Stabilization of p73 by Nuclear IκB Kinase-α Mediates Cisplatin-induced Apoptosis*

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In response to DNA damage, p53 and its homolog p73 have a function antagonistic to NF-κB in deciding cell fate. Here, we show for the first time that p73, but not p53, is stabilized by physical interaction with nuclear IκB kinase (IKK)-α to enhance cisplatin (CDDP)-induced apoptosis. CDDP caused a significant increase in the amounts of nuclear IKK-α and p73α in human osteosarcoma-derived U2OS cells. Ectopic expression of IKK-α prolonged the half-life of p73 by inhibiting its ubiquitination and thereby enhancing its transactivation and pro-apoptotic activities. Consistent with these results, small interfering RNA-mediated knockdown of endogenous IKK-α inhibited the CDDP-mediated accumulation of p73ε. The kinase-deficient mutant form of IKK-α interacted with p73α, but failed to stabilize it. Furthermore, CDDP-mediated accumulation of endogenous p73α was not detected in mouse embryonic fibroblasts (MEFs) prepared from IKK-α-deficient mice, and CDDP sensitivity was significantly decreased in IKK-α-deficient MEFs compared with wild-type MEFs. Thus, our results strongly suggest that the nuclear IKK-α-mediated accumulation of p73α is one of the novel molecular mechanisms to induce apoptotic cell death in response to CDDP, which may be particularly important in killing tumor cells with p53 mutation.

The NF-κB signaling pathway is activated by a variety of structurally and functionally unrelated stimuli, including inflammatory cytokines, ionizing radiation, viral and bacterial infection, and oxidative stress (reviewed in Refs. 1 and 2). Under normal conditions, NF-κB exists as heterodimeric complexes composed of p50 and p65 (RelA) subunits and is kept transcriptionally inactive through interaction with its inhibitory proteins such as IκB-α and IκB-β. IκB proteins mask the nuclear localization signal of NF-κB, thereby preventing its nuclear translocation. Upon certain stimulations, IκB proteins are rapidly phosphorylated at specific serine residues in the N-terminal their signal-responsive domain by upstream regulator IκB kinase (IKK)2 complex and subsequently polyubiquitinated and degraded in a proteasome-dependent manner (reviewed in Ref. 3). The high molecular mass IKK complex comprises two related catalytic subunits, IKK-α (also called IKK-1) and IKK-β (also called IKK-2), and one regulatory subunit with a scaffold function, IKK-γ (also called NEMO) (reviewed in Ref. 3). The proteolytic degradation of IκB proteins exposes the nuclear localization signal of NF-κB and results in translocation of NF-κB from the cytoplasm to the nucleus, allowing it to participate in transcriptional regulation of numerous target genes involved in immune responses, inflammatory reactions, cell adhesion, cell proliferation, apoptotic cell death, and other cellular processes. Therefore, the IKK complex represents one of the critical upstream regulators of the NF-κB signaling pathway.

In many experimental systems, the activation of NF-κB has been shown to play an important role in the control of survival processes, protecting cells from a variety of apoptotic signals (4–8). For example, tumor necrosis factor-α (TNF-α) simultaneously activates the NF-κB-mediated cellular protective mechanism against the pro-apoptotic effect of TNF-α through the induction of the NF-κB-responsive genes that function to block apoptosis. Additionally, inhibition of NF-κB has been shown to enhance sensitivity to chemotherapeutic agents (9, 10). Consistent with the well documented anti-apoptotic effect of NF-κB, high levels of NF-κB activity are detectable in various human tumors (11). On the other hand, NF-κB activation results in the promotion of apoptosis, depending on different stimuli and cell types. Huang and Fan (12) reported that the activation of NF-κB contributes to paclitaxel-induced apoptosis in human solid tumor cells. In addition, Bian et al. (13) found that NF-κB activation mediates doxorubicin-induced apoptosis in N-type neuroblastoma cells. In both cases, treatment of cells with the cytotoxic agents significantly down-regulated cytoplasmic IκB-α and then promoted the nuclear transloca-

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2 The abbreviations used are: IKK, IκB kinase; TNF-α, tumor necrosis factor-α; CDDP, cisplatin; siRNA, small interfering RNA; MEFs, mouse embryonic fibroblasts; MITT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT, reverse transcription; GST, glutathione S-transferase; HA, hemagglutinin; PIPES, 1,4-piperazinediethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; CBP, cAMP-responsive element-binding protein-binding protein.
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...tion of NF-κB; however, the molecular mechanism of the pro-apoptotic effect of NF-κB is still largely unknown.

p73 belongs to a small family of p53-related nuclear transcription factors. In accordance with their structural similarity, p73 functions in a manner analogous to p53 by inducing G1 cell cycle arrest or apoptosis in certain cancerous cells through transactivating an overlapping set of p53/p73 target genes (reviewed in Ref. 14). Like p53, endogenous p73 becomes stabilized as well as activated in cells exposed to certain genotoxic stimuli, including γ-irradiation and cisplatin (CDDP), and contributes to an apoptotic response to DNA damage (15–17). p73 is expressed as multiple isoforms that differ at their N and C termini, arising from alternative splicing and promoter usage (reviewed in Ref. 14). Among them, an N-terminally truncated form of p73 (ΔNp73) that lacks the transactivation domain of p73 has an oncogenic potential and exhibits dominant-negative behavior toward wild-type p73 as well as p53 (18–20). Of particular note, we (22) and others (21, 23) demonstrated that p73 functions in a manner analogous to p53 by inducing G1 cell cycle arrest or apoptosis in certain cancerous cells through transactivating an overlapping set of p53/p73 target genes, Birbach et al. (32) reported that one of its components (IKK-α) shuttles between the cytoplasm and nucleus of unstimulated cells, suggesting that IKK-α might have a novel nuclear role in controlling cell survival and death. Consistent with this notion, it has been shown that IKK-α accumulates in the cell nucleus in response to cytokine exposure and stimulates the expression of NF-κB-responsive genes through promoter-associated histone H3 phosphorylation (33, 34). In this study, we found that IKK-α accumulates in the cell nucleus during the CDDP-mediated apoptotic process. Moreover, IKK-α increased the stability of p73, but not p53, through direct interaction with p73 and enhanced p73-dependent transcriptional activity as well as pro-apoptotic function. Reduction of endogenous IKK-α by small interfering RNA (siRNA) against IKK-α resulted in the significant attenuation of the CDDP-induced accumulation of p73α. Similar results were also obtained in mouse embryonic fibroblasts (MEFs) derived from IKK-α-deficient mice (IKK-α−/− MEFs). Thus, our findings suggest that IKK-α has a novel nuclear role in regulating DNA damage-induced apoptosis, which is distinct from its cytoplasmic role in activating NF-κB.

EXPERIMENTAL PROCEDURES

Cell Lines and Transfection—African green monkey kidney COS-7 cells and human osteosarcoma U2OS cells were maintained in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, penicillin, and streptomycin (Invitrogen). Human lung carcinoma H1299, human neuroblastoma SK-N-AS, and mouse fibrosarcoma L929 cells were grown in RPMI 1640 medium, 10% fetal bovine serum, penicillin, and streptomycin. COS-7 cells were transfected with FuGENE 6 (Roche Applied Science) in accordance with the manufacturer’s specifications. H1299 and U2OS cells were transfected with Lipofectamine (Invitrogen) according to the manufacturer’s instructions. pcDNA3 (Invitrogen) was used as a blank plasmid to balance the amount of DNA introduced in transient transfection.

Cell Survival Assay—U2OS cells were seeded at 5 × 10⁴/well in a 96-well tissue culture dish with 100 µl of complete medium and allowed to attach overnight. CDDP was added to the cultures at a final concentration of 20 µM, and cell viability was determined by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at the indicated time points after the addition of CDDP as described (22).

RNA Extraction and Reverse Transcription (RT)-PCR—Total RNA was prepared from U2OS cells exposed to CDDP (20 µM) using an RNAeasy mini kit (Qiagen Inc.) according to the manufacturer’s protocol. For the RT-PCR, first-strand cDNA was generated using SuperScript II reverse transcriptase (Invitrogen) and random primers. PCR amplification was performed with Taq DNA polymerase (Takara, Ohtsu, Japan). The expression of glyceraldehyde-3-phosphate dehydrogenase was measured as an internal control.

Plasmids—The protein-coding region of IKK-α was amplified by PCR and inserted between the EcoRI and XhoI sites of pcDNA3-FLAG. The K44A mutation was introduced into wild-type IKK-α using PfuUltra™ high fidelity DNA polymerase (Stratagene) according to the manufacturer’s instructions. The nucleotide sequence of the PCR product was determined to verify the presence of the desired mutation and the absence of random mutations.

Immunoblotting, Immunoprecipitation, and Glutathione S-Transferase (GST) Pulldown Assay—For immunoblotting, cell lysates (50 µg of protein) were analyzed using anti-FLAG monoclonal antibody M2 (Sigma); anti-hemagglutinin (HA) monoclonal antibody (12CA5, Roche Applied Biosciences); anti-p73 monoclonal antibody (Ab-4, NeoMarkers, Fremont, CA); anti-p53 monoclonal antibody (DO-1, Oncogene Research Products, Cambridge, MA); anti-Bax monoclonal antibody (6A7, eBioscience, San Diego, CA); anti-IKK-α polyclonal (M-280), anti-IKK-β polyclonal (H-470), anti-IKK-γ...
polyclonal (FL-417), anti-p65 polyclonal (C-20), anti-ixB-α polyclonal (C-21), or polyclonal anti-p21WAF1 (H-164) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); or anti-actin polyclonal antibody (20–33, Sigma). After incubation with primary antibodies, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA), and immunoreactive proteins were finally visualized by the ECL system (Amer- sham Biosciences AB, Uppsala, Sweden). For immunopre- cipitation, cell lysates were precleared with 30 μl of protein G-Sepharose suspension (Amersham Biosciences AB) and then incubated with anti-HA polyclonal antibody (Medical and Biological Laboratories, Nagoya, Japan) or anti-FLAG monoclonal antibody for 2 h at 4 °C. Immunoblotting was performed with anti-FLAG or anti-p73 monoclonal antibody as described above. For GST pulldown assay, [35S]methio- nine-labeled FLAG-IKK-α was generated in the coupled transcription/translation system (Promega, Madison, WI) and mixed with GST or GST-p73 fusion proteins coupled to glutathione-Sepharose (Amersham Biosciences AB) for 2 h at 4 °C. 35S-Labeled bound proteins were analyzed by 10% SDS-PAGE and visualized by autoradiography.

Subcellular Fractionation and Immunofluorescence Analysis—To prepare nuclear and cytoplasmic extracts, cells were lysed in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture (Sigma) and centrifuged at 5000 rpm for 10 min to collect soluble fractions, which are referred to as cytosolic extracts. Insoluble materials were washed with the lysis buffer and further dissolved in SDS sample buffer to collect the nuclear extracts. The nuclear and cytoplasmic fractions were subjected to immunoblot analysis using anti-lamin B monoclonal antibody (Ab-1; Oncogene Research Products) or anti-α-tubulin monoclonal antibody (DM1A, Cell Signaling Tech- nology). For indirect immunofluorescence, U2OS cells were grown on coverslips and transfected with the indicated expression plasmids. Forty-eight hours after transfection, cells were fixed in 100% methanol for 20 min at −20 °C, blocked in 3% bovine serum albumin, stained with the corresponding antibodies, and examined with a laser scanning confocal micro- scope (Olympus, Tokyo, Japan). Nuclear matrix fractionation was performed as described previously (35, 36). In brief, cells were washed with ice-cold phosphate-buffered saline and lysed in 10 mM PIPES (pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 1 mM EGTA, 1 mM dithiothreitol, and 0.5% Triton X-100 containing a protease inhibitor mixture, and insoluble materials were separated from soluble proteins (fraction I) by centrifugation. The pellet fraction was treated with DNase I (at a final concentration of 1 ng/ml) for 15 min at 37 °C, and then ammonium sulfate was added to the reaction mixture (at a final concentration of 0.25 M). The pellet fraction was separated from the supernatant (fraction II) by centrifugation and further extracted with 2 M NaCl (fraction III). The remaining pellet was solubilized in 8 M urea, 0.1 M NaH2PO4, and 10 mM Tris-HCl (pH 8.0) to give fraction IV.

Protein Stability and Ubiquitination Assays—COS-7 cells were transfected with HA-p73α with or without IKK-α. Cells were harvested at different time points after pretreatment with cycloheximide (100 μg/ml), and cell lysates were processed for immunoblot analysis with anti-p73 or anti-actin antibody. Densitometry was used to quantify the amounts of HA-p73α that normalized to actin. Ubiquitination assay was performed as described previously (37). COS-7 cells were cotransfected with HA-p73α and His-tagged ubiquitin with or without IKK-α. Forty hours after transfection, cells were exposed to the proteasomal inhibitor MG132 (20 μM) for 6 h. Cells were resus- pended in 6 M guanidine HCl, 0.1 M NaH2PO4/Na2HPO4 (pH 8.0), and 10 mM imidazole, and ubiquitinated materials were recovered by nickel-nitrioltriacetic acid-agarose beads (Qiagen Inc.) and analyzed by immunoblotting with anti-HA antibody.

Luciferase Reporter and Apoptosis Assays—p53-deficient H1299 cells on 12-well plates were cotransfected with a p53/ p73-responsive element-driven luciferase reporter, an internal control vector for Renilla luciferase, and a combination of the indicated expression vectors. Both firefly and Renilla luciferase activities were assayed with the Dual-Luciferase reporter assay system (Promega). The firefly luminescence signal was normalized based on the Renilla luminescence signal. For apoptosis assay, H1299 cells on 6-well plates were cotransfected with β-galactosidase (50 ng) and HA-p73α (50 ng) with or without increasing amounts of IKK-α or IKK-β (100, 200, and 400 ng). Forty-eight hours after transfection, cells were stained with a 0.4% solution of trypan blue for 10 min at room temperature. Thereafter, cells were fixed in phosphate-buffered saline containing 2.5% glutaraldehyde, 1 mM MgCl2, and 2 mM EGTA for 10 min and then stained with Red-Gal for 2 h as described (11). Red-Gal was used as a marker to visualize the transfected cells and to assess the apoptotic frequency among the transfectants. Apoptotic cells were scored by rounding up of cells with dark pink-purple coloration due to double staining with Red-Gal and trypan blue.

In Vitro Kinase Assay—GST or GST-p73 deletion mutants were cotransfected with the active form of IKK-α (Upstate Bio- technology, Lake Placid, NY) in a solution containing 40 mM MOPS-NaOH (pH 7.0), 1 mM EDTA, 25 mM sodium acetate, and 0.25 mM ATP in the presence of [γ-32P]ATP at 30 °C for 10 min. After incubation, the reaction mixtures were sepa- rated by SDS-PAGE. The gel was then dried and subjected to autoradiography.

RNA Interference—To knock down endogenous IKK-α, the expression plasmid for siRNA directed against human IKK-α (GeneSuppressor, Imgenex Corp., San Diego, CA) was intro- duced into U2OS cells using Lipofectamine following the man- ufacturer’s instructions. Forty-eight hours after transfection, whole cell lysates were prepared and analyzed for the expression levels of IKK-α by immunoblotting.

Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitation assays were performed following a proto- col provided by Upstate Biotechnology (Lake Placid, NY). In brief, cells were cross-linked with 1% formaldehyde in medium for 10 min at 37 °C. Chromatin solutions were prepared and immunoprecipitated with anti-HA antibody. DNAs of the immunoprecipitates and control input DNAs were purified using a QIAquick PCR purification kit (Qiagen Inc.) and then analyzed by regular PCR using human Bax promoter-specific
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**RESULTS**

**Induction of IKK-α during CDDP-mediated Apoptosis in U2OS Cells**—To define the potential function(s) of IKKs in DNA damage-induced signaling, we first examined their expression levels in human osteosarcoma-derived U2OS cells exposed to the DNA-damaging chemotherapeutic drug CDDP. Under our experimental conditions, U2OS cells underwent apoptosis in a time-dependent manner as examined by cell survival assays (Fig. 1A). Similar results were also obtained by fluorescence-activated cell sorter analysis (data not shown). Immunoblot analysis demonstrated that p53 and its homolog p73α, which are major mediators in the DNA damage response (reviewed in Refs. 14 and 38), were significantly induced at protein levels in response to CDDP (Fig. 1B), whereas the expression of p53 and p73α mRNAs remained unchanged (Fig. 1C). Their accumulation was associated with several of their downstream effectors, including p21WAF1 and Bax. Notably, CDDP treatment led to a remarkable accumulation of IKK-α, and its induction was observed between 12 and 36 h after exposure to CDDP (Fig. 1B). Twelve hours after treatment with CDDP, the amount of IKK-γ (NEMO) was transiently increased at the protein level. By contrast, the amount of IKK-β was not significantly altered upon CDDP treatment.

RT-PCR analysis revealed that the expression levels of IKK-α and IKK-β mRNAs remained unchanged regardless of CDDP treatment, whereas a marked increase in the expression level of IKK-γ mRNA was detected in a time-dependent manner in response to CDDP (Fig. 1C). Intriguingly, immunoblot analysis also demonstrated that CDDP treatment caused a significant increase in the phosphorylated form of IκB-α, which is a well characterized substrate for the IKK complex (reviewed in Ref. 3). Taken together, these results suggest that DNA damage-induced accumulation of both p53 and p73α is associated with the up-regulation of IKK-α and IKK-γ and that a functional interaction might exist between them in DNA damage-mediated apoptotic pathways.

**Nuclear Accumulation of IKK-α in Response to CDDP**—It was shown recently that IKK-α shuttles between the nucleus and cytoplasm in a CRM1-dependent fashion (32). Nuclear IKK-α has the ability to transactivate NF-κB-responsive genes that control survival pathways after cytokine exposure (33, 34). In addition, Verma et al. (39) found that, like IKK-α, IKK-γ is present in both the nucleus and cytoplasm. These observations prompted us to examine whether the subcellular localization of endogenous IKKs can change in response to CDDP. For this purpose, nuclear and cytoplasmic extracts were prepared from U2OS cells exposed to CDDP or left untreated and then subjected to immunoblotting with the indicated antibodies. In agreement with previous results (33), IKK-α was localized in both the nucleus and cytoplasm, whereas IKK-β was expressed almost exclusively in the cytoplasm (Fig. 2A). The amounts of cytoplasmic IKK-α, IKK-β, and IKK-γ remained unchanged regardless of the treatment with CDDP. Of note, CDDP treatment led to a remarkable accumulation of IKK-α in the cell nucleus in a time-dependent manner, whereas IKK-β accumulated in the cell nucleus to a lesser degree. The temporal patterns of CDDP-mediated accumulation of nuclear IKK-α correlated with those of p73α. On the other hand, the transient nuclear accumulation of IKK-γ was detected 12 h after exposure to CDDP. Compared with the levels of nuclear IKK-α accumulated in response to CDDP, the amount of nuclear IKK-γ was small. Consistent with the enhanced phosphorylation of IκB-α in response to CDDP, cytoplasmic IκB-α was decreased in a time-dependent manner. However, CDDP treatment had little or no effect on the nuclear accumulation of the NF-κB p65 subunit (RelA), indicating that nuclear translocation of p65 might be inhibited in the presence of CDDP. Considering that, among IKKs, CDDP treatment promoted a significant nuclear accumulation of IKK-α, it is likely that IKK-α might have a certain nuclear function during CDDP-mediated apoptosis.

To investigate whether exogenously expressed IKK-α can reflect the behavior of endogenous IKK-α, we examined the intracellular distribution of exogenous IKK-α by immunoblotting and immunofluorescence staining. Nuclear and cytoplasmic fractions were prepared from U2OS cells transfected with the expression plasmid for FLAG-IKK-α or HA-p73α and subjected to immunoblotting with anti-FLAG or anti-p73α antibody, respectively. As shown in Fig. 2B, HA-p73α was localized exclusively in the cell nucleus, whereas FLAG-IKK-α was present in both the nucleus and cytoplasm. Surprisingly, immunofluorescence staining with anti-FLAG and anti-lamin B antibodies clearly showed that exogenous IKK-α was localized in...
Intriguingly, HA-p73 activity was determined. Or L929 (fraction IV). Our results suggest that nuclear IKK-α might interact with pro-apoptotic p73α and modulate its function.

As described above, the amounts of the nuclear transactivating p65 subunit remained unchanged in U2OS cells treated with CDDP. These findings prompted us to examine whether NF-κB activation can be detected in response to CDDP. To this end, U2OS cells transfected with the NF-κB reporter plasmid (40) were treated with CDDP, and their luciferase activity was determined. Consistent with the previous observations (13), CDDP treatment did not enhance NF-κB-dependent transcriptional activation (Fig. 3A, left panel). Under our experimental conditions, NF-κB-dependent transcriptional activation was detected within 2 h of exposure to TNF-α in the mouse fibrosarcoma cell line L929 (Fig. 3A, right panel), which is widely used to investigate TNF-α-dependent NF-κB activation (41). In addition, treatment of L929 cells with TNF-α caused a nuclear accumulation of p65 (Fig. 3B). Thus, it is likely that, the lack of a significant effect of CDDP on NF-κB-dependent transcriptional activation could be attributed to lack of the regulated nuclear accumulation of p65.

**IKK-α Interacts with p73**—To determine whether IKK-α can interact with p73 in cells, whole cell lysates prepared from transfected COS-7 cells were immunoprecipitated with anti-FLAG or anti-HA antibody and analyzed by immunoblotting using anti-p73 or anti-FLAG antibody, respectively. As shown in Fig. 4A, exogenously expressed FLAG-IKK-α and HA-p73α formed stable complexes in COS-7 cells. Similarly, HA-p73β was co-immunoprecipitated with FLAG-IKK-α (data not shown). Their interaction was further examined using endogenous materials. As shown in Fig. 4B, endogenous p73α formed a protein complex with endogenous IKK-α in U2OS cells exposed to CDDP. Similar results were also obtained in HeLa cells (data not shown). In contrast, immunoprecipitation of endogenous p53 followed by immunoblotting with anti-FLAG antibody did not detect co-immunoprecipitated FLAG-IKK-α (Fig. 4C), indicating that IKK-α interacts with p73, but not with p53, in cells. To identify the p73 determinants involved in the interaction with IKK-α, we generated several deletion mutants of p73α fused to GST and tested their ability to bind to FLAG-IKK-α in GST pulldown assays. These mutants were designed based on p73α, including the transactivation, DNA-binding,
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FIGURE 4. Interaction between IKK-α and p73α. A, FLAG-IKK-α was transiently coexpressed with HA-p73α in COS-7 cells as indicated. Whole cell lysates were subjected to immunoprecipitation (IP) with anti-FLAG or anti-HA antibody, followed by immunoblotting (IB) with anti-p73 or anti-FLAG antibody, respectively. B, whole cell lysates were prepared from U2OS cells exposed to CDDP and subjected to immunoprecipitation with normal mouse serum (NMS), anti-IKK-α antibody, or anti-p73 antibody, followed by immunoblotting with the indicated antibodies. C, FLAG-IKK-α was transiently transfected into COS-7 cells. Whole cell lysates were subjected to immunoprecipitation with normal mouse serum or anti-p53 antibody, followed by immunoblotting with anti-FLAG antibody. Input represents the 10% materials used for immunoprecipitation in B and C.

FIGURE 5. DNA-binding domain of p73α is required for interaction with IKK-α. A, shown is a schematic representation of the GST-p73 fusion proteins. TA, transactivation domain; DB, DNA-binding domain; OD, oligomerization domain; SAM, sterile α-motif domain. B, the DNA-binding domain of p73α is required for the interaction with IKK-α. 35S-Labeled FLAG-IKK-α was incubated with GST or the indicated GST-p73 fusion proteins, and bound radiolabeled proteins were recovered on glutathione-Sepharose beads and visualized by autoradiography. The 1/10 volumes of input sample (1/10 Input) of 35S-labeled FLAG-IKK-α used for pulldown assay were applied to the same gel (upper panel). Coomassie Blue-stained GST-p73 fusion proteins together with GST are also shown (lower panel).

IKK-α Increases p73 Stability—As described previously (16, 42), some p73-interacting protein kinases, including c-Abl and protein kinase Cδ, can stabilize p73. To investigate whether IKK-α can affect the stability of p73, COS-7 cells were transiently cotransfected with equal amounts of the HA-p73α expression plasmid with or without increasing amounts of the expression plasmid encoding IKK-α, and the protein level of HA-p73α was examined. As shown in Fig. 6A, the amount of HA-p73α was significantly increased in the presence of exogenous IKK-α, whereas IKK-α had no detectable impact on the stability of FLAG-p53. In addition, HA-p73β was also stabilized by IKK-α, but to a lesser degree compared with HA-p73α. p73α mRNA levels remained unchanged in the presence of IKK-α (p73α mRNA levels remained unchanged in the presence of IKK-α (Fig. 6A, lower panels), suggesting that IKK-α regulates p73 at the protein level. Next, we examined the possible effect of IKK-β on the stability of p73 and p53 by transient cotransfection. As shown in Fig. 6B, FLAG-IKK-β had negligible effects on the stability of both p73α and p53.

To investigate a possible role of endogenous IKK-α in the regulation of p73 stability, we employed RNA interference to block IKK-α expression. The enforced expression of siRNA against IKK-α in U2OS cells resulted in a significant reduction of endogenous IKK-α (Fig. 6C). We then tested the effect of knockdown of endogenous IKK-α on the CDDP-mediated accumulation of p73α. U2OS cells were transiently transfected with the expression plasmid for siRNA against IKK-α and exposed to CDDP for 36 h. As shown in Fig. 6C, down-regulation of endogenous IKK-α expression...
FIGURE 6. IKK-α increases p73α stability. A, the ectopic expression of IKK-α increases the stability of p73, but not of p53. COS-7 cells were transiently cotransfected with the indicated combinations of expression plasmids. Whole cell lysates and total RNA were prepared and subjected to immunoblotting (IB) (upper panels) or RT-PCR (lower panels), respectively. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. B, IKK-β does not affect the stability of p73. COS-7 cells were transiently cotransfected with the indicated combinations of expression plasmids. Whole cell lysates were subjected to immunoblotting with the indicated antibodies. C, the reduction of endogenous IKK-α results in the attenuation of the CDDP-mediated accumulation of p73α. U2OS cells were transiently transfected with the expression plasmid for siRNA against IKK-α (siIKK-α) or with the scrambled control. Forty-eight hours after transfection, whole cell lysates were analyzed by immunoblotting with anti-p73 antibody. Upper panels, actin levels were used to monitor loading. Lower panels, U2OS cells were transiently transfected with the expression plasmid as in the upper panels. Twenty-four hours after transfection, cells were exposed to CDDP (at a final concentration of 20 μM) for 36 h or left untreated. Equal amounts of whole cell lysates were subjected to immunoprecipitation with anti-p73 antibody, followed by immunoblotting with anti-p73 antibody. Actin was included as a loading control. D, the CDDP-mediated accumulation of p73α is not detectable in IKK-α/MEFs. Wild-type (WT) and IKK-α/MEFs were treated with or without CDDP (at a final concentration of 20 μM) for 24 h. Equal amounts of whole cell lysates were subjected to immunoprecipitation (IP) with anti-p73 antibody, followed by immunoblotting with anti-p73 antibody. Left panels, actin was included as a loading control. Right panel, shown are the results of MTT assay. Wild-type and IKK-α/MEFs were treated with CDDP (at a final concentration of 20 μM). At the indicated time periods after CDDP treatment, their viability was examined by MTT assay. KO, knock-out.

FIGURE 7. IKK-α increases the half-life of p73α. A and B, IKK-α or the FLAG-IKK-β expression plasmid, respectively, was transiently transfected into COS-7 cells with the expression plasmid for HA-p73α for 24 h. Cells were treated with cycloheximide (CHX) and harvested at the indicated time periods, followed by immunoblotting (IB) with anti-p73 antibody. The intensity of the bands was quantified by densitometry, and the HA-p73α remaining is indicated graphically. C, IKK-α inhibits the ubiquitination of p73. COS-7 cells were transiently cotransfected with the indicated combinations of expression plasmids and treated with MG132. Ubiquitinated products were recovered on nickel-agarose beads and separated by SDS-PAGE, followed by immunoblotting with anti-HA antibody. The brackets indicate slowly migrating ubiquitinated (Ub) forms of HA-p73α.
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A

Fold activation

p21WAF1

HA-p73α
IKK-α

p53
IKK-α

Bax

Fold activation

MDM2

HA-p73α
IKK-α

B

Fold activation

p21WAF1

p53
IKK-α

HA-p73α
IKK-β

Bax

Fold activation

MDM2

HA-p73α
IKK-α

C

Fold activation

p21WAF1

p53
IKK-α

HA-p73α
IKK-β

Bax

Fold activation

MDM2

HA-p73α
IKK-α

D

IB: Anti-p73

IB: Anti-FLAG

IB: Anti-p21WAF1

IB: Anti-Actin

HA-p73α

FLAG-IKK-α

E

HA-p73α

Input

IB: Anti-p73

IB: Anti-FLAG

IB: Anti-Actin

HA-p73α

FLAG-IKK-α

Actin
markedly inhibited the CDDP-mediated accumulation of p73α. To further confirm the effects of endogenous IKK-α, wild-type and IKK-α−/− MEFs were treated with or without CDDP for 24 h, and whole cell lysates were immunoprecipitated with anti-p73 antibody. As expected, CDDP-mediated accumulation of endogenous p73α was detected in wild-type, but not IKK-α−/−, MEFs, and CDDP sensitivity was decreased in IKK-α−/− MEFs compared with wild-type MEFs (Fig. 6D).

To explore whether IKK-α can modulate p73 turnover, we examined the decay rate of p73α in COS-7 cells. Twenty-four hours after transfection, cells were treated with cycloheximide. At the indicated time points, whole cell lysates were prepared and subjected to immunoblotting with anti-p73 antibody. As shown in Fig. 7A, the degradation rate of HA-p73α was slower in cells expressing both HA-p73α and IKK-α than that in cells expressing HA-p73α alone. In contrast, the half-life of HA-p73α was not prolonged in the presence of FLAG-IKK-β (Fig. 7B). An IKK-α-mediated increase in the half-life of endogenous p73α was also observed. Thus, IKK-α-mediated p73α stabilization resulted from the increase in the half-life of p73α. As described previously (43), the steady-state level of p73 is regulated at least in part by the protein degradation process through the ubiquitin/proteasome pathway. We then determined whether IKK-α can inhibit the ubiquitination of p73. To this end, COS-7 cells were transiently cotransfected with the expression plasmids for HA-p73α and His-ubiquitin with or without increasing amounts of the expression plasmid for IKK-α or FLAG-IKK-β. Forty-eight hours after transfection, whole cell lysates were prepared and analyzed by immunoblotting for the presence of His-ubiquitin-containing p73α. As shown in Fig. 7C, the amounts of the ubiquitinated forms of p73α were decreased in the presence of IKK-α, whereas FLAG-IKK-β inhibited the ubiquitination of p73α to a lesser degree. Taken together, these results strongly suggest that IKK-α inhibits the ubiquitination of p73α, thereby increasing the stability of p73α.

IKK-α Enhances p73-mediated Transactivation and Pro-apoptotic Functions in p53-deficient H1299 Cells—To address the functional implications of the interaction between IKK-α and p73, we first examined the effects of IKK-α on p73-mediated transcriptional activation. To this end, we cotransfected p53-deficient H1299 cells with the HA-p73α expression plasmid and the luciferase reporter construct under the control of the p21WAF1, bax, or MDM2 promoter with or without increasing amounts of the expression plasmid encoding IKK-α. As shown in Fig. 8A, ectopically expressed p73α successfully activated the transcription of each of these p53/p73-responsive reporters compared with the empty control plasmids, and IKK-α alone had little effect on luciferase activity. When HA-p73α was coexpressed with IKK-α, a marked increase in p73α-dependent transcriptional activation was observed in a dose-dependent manner. IKK-α also enhanced p73β-mediated transcriptional activation (data not shown). In contrast, there was no detectable IKK-α-induced increase in p53-dependent reporter gene activity (Fig. 8B). To examine the specificity of the IKK-α-mediated activation of p73-dependent transcription, we investigated whether IKK-β can enhance p73 transcriptional activity for the p53/p73-responsive promoters. As shown in Fig. 8C, no significant changes in p73α-dependent transcriptional activation were found with FLAG-IKK-β. In addition, the ectopic expression of IKK-α had no detectable effects on luciferase activity in p73-deficient neuroblastoma SK-N-AS cells bearing a mutant form of p53 (15, 44). Furthermore, the exogenous expression of IKK-α in H1299 cells resulted in a significant up-regulation of the p73α-mediated induction of endogenous p21WAF1 (Fig. 8D). To address whether the amounts of p73α associated with the p53/p73-responsive promoter can be increased in the presence of exogenous IKK-α, H1299 cells were transiently cotransfected with the expression plasmid for HA-p73α with or without the FLAG-IKK-α expression plasmid and subjected to chromatin immunoprecipitation analysis. As shown in Fig. 8E, the IKK-α-inducible association of p73α with the bax promoter was detected. Taken together, these results strongly suggest that IKK-α specifically enhances the transcriptional activity of p73.

We next investigated the potential impact of IKK-α on p73-dependent biological functions such as the regulation of apoptosis. H1299 cells were transiently cotransfected with a constant amount of HA-p73α and β-galactosidase expression plasmids with or without increasing amounts of the expression plasmid for IKK-α or FLAG-IKK-β. The β-galactosidase expression plasmid was used to identify the transfected cells. Forty-eight hours after transfection, cells were subjected to double staining with trypan blue (nonviable cells) and Red-Gal (transfected cells), and the number of cells with purple coloration was scored as described previously (11). As shown in Fig. 9 (A and B), the coexpression of IKK-α with HA-p73α resulted in an increase in the number of apoptotic cells compared with the expression of HA-p73α alone. In contrast, the coexpression of FLAG-IKK-β had no significant effect on p73α-dependent apoptosis (Fig. 9C). These data are consistent with the positive effect of IKK-α on p73-dependent transcriptional activation.

Kinase-deficient Mutant IKK-α Fails to Stabilize p73—To examine whether the intrinsic kinase activity of IKK-α is required for the stabilization of p73, we generated a mutant form of IKK-α (IKK-α(K44A)) in which Lys44 within the ATP-binding motif was replaced with Ala. As described previously (45), mutation of this site impairs the kinase activity of IKK-α.

**FIGURE 8.** IKK-α enhances the transcriptional activity of p73α. A and B, p53-deficient H1299 cells were transiently cotransfected with the expression plasmid for HA-p73α or p53, respectively, with the indicated p53/p73 luciferase reporter construct in the presence or absence of the IKK-α expression plasmid, followed by reporter assay. C, IKK-β does not affect p73-mediated transcriptional activation. H1299 cells were transiently cotransfected with the expression plasmid encoding HA-p73α and the indicated reporter constructs with or without the IKK-β expression plasmid, followed by reporter assay. D, shown are the results from analysis of endogenous p21WAF1. H1299 cells were transiently cotransfected with the indicated expression plasmids. Whole cell lysates were subjected to immunoblotting (IB) with the indicated antibodies. E, IKK-α increases the amount of p73α associated with the human bax promoter. H1299 cells were transiently cotransfected with the indicated combinations of expression plasmids. Forty-eight hours after transfection, cells were cross-linked with 1% formaldehyde and subjected to chromatin immunoprecipitation assays, followed by PCR analysis as described under “Experimental Procedures” (upper panels). Immunoblotting of the indicated proteins is also shown (lower panels).
**Functional Interaction between IKK and p73**

**A**

![Control](image1)

![HA-p73 α](image2)

![IKK- α](image3)

![HA-p73 α +IKK- α](image4)

**B**

![Graph](image5)

**C**

![Graph](image6)

**FIGURE 9. IKK-α enhances the pro-apoptotic function of p73 α.** A and B, H1299 cells were transiently cotransfected with the expression plasmids for HA-p73 α and β-galactosidase with or without IKK-α. Control transfection was performed with the empty plasmid plus the β-galactosidase expression plasmid. Forty-eight hours after transfection, cells were double-stained with trypan blue (blue) and Red-Gal (red) (A), and the number of transfected cells (positive for β-galactosidase) and transfected apoptotic cells (dark pink-purple) in at least three different fields (>300 transfected cells) was measured. The percentage of transfected apoptotic cells is indicated (B). C, H1299 cells were transiently cotransfected with the indicated combinations of expression plasmids plus the β-galactosidase expression plasmid and processed for double staining as described above. The percentage of transfected apoptotic cells is indicated.

Immunoprecipitation analysis indicated that IKK-α(K44A) retained the ability to form a complex with p73 α in cells (Fig. 10A). In sharp contrast to wild-type IKK-α, the coexpression of FLAG-IKK-α(K44A) had little or no effect on the intracellular level of exogenously expressed HA-p73 α (Fig. 10B). To examine the effect of kinase-deficient IKK-α on endogenous p73, U2OS cells were transiently transfected with the empty plasmid or the FLAG-IKK-α(K44A) expression plasmid and then exposed to CDDP for 24 h or left untreated. Whole cell lysates and total RNA were prepared and subjected to immunoblotting and RT-PCR, respectively. As shown in Fig. 10C, the CDDP-mediated stabilization of endogenous p73 α was markedly inhibited in U2OS cells transfected with the FLAG-IKK-α(K44A) expression plasmid, whereas FLAG-IKK-α(K44A) had no significant effect on the amount of endogenous p53. In good agreement with the observations above, CDDP-induced apoptosis was significantly inhibited in the presence of FLAG-IKK-α(K44A) (Fig. 10D). Similar results were also obtained in H1299 cells (Fig. 10, E and F). Thus, the kinase activity of IKK-α appears to be required for the stabilization of p73 α in response to CDDP-induced DNA damage.

**IKK-α Has the Ability to Phosphorylate p73 α—** To address whether IKK-α can phosphorylate p73 α, we performed an in vitro kinase assay. GST alone or the indicated GST-p73 α deletion mutants (Fig. 11) were incubated with the active form of IKK-α in the presence of [γ-32P]ATP. After incubation, the reaction mixture was separated by SDS-PAGE, followed by autoradiography. As shown in Fig. 11, GST-p73 -(1-62) was phosphorylated by IKK-α, suggesting that the N-terminal region of p73 α is phosphorylated by the active form of IKK-α.

**DISCUSSION**

Until recently, the IKK complex has been thought to participate in the cytoplasmic signaling pathway that activates NF-κB. However, this viewpoint has been challenged with the findings of the nuclear accumulation and function of IKK-α in response to cytokine exposure (33, 34). According to the previous results, nuclear IKK-α contributes to the induction of NF-κB-dependent gene expression through histone H3 phosphorylation. In this study, we found that CDDP treatment (DNA cross-linking) leads to a remarkable accumulation of IKK-α in the cell nucleus. We also demonstrated that IKK-α directly binds to the sequence-specific DNA-binding domain of p73 α and has positive effects on its stability as well as pro-apoptotic function. The CDDP-induced stabilization of p73 α was dependent on IKK-α as examined by siRNA-mediated knockdown and using IKK-α/−/− MEFs. In addition, chromatin immunoprecipitation assays showed that the IKK-α-dependent stabilization of p73 α correlates with an increase in the amounts of p73 α recruited onto the human bax promoter. Our present findings therefore imply not only a novel nuclear role of IKK-α in regulating the DNA damage response, which is distinct from NF-κB activation, but also a new regulatory pathway of pro-apoptotic p73 α.
Previous studies have suggested that endogenous p73 is both stabilized and activated for apoptosis in response to CDDP and γ-irradiation through a pathway that depends on the nuclear non-receptor tyrosine kinase c-Abl (15–17). c-Abl binds to p73 via the P
XX
P motif of p73 and the c-Abl SH3 (Src homology 3) domain and phosphorylates p73 at Tyr99. The phosphorylated form of p73 undergoes nuclear redistribution and becomes associated with the nuclear matrix in a c-Abl-dependent manner (36). In addition, HIPK2 (homeodomain-interacting protein kinase-2), which interacts with p73 and enhances its function, co-localizes with p73 in nuclear body-like structures (46). Mittnacht and Weinberg (47) reported that the retinoblastoma protein pRb differentially associates with the nuclear matrix, depending on its phosphorylation status or the integrity of the protein. The underphosphorylated form of pRb, which is active in growth control, remains tightly associated with the nuclear matrix, whereas the hyperphosphorylated form and a mutant form are detected largely in the nucleoplasm. In this study, we have demonstrated that IKK-α has the ability to stabilize and activate p73α and co-localizes with p73α in the nuclear lamina. Our findings, together with those previous observations, suggest that the nuclear structures including the nuclear matrix and/or nuclear body might provide an important subnuclear locale for p73 function.

As expected from their extensive amino acid sequence similarity, both IKK-α and IKK-β display IkB kinase activity in vitro (48), suggesting that their biochemical and biological functions seem to be redundant and overlapping with regard to NF-κB activation. On the other hand, genetic disruption studies in mice have demonstrated that IKK-α and IKK-β might have distinct regulatory functions. In IKK-α-deficient mice, inflammatory cytokine-induced activation of the NF-κB pathway is not severely impaired, although various developmental abnormalities, including defective epidermal differentiation, are detected (49–52). In contrast, mice lacking IKK-β die immedi-

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**FIGURE 10.** Kinase-deficient IKK-α fails to stabilize p73α. A, shown is the physical interaction between IKK-α(K44A) and p73α. COS-7 cells were transiently cotransfected with the indicated expression plasmids. Whole cell lysates were subjected to immunoprecipitation (IP) with anti-FLAG or anti-HA antibody, followed by immunoblotting (IB) with anti-p73 or anti-FLAG antibody, respectively. B, IKK-α(K44A) has no detectable effect on the amount of p73α. COS-7 cells were transiently cotransfected with the indicated combinations of expression plasmids. Equal amounts of the lysates were subjected to immunoblotting with anti-p73 or anti-FLAG antibody. C, IKK-α(K44A) suppresses endogenous p73 in response to CDDP. U2OS cells transiently transfected with or without FLAG-IKK-α(K44A) were left untreated or treated with CDDP for 24 h. Whole cell lysates and total RNA were prepared and subjected to immunoblotting (upper panels) or RT-PCR analysis (lower panels). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. D, IKK-α(K44A) inhibits CDDP-induced apoptosis. U2OS cells transiently cotransfected with the β-galactosidase expression plasmid with or without the FLAG-IKK-α(K44A) expression plasmid were treated with CDDP for 24 h or left untreated. Cells were then subjected to double staining as described in the legend to Fig. 9. The percentage of transfected apoptotic cells is indicated. E and F, transfected H1299 cells were exposed to CDDP or left untreated and subjected to immunoprecipitation, followed by immunoblotting (E, upper panels), RT-PCR (E, lower panels), and apoptosis assays (F).
Functional Interaction between IKK and p73

FIGURE 11. Active form of IKK-α phosphorylates p73 in vitro. Shown is the expression of GST-p73 deletion mutants. Upper panel, purified GST and the indicated GST-p73 deletion mutants were analyzed by SDS-PAGE, followed by Coomassie Brilliant Blue staining. Lower panel, shown are the results from the in vitro kinase reaction. Equal amounts of GST and the indicated GST-p73 deletion mutants were incubated with the active form of IKK-α in the presence of [γ-32P]ATP. After incubation, the reaction mixtures were separated by SDS-PAGE and subjected to autoradiography.

ate after birth because of uncontrolled hepatic apoptosis and exhibit an extensive defect in the activation of the NF-κB pathway (49). Thus, it is likely that IKK-β is absolutely critical in the regulation of inducible IkB degradation and the subsequent activation of the NF-κB pathway in response to inflammatory stimuli, whereas IKK-α is not required for this process. Alternatively, other studies suggest that IKK-α is involved in NF-κB activation through a second pathway that leads to the processing of the NF-κB2 (p100) precursor (53, 54). Under our experimental conditions, IKK-β bound to p73α as determined by immunoprecipitation analysis (data not shown); however, it failed to stabilize p73α and to enhance its transactivation as well as pro-apoptotic activity. Unlike IKK-α, the amounts of endogenous nuclear IKK-β remained almost unchanged in response to CDDP. This is in good agreement with recent observations showing that IKK-α, but not IKK-β, has the ability to shuttle between the nucleus and cytoplasm (32). Taken together, our findings suggest that their functional divergence might be attributed at least in part to their differential interaction with p73.

Another finding of this study is that CDDP treatment results in a marked phosphorylation of IkB-α in association with a significant down-regulation of cytoplasmic IkB-α; however, the amounts of nuclear p65 remains unchanged. Consistent with these results, CDDP treatment did not enhance NF-κB transcriptional activity in U2OS cells; however, p65 was induced to accumulate in the nucleus of L929 cells in response to TNF-α. Bian et al. (13) also reported that no change in NF-κB-dependent transcriptional activation is observed in certain neuroblastoma cells in response to CDDP. It is therefore likely that, during the CDDP-dependent apoptotic process, the survival pathway mediated by NF-κB might be impaired. As described previously (55), nuclear c-Abl is activated by DNA-damaging agents, including CDDP, but not by TNF-α, indicating that the differential behavior of p65 in response to CDDP or TNF-α might be due to the presence or absence of the activated form of c-Abl, respectively. In sharp contrast to CDDP, various anticancer agents, including camptothecin (topoisomerase I inhibition), paclitaxel (microtubule depolymerization inhibition), and doxorubicin (topoisomerase II inhibition), significantly induce the down-regulation of IkB-α and promote the nuclear translocation of NF-κB, followed by subsequent NF-κB-dependent transcriptional activation (13, 56, 57). Considering that the cytoplasmic retention of NF-κB by IkB is the major molecular mechanism that controls its activity as well as cell fate determination (reviewed in Ref. 3), the CDDP-mediated attenuation of the nuclear accumulation of p65 might be required at least in part for apoptotic cell death in response to CDDP. In this connection, it is worth noting that NF-κB promotes T cell survival by reducing the transcription of p73 following antigenic stimulation (27). Currently, it is not clear how the nuclear accumulation of p65 is blocked in cells exposed to CDDP, even though cytoplasmic IkB-α is significantly decreased in our system. Future experiments will be necessary to clarify the underlying mechanistic details of this phenomenon.

It has been shown that p73 stability is regulated in a ubiquitination-dependent and -independent manner (43, 58). p73 is stabilized by coexpression with c-Abl or protein kinase Cδ, which phosphorolyses p73 at Tyr99 or Ser289, respectively (16, 42). According to our results, IKK-α had the ability to stabilize p73, whereas the kinase-deficient mutant form of IKK-α does not, suggesting that the kinase activity of IKK-α is required for the stabilization of p73. As described previously (reviewed in Ref. 59), the amino acid sequence DSGΨXS (where Ψ is a hydrophobic amino acid and X is any amino acid) has been identified as a consensus motif for the IKK-dependent phosphorylation of IkB proteins. During the search for a putative phosphorylation site(s) targeted by IKK within the amino acid sequence of p73α, we failed to find a related motif. Of note, it has been shown that IKK-α, but not IKK-β and the kinase-deficient IKK-α mutant, phosphorolyses histone H3 at Ser10, which has no IKK phosphorylation consensus sequence (33, 34). According to our in vitro kinase reaction, the active form of IKK-α has the ability to phosphorylate the N-terminal region of p73α. Because IKK-α, but not the kinase-deficient IKK-α mutant, has the ability to stabilize p73α, it is important to determine whether IKK-α can phosphorylate p73 in cells exposed to CDDP. In addition, it will be interesting to identify the signaling component(s) upstream of IKK-α that could receive the nuclear signal in response to CDDP-mediated DNA damage.
As described previously (60), the transcriptional coactivator p300 and CBP interact with p73 and enhance its function. Costanzo et al. (61) reported that DNA damage induces the acetylation of p73 by p300 in a c-Abl-dependent manner. In addition, Hap7 et al. (62) found that p300-mediated acetylation results in p73 stabilization. It is worth noting that IKK-α, but not IKK-β, has the ability to interact with CBP (33). According to the previous results, IKK-α is required for the cytokine-induced phosphorylation and subsequent acetylation of histone H3. Although the precise molecular mechanism behind the IKK-α-dependent stabilization of p73 remains unknown, it is likely that a functional interaction might exist among c-Abl, p300/CBP, IKK-α, and p73. Our preliminary results suggest that the kinase-deficient form of c-Abl inhibits the IKK-α-dependent stabilization of p73α (data not shown). This issue is currently under investigation in our laboratory.

Upon CDDP treatment, endogenous p73α and p53 are significantly induced at the protein level in U2OS cells bearing wild-type p53 (45). Unlike p73, p53 is targeted for degradation by MDM2 through the ubiquitination-dependent proteasome pathway (reviewed in Ref. 38). It is well known that, in response to DNA damage, p53 is phosphorylated at multiple sites, including Ser15 and Ser20, and that these phosphorylation events stimulate p53 stabilization by preventing the interaction with MDM2. Alternatively, Li et al. (63) found that HAUSP (herpesvirus-associated ubiquitin-specific protease) participates in the deubiquitination and subsequent stabilization of p53. Because the kinase-deficient IKK-α mutant inhibited the CDDP-induced stabilization of endogenous p73, but not of p53, the IKK-α-dependent stabilization appears to be highly specific to p73. Recently, Rossi et al. (64) reported that the HECT-type ubiquitin-protein isopeptide ligase Itch binds to and ubiquititates p73, but not p53. Their study demonstrated that CDDP treatment results in a rapid reduction of Itch protein levels, indicating that Itch could contribute to the IKK-α-mediated stabilization of p73α.

Furthermore, it has been shown that p53-dependent apoptosis requires the indirect contribution of at least one of the other p53 family members, p73 or p63, whereas p73 is sufficient in the absence of p53 to induce apoptosis (65). p53 is the most frequent target for genetic alterations in human cancers, leading to loss of its pro-apoptotic function (66). In addition to mutation of p53 itself, many cancers bearing wild-type p53 may harbor the other defects in the p53 pathway (reviewed in Ref. 38). In contrast to p53, p73 is infrequently mutated in many human cancers (67). Given the specific induction and activation of p73 by IKK-α, it is likely that the IKK-α-mediated induction of p73 substitutes for the downstream defects in the p53 pathway and/or enables p53 to cooperate with p73 to induce apoptosis.

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