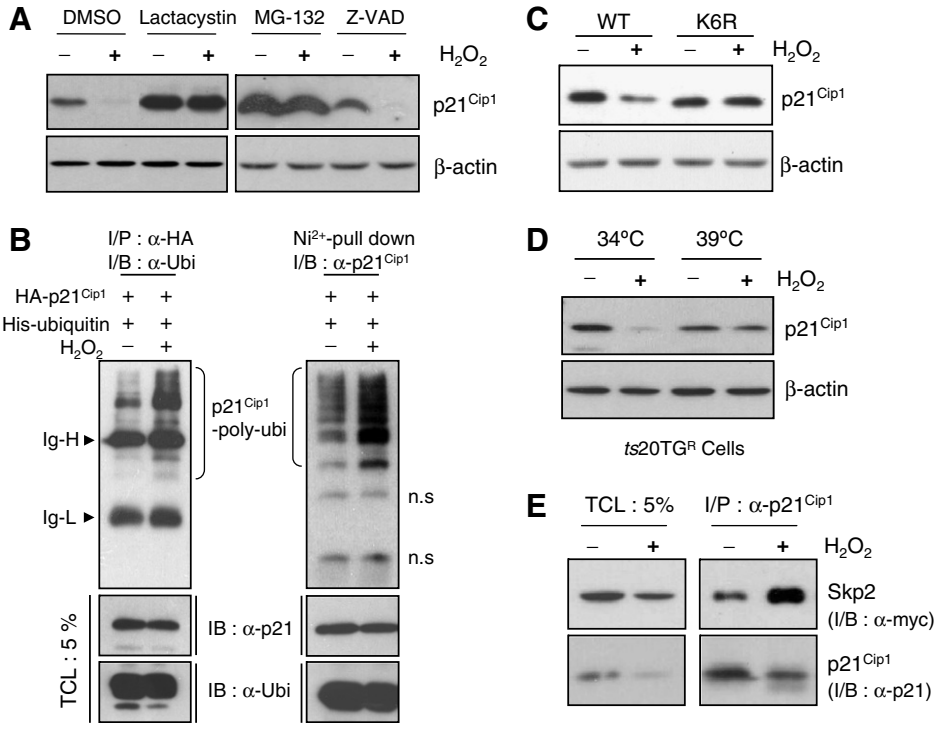

Fig. 2. Proteasome- and ubiquitin-dependent degradation of $p21^{Cip1}$ by mild oxidative stimulation. (A) HeLa cells were pretreated with the proteasome inhibitors, lactacystin and MG-132, and pan-caspase inhibitor, Z-VAD, or a vehicle (DMSO) for 2 h prior to H_2O_2 treatment. Cells were then treated with H_2O_2 (0.2 mM) or left untreated for 30 min prior to preparation of the cell lysates. Equal amounts of the proteins were immunoblotted with anti-$p21^{Cip1}$ and anti-β-actin antibodies. (B) H_2O_2 induced polyubiquitination of $p21^{Cip1}$. HEK293T cells were cotransfected with 2 μg of a plasmid encoding HA-tagged $p21^{Cip1}$-WT and 500 ng of a plasmid encoding His-ubiquitin. After 36 h, MG-132 was added and the cells were either treated with H_2O_2 (0.2 mM) or left untreated for 30 min prior to preparation of the cell lysates. Left, HA-$p21^{Cip1}$ proteins were immunoprecipitated with anti-HA antibody and ubiquitination was analyzed by immunoblotting with an anti-ubiquitin antibody. Right, His-ubiquitin was purified with nickel-affinity agarose and ubiquitinated $p21^{Cip1}$ was detected by immunoblotting with anti-$p21^{Cip1}$ antibody. n.s., nonspecific. (C) Ubiquitination-deficient $p21^{Cip1}$-K6R mutant was not degraded after H_2O_2 treatment. HEK293T cells were transfected with 2 μg of $p21^{Cip1}$-WT or $p21^{Cip1}$-K6R mutant. At 36 h posttransfection, the cells were either treated with 0.2 mM H_2O_2 or left untreated for 30 min. Cell lysates were analyzed by immunoblotting with an anti-$p21^{Cip1}$ antibody. (D) H_2O_2-induced degradation of $p21^{Cip1}$ was prevented by inactivation of the E1 enzyme. *ts*20TG^R cells expressing inactive E1 at the restrictive temperature (39 °C) were treated with 0.2 mM H_2O_2 or left untreated for 30 min at 34 °C and 39 °C. Cell lysates were analyzed by immunoblotting with anti-$p21^{Cip1}$ antibody. (E) Increased association between Skp2 and $p21^{Cip1}$ after H_2O_2 treatment. HeLa cells transfected with Myc-tagged Skp2 were either treated with 0.2 mM H_2O_2 or left untreated for 30 min. Cell lysates were immunoprecipitated with anti-$p21^{Cip1}$ antibody and immunoblotted with anti-Myc and anti-$p21^{Cip1}$ antibodies.

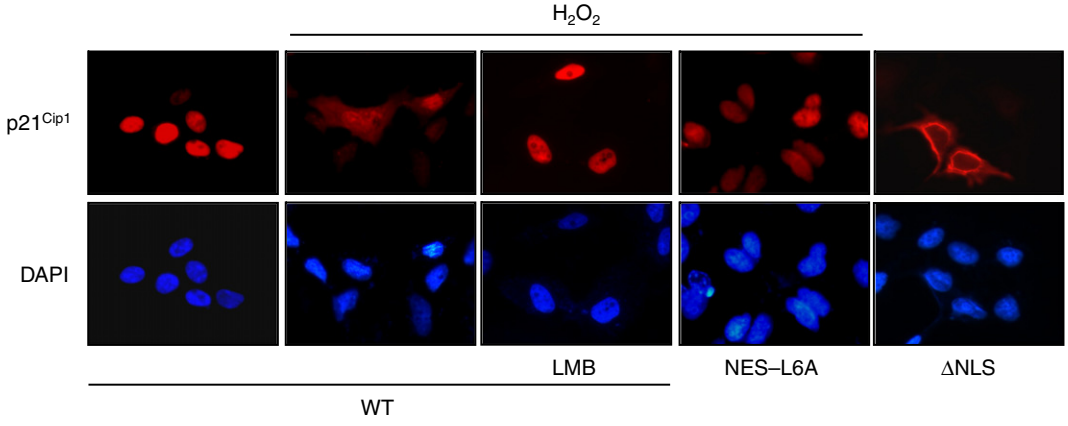

Fig. 3. Effect of H_2O_2 treatment on $p21^{Cip1}$ localization. HeLa cells were transfected with HA-tagged $p21^{Cip1}$-WT, $p21^{Cip1}$-L6A, or $p21^{Cip1}$-ΔNLS. Cells were pretreated with leptomycin B (LMB; 20 nM for 1 h) or left untreated prior to H_2O_2 exposure for 15 min. Cellular localization of $p21^{Cip1}$ was detected using an anti-HA antibody. After extensive washing, samples were further incubated with TRITC-conjugated rabbit anti-mouse IgG plus DAPI and examined by fluorescence microscopy.

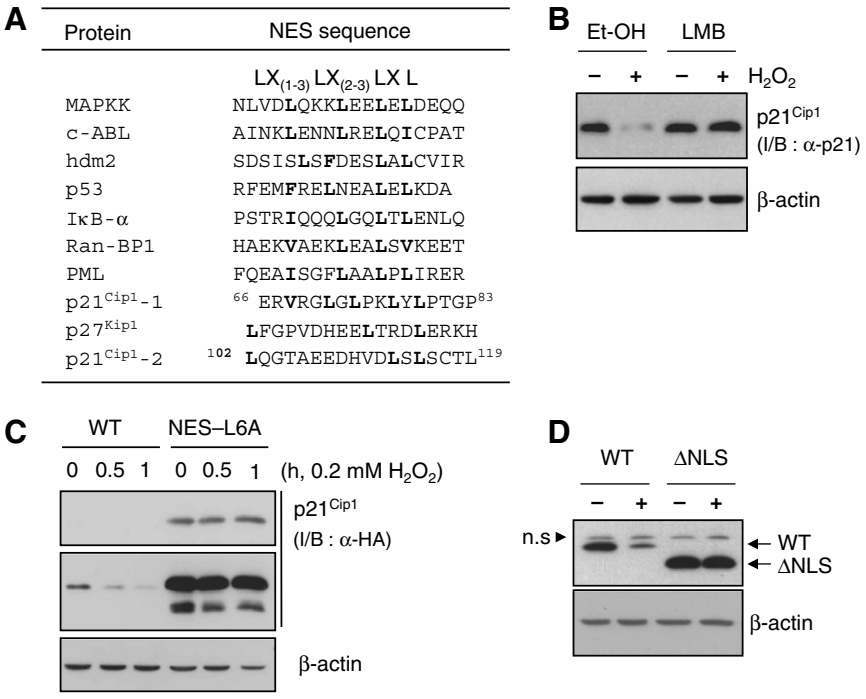

Fig. 4. Effect of $p21^{Cip1}$ localization on degradation. (A) Putative NESs of $p21^{Cip1}$ ($p21^{Cip1}$-1 and $p21^{Cip1}$-2) aligned with typical NESs from mitogen-activated protein kinase kinase 2 (MAPKK), Abelson tyrosine kinase (c-ABL), the human homolog of murine mdm2 (hdm2), inhibitor of nuclear factor-κB alpha (IκB-α), Ran GTP-binding protein 1 (Ran-BP1), promyelocytic leukemia protein (PML), and atypical NES ($p27^{Kip1}$). Conserved leucines (sometimes valine) are boldfaced. (B) Inhibition of nucleocytoplasmic translocation blocks endogenous $p21^{Cip1}$ degradation. HeLa cells pretreated with LMB for 1 h were exposed to 0.2 mM H_2O_2 for 30 min. Cell lysates were analyzed by immunoblotting with an anti-$p21^{Cip1}$ antibody. (C,D) HEK293T cells were transfected with HA-tagged $p21^{Cip1}$-WT, $p21^{Cip1}$-L6A (C), or $p21^{Cip1}$-ΔNLS (D) and either treated with 0.2 mM H_2O_2 or left untreated for 30 min or the indicated times. Cell lysates were analyzed by immunoblotting with an anti-HA antibody. Upper and middle panels show short and long film exposure of the same chemiluminescent blots, respectively (C).

treatment (Fig. 3, second panels). Endogenous $p21^{Cip1}$ was distributed in a similar way (data not shown), suggesting that ROS induce nucleocytoplasmic translocation of $p21^{Cip1}$.

To determine whether the H_2O_2-induced cytoplasmic localization of $p21^{Cip1}$ is controlled by the NES, we used a chemical inhibitor of NES and a mutant $p21^{Cip1}$ construct with a malfunctioned NES. We examined $p21^{Cip1}$ distribution after a shorter H_2O_2 exposure (15 min) to reduce the chance of excess $p21^{Cip1}$ degradation. Leptomycin B, a specific inhibitor of NES/CRM1-dependent nuclear export, suppressed the H_2O_2-induced cytoplasmic localization of $p21^{Cip1}$ (Fig. 3, middle panels). Previously, a putative NES (^{68}VRGLGLPKLYL) was predicted within the $p21^{Cip1}$ and identified as the weakest NES using an artificial reporter assay [18]. We predicted an additional NES (^{102}LQGTAEEDHVDLSLSCTL) on $p21^{Cip1}$ using the NetNES program available at http://www.cbs.dtu.dk/[19] (Fig. 4A). Simultaneous mutation of six leucines within two putative NESs to alanine (L71A/L73A/L76A/L78A/L113A/L115A, called $p21^{Cip1}$-L6A) abolished H_2O_2-induced $p21^{Cip1}$ translocation (Fig. 3). These results together indicate that NES is required for H_2O_2-induced $p21^{Cip1}$ translocation.

To determine whether nuclear export is required for $p21^{Cip1}$ degradation induced by H_2O_2, we also examined $p21^{Cip1}$ protein levels in cells that were pretreated with leptomycin B and in cells transfected with $p21^{Cip1}$-L6A following H_2O_2 treatment. Disruption of NES function by leptomycin B or the L6A mutation abolished H_2O_2-induced $p21^{Cip1}$ degradation (Fig. 4B and C), indicating that NES-dependent translocation is required for $p21^{Cip1}$ degradation in response to oxidative stimulation.

To determine whether cytoplasmic localization is sufficient for $p21^{Cip1}$ degradation, we used a cytoplasm-retention mutant of $p21^{Cip1}$. Deletion of an NLS sequence (amino acids 140–155, [20]) from $p21^{Cip1}$, that allowed $p21^{Cip1}$ cytoplasmic localization (Fig. 3), abolished H_2O_2-induced degradation (Fig. 4D). This result indicated that nuclear localization needs to precede cytoplasmic localization in order for $p21^{Cip1}$ degradation to occur. We speculate that some modification of $p21^{Cip1}$ inside the nucleus is a prerequisite for degradation. Taken together, our findings suggest that mild oxidative stimulation triggers NES-dependent cytoplasmic translocation and subsequent ubiquitin-dependent degradation of $p21^{Cip1}$, thus regulating the cell cycle-inhibitory role of $p21^{Cip1}$.

Discussion

Several studies indicate that $p21^{Cip1}$ is sensitive to redox status, but the underlying mechanism remains

controversial. $p21^{Cip1}$ levels increased in response to sublethal doses (<500μM) of H_2O_2, inducing G2/M-phase and multi-phase cell cycle arrest in human lung carcinoma H1299 cells [21] and mouse fibroblasts [22], respectively. In this report we found that that mild oxidative stimulation lead to rapid nuclear export and a transient decrease in $p21^{Cip1}$ levels in a variety of cell types. While $p21^{Cip1}$ responses to ROS were shown to be complex in previous studies [12,13,21,22], we concluded that $p21^{Cip1}$ levels are sensitive to redox status and suggest that a redox balance is required for the maintenance of appropriate $p21^{Cip1}$ levels during the cell cycle. Other CDK inhibitors, like $p27^{Kip1}$ and $p16^{INK4}$, are also thought to be redox regulated. We and others have demonstrated that Jab1 controls $p27^{Kip1}$ and is regulated by the redox enzymes, thioredoxin [23] and MIF [24]. Overexpression of the antioxidant enzyme, manganese superoxide dismutase, in confluent normal fibroblasts suppressed $p16^{INK4}$ protein levels and was thought to play a role in maintaining proliferative capacity [25].

It was reported that endogenous levels of ROS increase as cells progress from the early G1 to S phase, and decrease as cells transit from mitosis into early G1 [11]. Another report showed that Skp2 levels oscillate in the cell cycle, reaching a maximum in the S phase [4]. In this report, we identified that ROS induce $p21^{Cip1}$ degradation through a Skp2-mediated pathway, speculating that the elevated ROS and Skp2 levels play cooperative roles in $p21^{Cip1}$ degradation during the S phase.

We found that NES-dependent cytoplasmic localization is required for ROS-induced Skp2- and ubiquitin-dependent degradation of $p21^{Cip1}$. We verified that two putative sequences within $p21^{Cip1}$ comprise a functional NES. While simultaneous disruption of the two NESs blocked H_2O_2-induced $p21^{Cip1}$ translocation and subsequent degradation (Figs. 3 and 4C), disruption of only one did not completely abolish $p21^{Cip1}$ translocation and subsequent degradation upon oxidative stimulation (data not shown). Thus, both NESs are required for complete translocation and degradation of $p21^{Cip1}$.

This study poses interesting questions regarding the mechanisms by which NESs become activated and Skp2-$p21^{Cip1}$ binding is increased in response to oxidative stimulation. Our report describes the novel finding that two NESs within $p21^{Cip1}$ play a critical role in ROS-induced $p21^{Cip1}$ translocation and subsequent Skp2- and ubiquitin-dependent degradation, and suggests how altering the intracellular redox state may potentially function in cell cycle regulation.

Acknowledgments

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