Cooperative Roles of c-Abl and Cdk5 in Regulation of p53 in Response to Oxidative Stress

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The p53 tumor suppressor protein, a critical modulator of cellular stress responses, is activated through diverse mechanisms that result in its stabilization and transcriptional activation. p53 activity is controlled by transcriptional, translational, and post-translational regulation. The major mechanisms of p53 regulation occur primarily through interactions with HDM2, an E3 ubiquitin ligase that leads to p53 nuclear export and degradation. Here, we demonstrate that hydrogen peroxide-induced oxidative stress elicits down-regulation of HDM2. c-Abl mediates down-regulation of HDM2, leading to an increase of p53 level. Moreover, Cdk5 (cyclin-dependent kinase 5), a proline-directed Ser/Thr kinase, additionally increases p53 stability via post-translational modification of p53 in response to hydrogen peroxide. The p53 protein stabilized by c-Abl and Cdk5 is transcriptionally active; however, transcription of its target gene is differentially regulated with selective binding of Cdk5 on promoter regions of its target genes by c-Abl. In addition, c-Abl modulates Cdk5 activity via phosphorylation of tyrosine 15 in cooperation with cleavage of p35 to p25. Our results show that c-Abl and Cdk5 cooperatively regulate maximal activation of p53, resulting in neuronal death in response to oxidative stress by hydrogen peroxide. These findings aid in clarifying the mechanism underlying the occurrence of neuronal apoptosis as a result of c-Abl and Cdk5-mediated p53 stabilization and transcriptional activation.

The p53 tumor suppressor is an important transcription factor that regulates cellular functions in response to numerous stress signals, and it is also implicated in the regulation of apoptotic cell death (1, 2). p53 protein is kept at basal levels under unstressed conditions with a short half-life induced by HDM2-mediated continuous degradation. HDM2, human Mdm2, works as a major negative regulator of p53 due to its E3 ubiquitin ligase activity. HDM2-mediated p53 ubiquitination results in proteasome-mediated rapid degradation of p53, preventing its accumulation under normal conditions (3, 4). HDM2 additionally inhibits transcriptional activation of p53 by direct binding to the N-terminal transactivation domain and preventing its interaction with basal transcription machinery (5). Upon a variety of stresses, p53 stability is increased and accumulates in the nucleus, where it is activated as a transcription factor, resulting in cell cycle arrest and/or apoptosis in many cell types, including neurons. The accumulation and activation of p53 largely relies on post-translational events, such as phosphorylation and acetylation (6, 7). Phosphorylation of p53 at multiple sites increases p53 stability through uncoupling p53 from HDM2-mediated proteasomal degradation. Phosphorylation-driven acetylation of p53 on C-terminal regions activates its binding on specific promoter regions, resulting in transcription-dependent apoptosis. E3 ubiquitin ligase activity of HDM2 is also modulated by various cellular proteins via posttranslational modification, including phosphorylation and ubiquitination (8). In addition, the inhibitory activities of HDM2 are neutralized by the action of binding partners, such as ARF, MdmX, and deubiquitinase (HAUSP) (8, 9). Although accumulating evidence suggests the signaling pathway, resulting stabilization, and activation of p53, the mechanisms related to p53 activation still require further analysis.

c-Abl, a member of the nonreceptor tyrosine kinase family, is a ubiquitously expressed protein that mediates cellular signals in response to stimuli. Because of its cellular homologue of the transforming element of Abelson murine leukemia virus, c-Abl has been studied in the context of oncogenesis, and its participation in neuronal functions has been recently discussed (10, 11). c-Abl has been implicated in cell growth arrest and apoptosis, through multiple protein-protein interactions and its tyrosine kinase activity (12, 13). Upon exposure to stress, such as DNA damage, ATM phosphorylates and activates c-Abl, leading to cell growth arrest and apoptosis. The apoptotic activity of c-Abl is mediated by p73, a member of the p53 family, and through a p53-dependent pathway. c-Abl phosphorylates p73 at the tyrosine residue and drives it to the nucleus, where p73 can activate p53-responsive genes and induce apoptosis (14, 15).

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–4.
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3 The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; siRNA, small interfering RNA; TUNEL, TdT-mediated dUTP nick end labeling; RT, reverse transcription; ChIP, chromatin immunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
c-Abl can also regulate the p53 protein levels, possibly by phosphorylating HDM2 and neutralizing the inhibitory effect of HDM2 on p53 (8, 12).

Cdk5 (cyclin-dependent kinase 5), a proline-directed serine/threonine kinase, is regulated through association with its specific activator, including p35, p39, and p67. Ubiquitously expressed Cdk5 is mostly active in the central nervous system due to the selective localization of its activator proteins (16, 17). In addition to its regulatory roles in neuronal processes, such as neuronal differentiation, migration, axon outgrowth, and synaptogenesis (18–20), Cdk5 effects several neurodegenerative disorders, including Alzheimer disease, Parkinson disease, and amyotrophic lateral sclerosis (21, 22). Abnormal activation of Cdk5 has detrimental effects on neurons, leading to apoptosis under either physiological or pathological conditions, suggesting a pivotal role of Cdk5 in the molecular events during the pathological progression (23). Exposure of neurons to various insults, like ischemia and oxidative stress, leads to hyperactivation of Cdk5 with consequent neuronal apoptosis. Although accumulating evidence suggests the essential role of Cdk5 in the regulation of neuronal apoptosis, the mechanisms by which active Cdk5 facilitates apoptosis remain to be established.

Here, we provide conclusive molecular evidence to demonstrate the important role of cooperative action of c-Abl and Cdk5 on oxidative stress-induced cell death. c-Abl and Cdk5 cooperatively regulate the stability and activity of p53 and subsequently induce apoptotic cell death. HDM2 is down-regulated upon oxidative stress in a process requiring c-Abl, resulting in increased p53 stabilization. In addition, Cdk5 also efficiently stabilizes p53 via its phosphorylation of N-terminal regions. p53 stabilized by the action of c-Abl and Cdk5 is transcriptionally active; however, transcription of its target gene is differentially regulated through selective enhancement of p53 DNA binding. Moreover, we show that c-Abl cooperated with p25 formation to help activate Cdk5 via phosphorylation at tyrosine 15. Thus, the present findings define an important molecular mechanism of c-Abl- and Cdk5-mediated p53 stabilization and activation underlying apoptotic cell death induced by oxidative stress.

EXPERIMENTAL PROCEDURES

Materials—DMEM and penicillin/streptomycin were purchased from Invitrogen. Bovine calf serum and fetal calf serum were obtained from HyClone Laboratories (Logan, UT). Hydrogen peroxide was purchased from Sigma. A Cdk2/5 inhibitor, histone H1 peptide, and HDM2 (2A10) antibody were purchased from Calbiochem. c-Abl kinase inhibitor, STI571, was kindly provided by Novartis (Basel, Switzerland). Rabbit polyclonal antibodies raised against Cdk5 (C-8), p35 (C-19), and monoclonal p53 (DO-I) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal c-Abl antibody was obtained from Cell Signaling (Beverly, MA). Small interfering RNA (siRNA) against Cdk5, p53, and c-Abl and SMARTPool™ containing four pooled SMARTselected siRNA duplexes were purchased from Upstate Biotechnology (Dharmacon). Western blot detection reagent (SUPEX) was obtained from NEURONEX (Pohang, Korea). [α-32P]dATP was purchased from PerkinElmer Life Sciences.

Cell Cultures and Transfection of cDNA and siRNA

Experiments—Human neuroblastoma SH-SY5Y cells were grown in DMEM supplemented with 10% heat-inactivated bovine calf serum with 1% penicillin/streptomycin in a humidified atmosphere containing 5% CO2 at 37 °C. The DsRed-p53(R175H) expression construct was generated by cloning into the EcoRI/BamHI restriction sites of the pDsRed1-C1 plasmid. A constitutively active form of c-Abl-PP and a dominant negative mutant of c-Abl(K290R) were kindly provided by Dr. Daniela Barilà (Dulbecco Telethon Institute, Rome, Italy). Transient transfection with siRNA targeting Cdk5, p53, and c-Abl (SMARTpool™ containing four pooled SMARTselected siRNA duplexes) or scrambled control siRNA and plasmids was carried out by using the Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer’s instructions. The medium was changed with fresh medium 24 h after transfection, and then cells were collected for immunoblotting or treated with the indicated drugs for further experiments.

Western Blotting and Cdk5 Kinase Assay—Cells were treated with the indicated drugs, washed with chilled PBS, and then lysed for 45 min on ice, using Triton lysis buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 2 mM EDTA, 1 mM Na3VO4, 1% Triton X-100, 100 mM glyceral, 0.5 mM dithiothreitol, and protease inhibitors). The lysates were clarified by centrifugation at 15,000 × g for 15 min, and the supernatants were stored at –80 °C until use. The proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes, and the following procedures were performed as previously described (18). For Western blot analysis, bands were visualized using the Western blot detection kit (Pohang, Korea). For the Cdk5 kinase assay, immunoprecipitated endogenous Cdk5 was mixed with 8 μg of histone H1 peptide as a substrate in kinase reaction buffer containing 25 mM HEPES, pH 7.4, 25 mM β-glycerophosphate, 25 mM MgCl2, 100 μM Na3VO4, 500 μM dithiothreitol, and 1 mM [γ-32P]ATP. The reaction was allowed to proceed at 30 °C for 30 min as described previously (24), and radioactivity was measured by autoradiography.

Immunocytochemical Analysis—SH-SY5Y cells were fixed with 4% paraformaldehyde and then incubated with blocking solution (2.5% bovine serum albumin and 2.5% equine serum in phosphate-buffered saline) for 1 h at room temperature. The samples were incubated overnight at 4 °C with the indicated antibodies, followed by incubation with fluorescein isothiocyanate-conjugated anti-rabbit IgGs. Slides were mounted and visualized by fluorescence microscopy (Axioplan2; Zeiss, Oberkochen, Germany).

Cell Viability Assay and TdT-mediated dUTP Nick End Labeling (TUNEL) Assay—To evaluate cell survival, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed with a modification (25). In brief, cultures were incubated with a final concentration of 1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium salt at 37 °C for 2 h. The reaction was stopped by the addition of lysis buffer containing 20% SDS and 50% N,N-dimethylformamide, pH 4.5; samples were incubated overnight at 37 °C; and absorbance was measured photometrically at 570 nm. Apoptotic cells were further visualized using the modified TUNEL
assay offered by the DeadEnd colorimetric detection system (Promega, Madison, WI), according to the manufacturer’s instructions.

RNA Extraction and Quantitative Real Time Reverse Transcription (RT)-PCR—Total RNA was extracted using Trizol reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer’s protocol. For quantitative real time RT-PCR, total RNA was reverse transcribed by using the iScript™ CDNA synthesis kit (Bio-Rad) according to the manufacturer’s instructions. For detection and quantification, the MyiQ™ real time PCR detection system (Bio-Rad) was used. GAPDH mRNA served as a control for relative quantification. PCRs were performed using the iQ™ SYBR Green Supermix kit (Bio-Rad). PCR was carried out in a final volume of 20 μl using a 0.5 μM concentration of each primer, cDNA, and 10 μl of the supplied enzyme mixture containing the DNA double strand-specific SYBR Green I dye to detect PCR products. PCR was performed with a 3-min preincubation at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. PCR products were verified by melting curve analysis, agarose gel electrophoresis, and DNA sequencing. The following primers were used: GAPDH, forward (5′-GCCATCAATGACCCCTTCATT-3′) and reverse (5′-GCTCCTGGAGATGTTCCG-TGG-3′); p53, forward (5′-GAGCCTTGGAGATGTTCCGAGGC-3′) and reverse (5′-GTCTGAGTCAGGCCCTTCTGATTTG-3′); BAX, forward (5′-AGAGGATGATTGCCGCC-AGAGC-3′) and reverse (5′-CAACACCTTGTGGAGCCA-AGCAC-3′); HDM2, forward (5′-GCCATTGAACCTTG-GCCATTGAACCTTG-3′) and reverse (5′-GGAAATAAGTTAGCACAATCATTTG-3′). The relative value of gene expression was analyzed using the 2^{-ΔΔCt} method (18).

Chromatin Immunoprecipitation (ChIP) Assay—We performed a ChIP assay using a previously published method (26). Briefly, SH-SY5Y cells were treated with drugs and cross-linked with 1% formaldehyde for 20 min, nuclei were collected, and the chromatin were sonicated to an average length of 700 bp. Five percent of the total lysate was used as an “input DNA” control. The remaining sheared chromatin was precleared with protein G-Sepharose that was blocked previously with 1 mg/ml salmon sperm DNA and was used for immunoprecipitation with 1 μg of an affinity-purified monoclonal antibody against p53. Immunoprecipitated DNA was subjected to semiquantitative PCR with specific primers (HDM2, forward (5′-GGGAGTTCAGGTGAAGGTCA-3′) and reverse (5′-CTTCTTACGATTGTGAGTTCG-3′); BAX, forward (5′-AGTGACTTGGACAGCCTCCT-3′) and reverse (5′-AGTCCCTGCTGGAAGCATGCTATT-3′)) to amplify a fragment of the human HDM2 or BAX promoter region. Each PCR band was purified and verified by DNA sequence analysis.

Statistical Analysis—All experiments, including the immunoblots and kinase assay, were independently repeated at least three times. All assays and blots presented are representative of more than three separate experiments. All quantitative data are presented as means ± S.D. Comparisons between two groups were analyzed via Student’s t test, and values of p < 0.05 were considered to be significant.

RESULTS

p53 Stabilization Occurs Post-translationally via HDM2 Down-regulation in Response to H₂O₂ Treatment—To examine the contribution of p53 to oxidative stress-induced neuronal death, we examined the effects of hydrogen peroxide (H₂O₂), a well known oxidative stress inducer, on cell viability and found that H₂O₂-induced oxidative stress caused both concentration- and time-dependent cell death in human neuroblastoma, SH-SY5Y cells (supplemental Fig. 1, A and B). In addition, TUNEL assay and Hoechst staining showed morphological characteristics of apoptosis, such as DNA fragmentation and nuclei condensation caused by hydrogen peroxide (supplemental Fig. 1C). Given the well known function of p53 in apoptotic cell death (1), we investigated whether p53 was essential for H₂O₂-induced neuronal death by suppressing its activity with dominant-negative p53(R175H). Dominant-negative p53(R175H) markedly decreased TUNEL staining, whereas ~57% of the H₂O₂-treated control cells were TUNEL-positive (supplemental Fig. 1D). Similar results were obtained in Western blotting analysis showing that suppression of p53 expression with siRNA abrogated caspase-3 cleavage, which is involved in mechanisms of apoptotic signaling (supplemental Fig. 1E). These results collectively suggest that p53 is an important cellular factor involved in oxidative stress-triggered neuronal death. To test the p53 response to oxidative stress, p53 protein levels were monitored by Western blot and immunocytochemical analysis. A significant induction of endogenous p53 was detected in SH-SY5Y cells when H₂O₂ was treated (Fig. 1A). Immunocytochemical analysis also showed that p53 is mainly accumulated specifically in the nuclear region (supplemental Fig. 2A). Given that p53 regulation is largely dependent on the activity of HDM2, we also determined HDM2 expression. Compared with p53, hydrogen peroxide led to a rapid drop in the HDM2 protein level, which then increased again at later time points. To investigate whether protein expression of p53 and HDM2 was primarily induced by an increase of transcript levels, the mRNA transcript levels were determined by quantitative real time RT-PCR analysis. In contrast to the changes observed in protein expression, the p53 mRNA level remained unchanged, suggesting that the p53 increase is likely to occur post-translationally. HDM2 mRNA levels were not down-regulated at early times, suggesting that the decreased level is not the result of down-modulation of HDM2 mRNA. However, HDM2 transcript levels increased at later times, indicating that protein induction is possibly subsequent to the mRNA increase, in contrast to p53 transcript levels (Fig. 1B). HDM2 is a p53 target gene, so increased HDM2 mRNA at later times might be the result of p53 stabilization. To further examine whether p53 and HDM2 regulation occur at the post-translational level, we determined their degradation rates in the presence or absence of hydrogen peroxide when de novo protein synthesis was blocked with cycloheximide. The rate of p53 degradation significantly decreased in the presence of H₂O₂ compared with the vehicle, strongly suggesting that oxidative stress increases the p53 level by stabilization without transcriptional modulation. However, HDM2 levels significantly decreased in the presence of H₂O₂ compared with the
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FIGURE 2. HDM2 destabilization is regulated by c-Abl. A and B, c-Abl-mediated HDM2 destabilization. A, SH-SYSY cells were pretreated for 1 h with 10 μM STI571 or an equal volume of vehicle and then incubated with 60 μg/ml cycloheximide for the indicated times in the presence of 200 μM H2O2 or an equal volume of vehicle. Cycloheximide was simultaneously treated with H2O2. The amounts of HDM2 and GAPDH were determined by immunoblotting. A representative blot of data from three independent experiments is presented on the left. Protein levels are quantified on the right. B, SH-SYSY cells were transiently transfected with a mock vector or vector encoding c-Abl(K290R) and then treated with 200 μM H2O2 for 1 h or not. Cell lysates were subjected to immunoblotting with the indicated antibodies. C, regulation of p53 expression by c-Abl. SH-SYSY cells were pretreated for 1 h with 10 μM STI571 or an equal volume of vehicle and then treated with 200 μM H2O2 for the indicated times or not. The amounts of p53 and GAPDH were determined by immunoblotting.

FIGURE 1. Expression of p53 increases post-translationally, but HDM2 is down-regulated in response to oxidative stress. A, change of p53 and HDM2 protein level upon H2O2 treatment. SH-SYSY cells were treated with 200 μM H2O2 for the indicated time periods and then subjected to immunoblotting with the indicated antibodies. B, SH-SYSY cells were treated in an experimental paradigm similar to that of A for the indicated times, total RNA was isolated, and quantitative real-time RT-PCR was performed. The results for HDM2 (i) or BAX (ii) are from three independent experiments and are presented as the means ± S.D. values. Each experiment was performed in triplicate. C, SH-SYSY cells were incubated with 60 μg/ml cycloheximide for the indicated times in the presence of 200 μM H2O2 or an equal volume of vehicle. Cycloheximide is simultaneously treated with H2O2. The amounts of p53, HDM2, and GAPDH were determined by immunoblotting.

vehicle, indicating that oxidative stress decreases the stability of the HDM2 protein (Fig. 1C). These results also support the notion that p53 stabilization upon oxidative stress is mediated by HDM2 down-regulation at least in the early response phase. We also investigated whether stabilization of p53 is related to the proteasome-dependent degradation pathway. To explore the involvement of proteasomal pathway, p53 levels were measured following oxidative stress in the presence of the proteasomal inhibitor MG132. As shown in supplemental Fig. 2B, the basal level of p53 was increased by pretreatment with MG132; however, its levels were not further up-regulated by hydrogen peroxide, indicating that the proteasome-dependent degradation machinery is responsible for the H2O2-mediated p53 stabilization.

c-Abl Mediates Selective Transactivation of p53—The consequence of p53 stabilization is transcriptional activation (28). Given that p53 enhances transcription of its target genes in response to cellular stress (29), we investigated whether c-Abl
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could affect p53 transactivation in addition to its stabilization. We analyzed mRNA levels of p53 target genes, HDM2 and BAX, by quantitative real-time RT-PCR. Oxidative stress triggered an increase in HDM2 and BAX transcript levels, whereas suppression of c-Abl expression with siRNA significantly impaired the induction of HDM2. However, mRNA levels of BAX were not affected by suppression of c-Abl, suggesting selective transcriptional activation of p53 by c-Abl (Fig. 3A). A recent study demonstrated that c-Abl selectively stimulate binding of p53 on the promoters of its target genes (30). To determine whether c-Abl regulates specific DNA binding of p53 in vivo in response to oxidative stress, we performed a ChIP assay using promoters of HDM2 and BAX. An IgG was used as a negative control. As illustrated in Fig. 3B, cells treated with hydrogen peroxide yielded a strongly amplified product, indicating increased p53 binding to the HDM2 and BAX promoter regions. However, inhibition of c-Abl activity significantly reduced binding of p53 on the HDM2 promoter region but had no effect on p53 binding to the BAX promoter. These data imply that the p53 protein stabilized by oxidative stress is transcriptionally active; however transcription of its target genes is differentially regulated by c-Abl through selective enhancement of DNA binding of p53.

Cdk5 Mediates Stabilization of p53 via Phosphorylation—We recently reported that Cdk5 increased p53 stabilization via N-terminal phosphorylation (31). In view of the finding that p53 was not totally eliminated by c-Abl inhibition (Fig. 2C and supplemental Fig. 3B), we investigated whether Cdk5 also mediates p53 stabilization upon H2O2 treatment. Down-regulation of Cdk5 activity with the Cdk2/5 inhibitor N\textsuperscript{\textregistered}-(6-aminopyrimidin-4-yl)-sulfanilamide or suppressed expression of Cdk5 with siRNA showed similar inhibitory effects on p53 stabilization induced by oxidative stress (Fig. 4A and supplemental Fig. 4A). However, basal expression of p53 was not affected by the Cdk2/5 inhibitor or siRNA against Cdk5 in the absence of H2O2. To further define p53 regulation by Cdk5, we determined the degradation rate of p53 in the presence or absence of the Cdk5 inhibitor when de novo protein synthesis was blocked with cycloheximide. Cellular extracts were prepared at different times after the cycloheximide addition, and the p53 level was determined by immunoblot analysis. The blot data show a rapid decline in the degradation rate of p53 in the presence of Cdk5 inhibitor in contrast to that of the untreated control, strongly suggesting Cdk5-mediated sustained p53 stabilization (Fig. 4B). Since phosphorylation is an important element of p53 regulation, and our previous reports showed that Cdk5 induced...
oxide caused significantly increased phosphorylation at N-terminal regions; however, these phosphorylations were dramatically reduced when Cdk5 was blocked with inhibitor or suppressed with siRNA (Fig. 4C and supplemental Fig. 4B). These data demonstrate that Cdk5 is also responsible for p53 phosphorylation and subsequent stabilization in response to oxidative stress. Next, we explored whether Cdk5 also affects transactivation of p53 by quantitative real time RT-PCR analysis. In contrast to c-Abl siRNA, the transcript levels of both HDM2 and BAX were affected as a result of reduced Cdk5 expression by siRNA, although the inhibition of HDM2 expression by Cdk5 suppression was not as dramatic as the inhibition of c-Abl (Fig. 4D). This result suggests that transcriptional activity of p53 can be modulated by Cdk5 activity; however, transcription of its target gene is different from the c-Abl-mediated response. Marginal suppression of HDM2 mRNA by Cdk5 siRNA is probably due to the c-Abl-mediated selective transactivation of p53 as shown in Fig. 3, A and B.

We next explored whether simultaneous inhibition of c-Abl and Cdk5 has a more significant effect on p53 expression compared with inhibition of c-Abl or Cdk5 alone. Immunoblotting and immunocytochemical analysis showed that STI571 and Cdk5 inhibitor resulted in a more significant decrease in H2O2-induced p53 up-regulation than STI571 or Cdk5 inhibitor alone (Figs. 2C, 4A, and 5A), implying cooperative roles of c-Abl and Cdk5 in regulation of p53. Because p53 has been well implicated in neuronal death, we investigated whether the inhibition of both c-Abl and Cdk5 has more protective effects on neuronal death triggered by hydrogen peroxide. Similar to the effects on p53, the protective effect of STI571 was further enhanced by Cdk5 inhibition (Fig. 5B). Corresponding results were also obtained in Western blotting analysis, which showed that co-suppression of c-Abl and Cdk5 activity more dramatically abrogated caspase-3 cleavage (Fig. 5C). In addition, significantly reduced TUNEL staining, from ~56% in control cells to 18% in cells

p53 phosphorylation, we next investigated whether Cdk5 also affects p53 through phosphorylation upon H2O2 treatment by using phosphorylation site-specific antibodies. Hydrogen peroxide caused significantly increased phosphorylation at N-termin...
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with co-inhibition of c-Abl and Cdk5, further verified that c-Abl and Cdk5 have cooperative roles in p53 regulation and the subsequent apoptotic death in response to oxidative stress (Fig. 5D).

c-Abl Modulates Cdk5 Activity via Tyrosine Phosphorylation—Calcium-dependent cysteine protease, calpain, is known to induce cleavage of p35 to p25 in response to oxidative stress, leading to increased Cdk5 activity (23). As a previous study, we found that hydrogen peroxide triggered the cleavage of p35 to p25 and subsequently increased Cdk5 activity in a time-dependent manner (Fig. 6A). In order to examine whether p35 transcripts also increase and mediate Cdk5 activation, quantitative real-time RT-PCR was performed. However, p35 mRNA levels remained unchanged, indicating that p25 formation without p35 increase is a major factor for Cdk5 activation in response to oxidative stress (Fig. 6B). However, a recent study demonstrated that p35 cleavage into p25 is insufficient to initiate Cdk5 kinase activation (32). The report speculated that c-Abl can contribute to the activation of Cdk5 via phosphorylation at the tyrosine 15 residue. We thus determined whether c-Abl can modulate Cdk5 activity under oxidative stress. As shown in Fig. 6A, we found that phosphorylation of Cdk5 at tyrosine 15 increased in response to oxidative stress. To test whether this phosphorylation is mediated by c-Abl, cells were treated with STI571 to block c-Abl activity. The presence of STI571, tyrosine 15 phosphorylation of Cdk5 was dramatically reduced, and Cdk5 activity was also reduced a little but significantly (Fig. 6C), suggesting that c-Abl modulates Cdk5 activity in addition to p25 formation by cleavage of p35. To further verify that c-Abl cooperates with p25 formation to activate Cdk5, calpastatin peptide, a calpain inhibitor, was used to block p35 cleavage in addition to STI571. Blockage of p35 cleavage significantly reduced Cdk5 activity, indicating an essential role of p25 formation in Cdk5 activity. Simultaneous inhibition of p35 cleavage and c-Abl further reduced Cdk5 activity, clearly suggesting the cooperative roles of c-Abl and p25 formation in Cdk5 activation upon oxidative stress (Fig. 6D). Moreover, p53 expression was further blocked upon co-treatment with calpastatin peptide and STI571, verifying the cooperative roles of c-Abl and Cdk5 in regulation of p53.

**DISCUSSION**

Since p53 is an important factor that regulates the fate of cells and is situated at a crossroad in the pathway implicated in the cellular response to a wide variety of stresses, appropriate and tight regulation of p53 stabilization and activation is critical in most cell types, including the neuron (1, 33). However, the mechanisms by which this is achieved have been challenging to elucidate. Therefore, the present study clearly provides evidence to reveal the cooperative roles of c-Abl and Cdk5 in p53 regulation, stabilization of a transcriptionally active p53 protein through post-translational, not transcriptional modulation. This study shows that c-Abl and Cdk5 appear to utilize different mechanisms to stabilize and activate p53. Two different mechanisms that may account for p53 stabilization are 1) down-regulation of HDM2 stabilization via c-Abl activation and 2) post-translational modification of p53, specifically N-terminal phosphorylation by activated Cdk5.

**FIGURE 6.** c-Abl regulates Cdk5 activity in cooperation with p25 in response to oxidative stress. A, increase of p25 formation and tyrosine 15 phosphorylation (pTyr15) of Cdk5 upon oxidative stress. SH-SY5Y cells were treated with 200 μM H₂O₂ for the indicated times, and cell lysates were subjected to immunoblotting. Cdk5 kinase activity was determined by autoradiography using histone H1 as a substrate. B, no increase of p35 mRNA levels upon oxidative stress. SH-SY5Y cells were treated with 200 μM H₂O₂ for the indicated time periods, total RNA was isolated, and quantitative real-time RT-PCR was performed. The results are from three independent experiments and are presented as the mean ± S.D. values. Each experiment was performed in triplicate. C, regulation of Cdk5 activity by c-Abl. SH-SY5Y cells were pretreated for 1 h with 10 μM STI571 or an equal volume of vehicle and then treated with 200 μM H₂O₂ for 3 h. Cell lysates were subjected to immunoblotting with the indicated antibodies. Cdk5 kinase activity was determined by autoradiography using histone H1 as a substrate. D, cooperative role of c-Abl and p25 in the regulation of Cdk5 activity. SH-SY5Y cells were pretreated with 25 μM calpastatin (CS) peptide alone or with 10 μM STI571, as indicated, and then treated with 200 μM H₂O₂ for 3 h. Cell lysates were subjected to immunoblotting with the indicated antibodies. Cdk5 kinase activity was determined by autoradiography using histone H1 as a substrate. E, proposed model for the collaborative role of c-Abl and Cdk5 in the regulation of p53 via down-regulation of HDM2 or phosphorylation of p53 in response to hydrogen peroxide. p53 stabilized by c-Abl is transcriptionally active; however, transcription of its target gene is differentially regulated by c-Abl activity through selective enhancement of p23 binding. The consequence of p53 stabilization and activation is the activation of the conserved death program and induction of neuronal death.
The present study showed that oxidative stress induced by hydrogen peroxide triggered down-regulation of HDM2 expression without transcriptional modulation, suggesting that at least p53 stabilization upon oxidative stress is mediated by HDM2 down-regulation. HDM2 maintains the amount of p53 at basal levels in nonstressed cells through its E3 ubiquitin ligase activity-dependent p53 degradation and direct binding-dependent transcriptional inactivation of p53 (3, 4). Thus, disruption of HDM2 function is a mechanism leading to stabilization of p53 and, subsequently, its transcriptional activation. A growing body of work shows that HDM2 function can be regulated by phosphorylation or binding to other factors, such as p19/p14ARF, MdmX, and Rb (34–37). Alternatively, HDM2 activity can be regulated by PML (promyelocytic leukemia) through its nucleolar localization (38) or by down-regulation of its expression. It has been reported that diverse stresses can down-regulate HDM2 levels through both transcriptional and post-translational mechanisms (4, 39, 40), resulting in the increase and activation of p53. Down-regulation of HDM2 in transcriptional levels has been observed in response to UV and other DNA-damaging agents. Phosphatidylinositol 3-kinase family members, including ATM, ATR, and DNA-PK, are reported to induce HDM2 destabilization through HDM2 phosphorylation at multiple sites. The present study showed that HDM2 destabilization requires the activity of c-Abl. Recent studies demonstrated that c-Abl can phosphorylate HDM2 at tyrosine 276 and 394 residues and modulate its activity (8, 12). Based on this effect, c-Abl-mediated HDM2 destabilization may be induced by phosphorylation at these residues or other unidentified residues. Further studies will be required to clarify how c-Abl affects HDM2 destabilization.

In addition to the function of c-Abl on HDM2 destabilization, we found that c-Abl can mediate selective transcriptional activation of p53. c-Abl is known to interact with p53 and then to stabilize its tetrameric conformation. As a consequence, it is thought to specifically stimulate DNA binding of p53 on the promoters with perfect binding sequences (30, 41). Since c-Abl regulates stabilization of p53, all of the target genes should be affected to some extent by c-Abl. However, our quantitative real time RT-PCR data revealed that transcription of HDM2 and BAX, p53 target genes, is differently regulated by c-Abl (Fig. 5A). A ChIP assay further verified that c-Abl selectively regulates the DNA binding ability of p53 between the HDM2 and BAX promoter regions in response to oxidative stress (Fig. 5B). Recently, the extranuclear role of p53 in the cytoplasm was described. p53 has been shown to directly activate the proapoptotic Bcl-2 protein BAX, allowing for mitochondrial membrane permeabilization and apoptosis instead of transcriptional roles (42). Therefore, it is plausible that only some extent of the accumulated p53 is transcriptionally active, and these active portions of p53 are differentially regulated by c-Abl in terms of DNA binding on the promoters. However, it is also possible that c-Abl regulates other molecules that subsequently regulate binding activity of p53. Further studies are warranted to clarify the c-Abl-mediated selective transcriptional activation of p53.

Another candidate mechanism for the p53 stabilization and activation is the mediation of Cdk5-dependent phosphorylation. Expression and proapoptotic activity of p53 are achieved by post-translational modifications, protein-protein interactions, and protein stabilization by escaping ubiquitin-dependent degradation (18, 21, 43). In particular, phosphorylation of p53 at multiple sites is an important post-translational modification regulated by several different protein kinases, depending on cell types and extracellular stimuli. These phosphorylation reactions modulate p53 stability through inhibition of interactions with HDM2, transactivation, and specific targeting of various p53-regulated genes. DNA damage induces phosphorylation of p53 at Ser15 and Ser20, leading to reduced interactions of p53 with its negative regulator, HDM2, and consequently p53 accumulation (44, 45). HIPK2 (homeodomain-interacting protein kinase-2) phosphorylates Ser46 in response to UV radiation and drives an apoptotic response (46). In addition, we recently showed that Cdk5 regulates the stability and nuclear accumulation of transcriptionally active p53 protein through post-translational, not transcriptional, modulation of p53, especially phosphorylation at N-terminal regions (31). Consistent with previous reports, we demonstrated that Cdk5 caused p53 phosphorylation and, subsequently, stabilization in response to oxidative stress (Fig. 4, A–C, and supplemental Fig. 4, A and B). However, compared with the above mentioned reports, phosphorylation at Ser46 was marginally regulated by Cdk5 (data not shown). The phosphorylation pattern of p53 is determined by the particular set of available kinases, which depend on the cell type and extracellular stimulus. Therefore, it is possible that other signaling molecules are also involved in p53 phosphorylation and the following stabilization and activation. Further studies would be necessary to clarify the cooperative roles in terms of p53 regulation in response to hydrogen peroxide.

Cdk5 is prevalent in postmitotic neurons and has received considerable attention because of its crucial roles in diverse forms of apoptotic cell death (21). Evidence suggests that Cdk5 becomes an inducer of neuronal death and is involved in neurons treated with excitotoxins, β-amyloid, and oxidative stress as well as in animal models of stroke, Parkinson disease, and amyotrophic lateral sclerosis (23, 47, 48). In general, p25, a proteolytic fragment of p35, is suggested as a key molecule in Cdk5 activation in neurodegenerative conditions (49). Upon neurotoxic insults, calcium–dependent cysteine protease, calpain is activated and induces p35 cleavage into p25, resulting in mislocalization and prolonged deregulation of Cdk5 (50). In addition, our recent study suggested another possible mechanism in which Cdk5 activity can be elevated due to increased p35 expression without p25 formation in response to mitomycin C-induced DNA damage (51). However, the present study demonstrated that the aberrant activation of Cdk5 is induced by p25 formation without increases of p35 expression in response to oxidative stress (Fig. 6, A and B), suggesting that different mechanisms may be utilized to activate Cdk5 depending on the extracellular stimulus. Lin et al. (32) recently showed that c-Abl can also activate Cdk5 by phosphorylating Cdk5 at tyrosine 15 during β-amyloid (Aβ42)-initiated neurodegeneration. Cables has been reported to link c-Abl and Cdk5, leading to Cdk5 tyrosine phosphorylation, kinase up-regulation, and neurite outgrowth during brain development (52). Consistent with previous reports, the present study shows that c-Abl...
also induces Cdk5 activation by phosphorylation at tyrosine 15. Cooperative action of c-Abl and calpain-induced p25 formation deregulates Cdk5 activity and results in p53 activation and then neuronal death in response to oxidative stress. However, further studies are still needed to clarify the function of Cables as a mediator on the phosphorylation and activation of Cdk5 by c-Abl in response to oxidative stress.

We also showed that Cdk5 regulated transcriptional activation of p53; however, it did not show any transcriptional selectivity as a mediator on the phosphorylation and activation of Cdk5. Further studies are still needed to clarify the function of Cables in the cooperative action of c-Abl and calpain-induced p25 formation via phosphorylation at tyrosine 15. Activated Cdk5 also induces p53 stabilization with calpain-mediated p25 formation via phosphorylation-dependent translocation, cytochrome c release, and caspase activation. In parallel to the effects of p53, the present study also shows that consequent apoptotic neuronal death is regulated through collaboration of c-Abl with Cdk5 in response to H2O2-induced oxidative stress.

In conclusion, we provide strong evidence for the pathway that regulates oxidative stress-induced neuronal death depicted in Fig. 6E. HDM2 normally maintains p53 at low levels by ubiquitination-dependent degradation. In response to oxidative stress, c-Abl induced p53 stabilization by down-regulating HDM2 expression without transcriptional regulation of p53. p53 stabilized by c-Abl is transcriptionally active; however, transcription of its target gene is differentially regulated by c-Abl activity through selective enhancement of p53 binding to the promoter regions of its target genes. In addition, c-Abl promotes Cdk5 activity in cooperation with calpain-mediated p25 formation via phosphorylation at tyrosine 15. Activated Cdk5 also induces p53 stabilization and activation in a phosphorylation-dependent manner. These findings facilitate our understanding of the molecular mechanisms underlying neuronal death induced by oxidative stress, the cooperative action of c-Abl and Cdk5 on p53 stabilization, and consequent apoptotic cell death.

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