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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